AN ASSESSMENT OF THE POLLUTION OF KABUTHI RIVER BY WASTEWATER FROM DAGORETTI SLAUGHTERHOUSES COMPLEX IN KIAMBU DISTRICT OF KENYA

Joyce Wangari Thaiya (BVM)

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Department of Public Health, Pharmacology and Toxicology,
Faculty of Veterinary Medicine;
College of Agriculture and Veterinary Sciences
University Of Nairobi.

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DECLARATION

This project is my original work and has not been presented for a degree in any other University

Joyce Wangari Thaiya

Signature ---------------------------------- Date ----------------------------------

This project report has been submitted for examination with our approval as the University supervisors

Dr Gathura P. B. (B.V.M., MSc., PhD)

Signature ---------------------------------- Date ----------------------------------

Prof. Kagiko M. M. (B.V.M., MSc., PhD).

Signature ---------------------------------- Date ----------------------------------

Dr. Ogara W. O. (B.V.M., MSc., Ph.D.)

Signature ---------------------------------- Date ----------------------------------

Prof. Njeruh F. M. (B.V.M., MSc., PhD).

Signature ---------------------------------- Date ----------------------------------

DEDICATION

To my mother-in-law Peris and my mother Isabella

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TABLE OF CONTENTS

DECLARATION....2
DEDICATION....3
ACKNOWLEDGEMENT....4
TABLE OF CONTENTS....5
LIST OF TABLES....8
LIST OF FIGURES....9
ABSTRACT....10
CHAPTER 1....12
1. INTRODUCTION....12
CHAPTER 2....16
2. REVIEW OF LITERATURE....16
2.1 REVIEW ON SLAUGHTERHOUSES....16
2.2 CHARACTERISTICS OF WATER....16
2.2.1 BIOLOGICAL CHARACTERISTICS OF WATER....16
2.2.2 PHYSICAL CHARACTERISTICS OF WATER....17
2.2.3 CHEMICAL CHARACTERISTICS....18
2.2.4 ORGANICS....18
2.3 WATER POLLUTION....19
2.4. WASTEWATER TREATMENT....19
2.5 INDICATORS OF WATER POLLUTION....20
2.5.1. BIOLOGICAL INDICATORS....20
2.5.1.1. COLIFORM GROUP....21
2.5.1.2 FAECAL COLIFORM....22
2.5.1.3 ESCHERICHIA COLI....22
2.5.1.4 FAECAL STREPTOCOCCI....23
2.5.1.5 CLOSTRIDIUM PERFRINGENS....24
2.6.2 OTHER INDICATORS....24
2.6.2.1 BIOCHEMICAL OXYGEN DEMAND....24
2.7 STANDARD MICROBIOLOGICAL TECHNIQUES FOR TESTING WATER FOR MICROBIAL QUALITY....26
2.7.1 TOTAL VIABLE COUNT POUR PLATE METHOD....26
2.7.2 MULTIPLE TUBE TECHNIQUE (MTT)....27
2.7.3 MEMBRANE FILTRATION TECHNIQUE....28
2.8 ANTIBIOTIC RESISTANCE....28
2.9. SALMONELLA....30
CHAPTER 3....32
3. MATERIALS AND METHODS....32

3.1 AREA OF STUDY 32
3.2 COLLECTION OF SAMPLES 34
3.2.1 PREPARATION OF MEDIA AND GLASSWARE 34
3.2.2 COLLECTION OF SAMPLES FOR MICROBIOLOGY..34
3.2.3 COLLECTION OF SAMPLES FOR BIOCHEMICAL OXYGEN DEMAND 34
3.3 DETECTION OF INDICATOR ORGANISMS..35
3.3.1 TOTAL BACTERIAL COUNT BY POUR PLATE METHOD (ANON 1999C)..................35
3.3.2 TOTAL COLIFORM COUNTS USING MULTIPLE TUBE TECHNIQUE 35
3.3.3 CONFIRMATORY TEST FOR COLIFORM...36
3.3.4. BIOCHEMICAL TESTS FOR COLIFORM..36
3.3.4.1 TEST FOR INDOLE PRODUCTION 36
3.3.4.2. METHYL RED AND VOGES-PROSKEUR TEST...37
3.3.4.3. CITRATE TEST 37
3.3.5. FAECAL *E. COLI*..37
3.3.6 ANTIBIOTIC SUSEPTIBILITY TESTING FOR FAECAL *E. COLI* 37
3.3.7. ENUMERATION OF FAECAL STREPTOCOCCI USING THE MEMBRANE FILTRATION TECHNIQUE 38
3.4 ISOLATION AND IDENTIFICATION OF *SALMONELLA* SPP 39
3.4.1 PRE-ENRICHMENT 40
3.4.2 SELECTIVE ENRICHMENT 40
3.4.3 SELECTIVE PLATING...40
3.4.4. BIOCHEMICAL TESTS..40
3.4.4.1 REACTION ON TRIPLE SUGAR IRON AGAR...40
3.4.4.2 UREASE TEST 41
3.4.4.3 IDENTIFICATION USING ANALYTICAL PROFILE INDEX (API) 20E STRIPS.................41
3.4.4.3.1 PREPARATION OF STRIP 41
3.4.4.3.2 TEST READING..41
3.4.5. SEROTYPING 42
3.5 BIOCHEMICAL OXYGEN DEMAND (BOD) ANALYSIS 42
3.6 DATA ANALYSIS 42
CHAPTER 4 43
4.0 RESULTS 43
4.1 OVERVIEW OF THE SLAUGHTERHOUSES COMPLEX AND THE STUDY SITES 43
4.2 RESULTS OF MICROBIOLOGICAL AND ORGANIC QUALITY ANALYSIS 46
4.3 TOTAL BACTERIAL COUNT..48
4.4 TOTAL COLIFORM COUNTS...49
4.5 FAECAL STREPTOCOCCI 49
4.6 FAECAL ESCHERICHIA COLI..50

4.7 ANTIBIOTIC SENSITIVITY OF *E. COLI* ISOLATES 51
4.8 SALMONELLA 51
4.9. BIOCHEMICAL OXYGEN DEMAND 52
CHAPTER 5 54
5.0 DISCUSSIONS 54
CHAPTER 6 59
6.1 CONCLUSIONS 59
6.2 RECOMMENDATIONS 59
6.2.1 RECOMMENDATIONS FOR FURTHER STUDIES 61
REFERENCES 62
APPENDICES 79

LIST OF TABLES

Table 1: Mean values for the different parameters, ...46

Table 2: Total bacterial counts as colony forming units per ml of water. 48

Table 3: Total coliform counts....................49

Table 4: Faecal streptococci as colony forming units per 100 ml of water 50

Table 5: Results obtained for presence of faecal *E. coli* for the various sites 50

Table 6: Antibiotic resistance of faecal *E. coli*....................51

Table 7: Biochemical Oxygen Demand in mg/L. for the various sites....................53

LIST OF FIGURES

Figure 1 Map showing the location of the slaughterhouses 33

Figure 2 Flow Chart for Isolation and Identification of *Salmonella* spp 39

Figure 3. An elevated View of Dagoretti slaughterhouses 43

Figure 4 Sample Collection Sites 44

Figure 5 Other possible sources of pollution to the river 45

Figure 6 Mean values for the different parameters 47

ABSTRACT

Wastewater is an important source of environmental pollution. Both ground and surface water is polluted by wastewater from abattoirs, domestic, agricultural and industrial effluent. Wastewater from slaughterhouses contains both organic waste and micro-organisms. Organic waste results in reduction in oxygen demand of receiving water thus affecting the aquatic eco-system. Presence of micro-organisms may pose a public health risk to users of the receiving water body.

The current study sought to assess the quality of effluent from the Dagoretti slaughterhouses and its level of pollution in the receiving stream, the Kabuthi River.

Water samples were taken from taps in the slaughterhouses, effluent near the slaughterhouses, at the point of discharge into the river, 200 metres upstream from the point of discharge and 200 metres downstream from the point of discharge. Samples were taken at weekly intervals for a period of nine weeks. There was no weather change during the sampling period. The samples were analysed for total coliform, faecal coliform, faecal streptococci, presence or absence of faecal *Escherichia coli,* presence or absence of *Salmonella*, and for antibiotic resistance patterns of the isolated *E. coli*.

The water samples taken inside the slaughterhouse had insignificant counts of bacteria. Samples collected at the point of discharge and downstream from the point of discharge had very high counts of all micro-organisms considered. The total bacterial count was an average of 1.5×10^5 for samples taken 200 metres upstream from the point of discharge, 6.7×10^8 200 metres downstream from the point of discharge, and 4.0×10^8 at the point of discharge into the river.

There was a significant difference ($p>0.05$) between bacterial count of samples collected upstream from the point of discharge and those at the point of discharge and downstream

from the point of discharge but there was no significant difference in counts from other sample collection sites. Faecal streptococci counts were 3.3×10^3 for samples taken upstream from the point of discharge, 1.3×10^7 downstream to the point of discharge, 2.4×10^7 at the point of discharge into the stream during peak operation; 1.1×10^7 where effluent from Nyongara, Thiani and Mumu slaughterhouses meet and 1.4×10^5 for the point of discharge into the stream before start of operations in the slaughterhouses.

Fifteen (15) samples (28.8%) were positive for faecal *E. coli*. Of the *E. coli* isolates, 13(65%) were resistant to one or two of the 12 antimicrobials tested while 4(25%) were resistant to five or more antimicrobials. The isolates were resistant to ampicilin (25%), sulphamethoxazole (25%), streptomycin (20%), tetracycline (20%), chloramphenicol (10%), amoxyllin/clavulanic acid (10%) and nalidixic acid (5%).

There was no significant difference ($p>0.05$) in 5-day Biochemical Oxygen Demand (BOD_5) between samples taken from the different sites except for samples collected upstream from the point of discharge where the BOD_5 level was below detectable level as compared to BOD_5 at the point of discharge (2944 mg/l).

Salmonella was isolated from the site downstream to the point of discharge and one isolate was identified as *Salmonella typhimurium*.

CHAPTER 1

1. INTRODUCTION

Wastewater before or after treatment enters the hydrologic cycle again as ground or surface water. The quality characteristics of wastewater are important from a public health or a biological point of view. The main pollutants are living organisms, organic material, nitrates and phosphates while the main sources of these pollutants are agriculture, industry and domestic wastewaters (Anon, 1996a).

The organisms in surface water could be bacteria, viruses, protozoa and helminthes. The type of organisms depends on the source of pollution: domestic waste would contain organisms found in the human gastrointestinal tract, while wastewater from animal enterprises would contain organisms found in animal gastrointestinal tract. Since a number of organisms are transmitted between animals and man, wastewater is likely to be a source of infection to both man and animals if it contaminates food, feed or water. Pathogens in abattoir waste pose a risk to the workers who in turn would contaminate the carcasses thus leading to spread of disease to consumers. Micro-organisms found in wastewater may or may not be pathogenic but the presence of micro-organisms normally found in the intestines is an indication of the likelihood of having pathogenic organisms in the same wastewater. Faecal coliform and faecal streptococci are some of the organisms used as indicators of faecal pollution and their numbers is an indication of the level of pollution (Anon, 1989). In as much as indicator organisms are used to predict presence or absence of pathogenic organisms in water, isolation of pathogens and their characterization can be a good indication of the actual risk of disease in populations coming into contact with the contaminated water.

Characterization of antimicrobial resistance is an indicator of antimicrobial consumption within the population and can be used to detect spread of resistance in bacteria in the environment and in animal populations. This could in turn reflect possible resistance in the human population and could be used to monitor emergence and spread of antimicrobial resistance and plan antibiotic treatment regimes (Anon, 2001).

The Kenya Environment Management and Co-ordination Act (EMCA) has set standards for effluent discharged into water bodies from industrial enterprises but there is no baseline data to indicate the quality of the wastewater being discharged into the environment.

Organic pollution in surface water can cause a serious problem due to increasing oxygen demand. When micro-organisms degrade organic material in water, they utilise the dissolved oxygen. When the dissolved oxygen is depleted at rates higher than can be replenished by absorption on water surface and photosynthesis, there is interference with aquatic life. The more the material for degradation, the higher the oxygen demand. Slaughterhouse and meatpacking wastewaters contain high proportion of blood as well as excreta, undigested food, grease and hairs. All these contribute to very high BOD in the receiving water bodies (Edwards *et al*, 1997). For example, blood from beef cattle has an average BOD_5 of 156,000 mg/l (Grady and Lim, 1980). The generally accepted value for the BOD_5 of beef cattle manure is approximately 27,000 mg/kg of excreted manure, (Anon, 2002).

This implies that untreated slaughterhouse effluent discharged into municipal sewerage would have a far higher BOD than most sewage treatment plants can handle. Most sewage treatment plants are designed to handle wastewater of domestic sewage quality hence they are likely to fail when such wastewater is discharged into them without prior treatment.

The polluting chemicals of most concern in fresh water are nitrates, nitrites and phosphates. Nitrates are mainly derived from wastewater from livestock enterprises including

slaughterhouses and meat packing plants, while phosphates are from agriculture, domestic effluent and industries. These chemicals are potentially very destructive by interfering with the aquatic ecosystem (Harper, 1991; Hellawel, 1988).

The Kenya Environmental Management and Coordination Act (EMCA) 1999(Anon, 1999a), has mandated the Standards and Enforcement Committee of the National Environment Management Authority (NEMA) to recommend minimum effluent quality standards. This Act stipulates that all effluent from any industrial undertaking should be discharged into the local authority sewerage system where that is available and that the effluent should be treated before being discharged into the sewerage system or the environment. It also stipulates that standards for the waste and the disposal methods should be prescribed.
The problem of wastewater treatment arises from the question of how to justify on economic grounds the treatment of wastewater including effluent. Though this may not be estimated in quantitative terms the gains from cleaner surface water, reduction in the risk of waterborne diseases and improved public health and usability of surface waters are real benefits even though they defy quantification. Even though water pollution from industrial effluent has been recognized in most developing countries and laws to prevent it have been passed, systematic measurement of pollution aimed at coordinating relevant information for remedial work has not been done (Anon, 1993).
An analysis of wastewater is necessary to assess the potential toxicity and disease hazard, toxicity to fish and other aquatic life and the trophic properties of sewage algae. This knowledge is necessary especially in the light of the growing need for reuse and conservation of water, to help decide how best to treat effluent further before reuse (der Hoek *et al*, 2001).

Most slaughterhouses in the country do not have any form of effluent pre-treatment before the effluent is discharged into the environment, mainly streams, which in most rural areas are used down stream for domestic purposes, irrigation and aquaculture. Those that have any pre-treatment use mainly soak pits. The impact of this on the surface and ground water has not been evaluated. There are a few slaughterhouses within the urban areas, which discharge their wastewater into the local authority sewerage systems. The effluent needs to be evaluated to determine if the pre-treatment meets the minimum required standards for discharge into the municipal sewerage system.

The main objective of the present study is to evaluate the microbiological and organic qualities of effluent from the Dagoretti slaughterhouses complex.

SPECIFIC OBJECTIVES

1. To evaluate the microbiological quality of slaughterhouse wastewater by use of total bacterial counts, coliform and faecal streptococci as indicator organisms
2. To examine the presence of faecal *Escherichia coli* and *Salmonella* in the slaughterhouse wastewater
3. To evaluate antibiotic resistance in isolated *E. coli*
4. To evaluate the level of organic matter contamination by determining the biochemical oxygen demand.

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 REVIEW ON SLAUGHTERHOUSES

Currently there are about 2500 licensed slaughtering points in Kenya. About 100 of these are modern or modernised while the rest are mainly small slaughter slabs or gantries (DVS 2004). Most of these lack basic facilities including adequate water. The average daily kill for these slabs is less than five animals in a week, most slaughtering only on market days (DVS 2004). Due to the unavailability of water, these slaughtering points use very little water and though they may not have any effluent treatment, their impact on environment in terms of water pollution may be minimal.

The larger slaughterhouses slaughter on average 5 to 20 animals per day (DVS 2004). These, for hygiene reasons, use large amounts of water. Some of them have effluent treatment plants which mainly consist of sedimentation and facultative ponds. The efficiency of these effluent treatment systems has however not been assessed. This class of slaughterhouses are the ones likely to cause water pollution.

2.2 CHARACTERISTICS OF WATER

2.2.1 BIOLOGICAL CHARACTERISTICS OF WATER

Natural and wastewater contain a large variety of living organisms. These include bacteria, viruses, and metazoa such as helminthes and protozoa. These organisms are either free-living or pathogenic. Pathogenic bacteria include *Salmonella* spp, *Shigella, Vibrio cholerae,* Enteropathogenic *Escherichia coli* and *Leptospira spp*. Enteroviruses such as polio and Coxsackie virus, adenovirus and rhinovirus. Protozoa include *Entamoeba histolytica, Giardia lamblia, Cryptosporidium spp* and *Balantidium coli.* These organisms have all been associated with drinking water outbreaks. (Craun, 1979a; Craun, 1979b). Helminthes

include *Schitosoma* spp and *Dracunculus medinensis.* (Anon, 1984). Contamination with these pathogens occurs from human and animal excrement, sewage and sewage effluent and washing from soil (Anon, 1987).

A number of diarrheal diseases are water washed and are transmitted through faecal-oral route or are waterborne. Although human faeces present the greatest health risk from these water-washed diseases, animals play an important role as reservoirs of these diseases. The most common and widespread danger associated with drinking water is contamination, either directly or indirectly by sewage, human and animal excrement and other waste. Should some of the contributors of the contamination be carriers of communicable or zoonotic enteric diseases, some of the living causal agents may be present. Such water if used for drinking or in preparation of certain foods may result in further cases of infection.

2.2.2 PHYSICAL CHARACTERISTICS OF WATER

The commonly evaluated characteristics of water are turbidity, colour, taste and odour. Turbidity is the ability of a medium to absorb or scatter light. Turbidity results from colloidal or suspended matter such as clay, silt, fine organics and inorganic matter, soluble coloured organic compound, planktons and other micro-organisms. Colour in water is due to soluble matter which could be natural metallic ions such as iron and manganese, humus, planktons, and weeds or agricultural and industrial waste. Taste and odour mainly result from dissolved organic or inorganic chemicals. These may originate from natural processes such as erosion or decomposition of vegetable matter by micro-organisms or from wastewater discharges (Anon, 1989).

2.2.3 CHEMICAL CHARACTERISTICS

The commonly measured chemical parameters include pH, hardness, dissolved oxygen, nutrients and biochemical oxygen demand. The main cause of alkalinity is the presence of carbonates and bicarbonates of calcium, magnesium, potassium and sodium. Acidity is the quantitative capacity to react with a base at designated pH levels (Anon, 1989) in water. It results from free carbon dioxide while excessive acidity results from industrial pollution. Hardness results from a concentration of multivalent cations especially Ca^{2+} and Mg^{2+} (Peary *et al*, 1985) and nutrients mainly nitrogen and phosphorous from natural deposits, domestic, agricultural and industrial waste.

2.2.4 ORGANICS

Organic matter in water consists of decaying plant and animal tissues and microorganisms. They get into water through natural sources and from pollution. Organic matter is susceptible to biological degradation in bodies of animals or by bacteria. When oxidized in water oxygen is taken up from water thus leading to depletion of dissolved oxygen. If water of a high oxygen demand flows into a river, the dissolved oxygen is consumed faster than it can be dissolved back from the air or re-aerated through photosynthesis. This leads to death of fish and other aquatic animals (Hach *et al,* 1997).

The concentration of organic waste in wastewater is determined by the Biochemical oxygen demand, the Chemical oxygen demand (COD), which may or may not be similar to the BOD, the total suspended solids (TSS), nitrogen, often referred to as Total Kjeldahl Nitrogen (TKN), and phosphorus (Anon, 2002).

2.3 WATER POLLUTION

Water pollution is any change in water quality, natural or induced which renders it unacceptable for its intended use, reduces its usability, or renders it hazardous to health. The change may be chemical, physical or biological (Anon, 1987).

Studies carried out around Nairobi indicate high levels of faecal contamination. There seems to be a gradual increase in the levels of contamination over the years. Whereas Muhammed (1971) indicated that borehole water did not have any indication of faecal coliform, Khalid (1993) reported that 70% of boreholes showed faecal coliform. Githui-Kaba (1990) and Simiyu (1997) also reported increasing contamination. The rivers in Nairobi are polluted by domestic and industrial waste (Odundo, 1994). A survey done by the Nairobi City Council (Anon, 1997) found out that effluent from the Dagoretti slaughter complex was a major source of river pollution.

2.4. WASTEWATER TREATMENT

Some reports have indicated that improved water quality and sanitation facilities are not cost effective or efficacious in improving health (Walsh and Warren, 1979), but 67 studies from 28 countries found out that investment in water and sanitation reduced diarrhea morbidity and mortality by 21% and 22% respectively (Esrey *et al*, 1985). Less than 2% of cities in the world have treatment plants, and without proper treatment and dilution, untreated wastewater represents a health hazard (Anon, 1993).

In order to slow down the rapid deterioration of water quality and enhance availability of safe water, all countries should have in place water pollution control programmes based upon enforceable standards for major point-source discharges as well as major non-point sources of pollution (Anon, 1992). To prevent pollution of surface and ground waters,

stringent water quality standards are necessary and water reclamation for agriculture is highly desirable (Lohani and Thanh, 1978).

Stabilization ponds are the simplest form of wastewater treatment. Anaerobic lagoons have been one of the most extensively used systems for treating slaughterhouse wastewater in the USA and Australia, where climatic conditions and land availability permit the construction of large lagoons(Johns 1995; Rollag and Dornbuh 1966). Low capital, operational, and maintenance costs combined with a high in reducing polluting charges have all contributed to the popularity of lagoons. The disadvantages of lagoons include the large area requirement, odor problems, and the emission of methane, one of the major contributors to greenhouse gas, with a heat-trapping capacity 20 to 30 times that of carbon dioxide (Massé and Masse 2000). Although they are land intensive, they are the most effective way of removing pathogens especially for effluent used for irrigation, aquaculture or aquifer recharge (Anon, 1987).

The basic stages of wastewater treatment are: screening to remove coarse solids, grit separation to remove the dense solids, primary separation for removal of fine suspended material, biological oxidation of organic matter and the final sedimentation to remove residual suspended material before the treated water is discharged into surface water (Anon, 1993).

2.5 INDICATORS OF WATER POLLUTION

2.5.1. BIOLOGICAL INDICATORS

Water, because of its universal usage as the common carrier of waste materials, is an excellent vehicle for enteric disease causing agents. However, even though pathogenic organisms have been recovered from water using elaborate methods (Fair and Morrison,

1967) direct detection of many pathogens is time consuming and enumerations are often complex. Organisms whose presence in water reflects faecal contamination are instead used as the criterion of potability of water (Anon, 1999). Though the detection of such organisms in water does not necessarily indicate that enteric pathogens are present, the disease causing potential of the water is recognized. The indicator organism should be always present in faeces of man and animals and in large amounts. The organisms should be easily enumerated, identified, and genetically stable. The organisms should be more resistant to disinfectants and to aqueous environments than the pathogens and grow readily on relatively simple media (Anon, 1983)

2.5.1.1. COLIFORM GROUP

Coliform have for a long time been recognized as suitable microbial indicators of drinking water quality, because these micro-organisms are easy to detect and enumerate in water (Anon, 1984). This group includes organisms that vary in biochemical and serological characteristics and in their natural sources and habitat. The use of coliform group as indicators of faecal pollution has been limited by the existence of non-faecal bacteria that fit the definition of coliform (Geldreich *et al* 1978, Geldreich, 1978). Recent studies have shown that coliform differ significantly in ecology, prevalence and resistance to environmental stress from many of the pathogens which they are proxy for (Crichton and Old, 1979, Scott *et al*, 2002). Gordon and McFeters (1990) suggested the use of anaerobes or their phages, primarily due to the ability to survive outside the intestinal tract.

2.5.1.2 FAECAL COLIFORM

Faecal coliform are characterized by their ability to ferment lactose at 44 to 45 °C. Apart from the *E. coli* other group members are those belonging to genera *Citrobacter, Enterobacter* and *Klebsiella*. Some coliforms such as *Klebsiella* and *Enterobacter* species have been found to occur in decomposing matter and water soaked wood where there is no known faecal contamination (Anon, 1985). The use of faecal coliform apart from *E. coli* in assessing the water quality is acceptable and methods of detecting them are relatively simple and widely available (Anon, 1999c). Faecal coliform have been used for a long time to assess the presence of faecal contamination in water (Anon, 1985). Some studies have questioned the suitability of faecal coliform as indicators of faecal contamination (LeClerc *et al*, 2001, Edberg *et al,* 2000, Goyal, 1983). Some of the reasons advanced are the fact that other bacteria of non faecal origin meet the definition of faecal coliform (LeClerc *et al* 2001).

2.5.1.3 ESCHERICHIA COLI

Although other coliform may be of faecal origin, the primary habitat of *E. coli* is the gastrointestinal tract of mammals and birds. However *E. coli* is exclusively of faecal origin (Anon, 1985). This has made it an important indicator organism of faecal contamination of water. Also recent studies have shown a correlation between levels of faecal coliform with *E. coli* levels (Doòan-Halkman *et al* 2003). These studies may indicate faecal *E. coli* as a better indicator of faecal pollution than faecal coliform. However some studies have indicated that *E. coli* dies off faster than *Salmonella* spp and hence may not be a suitable indicator (Burton *et al*, 1987, Temple *et al*, 1980). Other studies have indicated that *E. coli* survives longer than *Salmonella* especially on sediments and may be re-suspended in

overlying waters (Burton *et al,* 1987, LaBelle *et al* 1980,). Studies have shown that the rate of gastrointestinal illnesses among swimmers was better correlated with *E. coli* concentrations in the water at the time of exposure than to faecal coliform concentrations (USEPA, 2002).

Presence and levels of *E. coli* may be used to supplement faecal coliform and other indicator tests in determination of presence of faecal contamination and how recent it may be. Also better model organism(s) may be required to monitor water quality.

2.5.1.4 FAECAL STREPTOCOCCI

The organisms in this group originate from the intestines of various animals and man, and belong to the genera *Streptococcus* and *Enterococcus*. They are regarded as specific indicators of faecal pollution of water. Faecal streptococci have been used as indicators of faecal pollution in assessment of water quality for a long time. Some studies have shown that faecal streptococci survive longer than coliform in water and as such more closely parallel the survival of viruses than faecal coliform (Dufour, 1984, Cabbielli, 1980 cited in Harwood *et al*, 2000). They also have greater resistance to unfavourable environmental conditions than faecal coliform (El Zanfaly, 1991). They have been shown to have a better predictive value of presence of pathogenic micro-organisms than coliform (Collin *et al*, 1988). Other workers have not found faecal streptococci to survive longer than faecal coliform (McFeters *et al*, 1974 as cited in Sinton *et al*, 1993). They can and have been used as supplementary material indicator in doubtful coliform results.

Several species in the faecal streptococci group are found in low numbers in faeces but are not adapted to persist in the digestive tract thus may not be considered faecal. Also other bacterial species such as *Pediococcus* and *Leuconostoc* grow on the conventional media

used to assess the presence of faecal streptococci (LeClerc *et al*, 1996). This reduces the predictive value of faecal streptococci in determining faecal pollution.

The ratio of faecal coliform to faecal streptococci may be useful in locating the origin of the faecal pollution in heavily contaminated sources of raw water provided sufficient data are collected (Anon, 1989). A ratio less than 0.7 suggest contamination from animal source while a ratio of greater than 4 (four) indicates human faecal contamination (Edwards *et al*, 1997).

2.5.1.5 CLOSTRIDIUM PERFRINGENS

The use of *C. perfrigens* as an indicator of faecal pollution was first suggested by Klein and Houston (1899). *Clostridium perfringens* is normally present in faeces of man and animals but occur in smaller numbers than *E. coli* (Fujioka and Shizumura, 1985). However *C. perfringens* survives longer than either coliform bacteria or streptococci and resist disinfection hence a good indicator of remote pollution (Edberg *et al*, 1997). However, it is rarely used since the spores can survive in water after all the other contaminants have been eliminated.

2.6.2 OTHER INDICATORS

2.6.2.1 BIOCHEMICAL OXYGEN DEMAND

Biochemical Oxygen Demand (BOD) is the amount of oxygen, expressed in mg/l that bacteria will take from water when they oxidize organic matter. It is determined by comparing the amount of dissolved oxygen in a sample at the end of a specified period with the amount known to be present at the beginning. The standard oxidation (or incubation) test period for BOD is 5 days at 20 degrees Celsius (BOD_5). The BOD_5 value has been used and

reported for many applications, most commonly to indicate the effects of sewage and other organic wastes on dissolved oxygen in surface waters. The 5-day value, however, represents only a portion of the total biochemical oxygen demand. Twenty days is considered, by convention, adequate time for a complete biochemical oxidation of organic matter in a water sample, but a 20-day test often is impractical when data are needed to address an immediate concern (Delzer and MacKenzie, 2003).

The BOD_5 test has limited value by itself in the assessment of stream pollution and does not provide all of the relevant information to satisfy every study objective. Additional analyses of water samples for chemical oxygen demand, faecal bacteria, and nutrients can aid in the interpretation of BOD_5 (Nemerow, 1974, Stamer *et al*, 1983, Hach *et al*, 1997). BOD is an index of the biodegradable organic material present in wastewater (Anon, 1989). BOD is exerted by three classes of material, organic material utilizable as a source of nutrients by aerobic organisms exerts a carbonaceous demand; nitrogenous oxygen demand is from oxidisable nitrogen compounds which serve as food for specific micro-organisms, and demand from oxidation of inorganic material such as ferrous iron and sulphides (Anon, 1989). The extent of oxidation of nitrogenous compounds depends on the presence of micro-organisms capable of carrying out this oxidation.

BOD test determines only the organic load readily utilised by microorganisms. Complete oxidation of organic material may require a period of incubation too long for practical purposes hence a 5-day period has been accepted as a standard. The test is of limited value in measuring the actual oxygen demand of surface water and extrapolation of the results to actual stream demand is questionable since the laboratory environment does not reproduce the stream conditions especially relating to temperature, sunlight, biological population,

water movement and oxygen concentration. (Velz and Gannon, 1963 as cited by Leclerc, *et al* (2002)

2.7 STANDARD MICROBIOLOGICAL TECHNIQUES FOR TESTING WATER FOR MICROBIAL QUALITY

2.7.1 TOTAL VIABLE COUNT POUR PLATE METHOD

The Total Viable Count (TVC) pour plate procedure is a quantitative estimate of the level of microorganisms present in the test sample. It is intended to indicate the level of microorganisms in a product. This involves counting the colonies produced by viable cells under favourable growth conditions. In pour-plate method, an aliquot of suitably diluted sample is mixed with nutrient agar at a temperature where it is liquid (Anon. 1984a). Plate count agar or nutrient agar is commonly used.

After incubation, the number of colony forming units (cfu) on each TVC pour plate are counted either manually or with the aid of a colony counter. The average the count from the duplicate dilution plates, then multiply by the dilution factor (inverse of dilution) to obtain the final cfu/g or ml in the test sample. Only plates with between 30 and 300 colonies are counted. Counts outside the normal 30-300 range may give erroneous indications of the actual bacterial composition of the sample. Dilution factors may exaggerate low counts (less than 30), and crowded plates (greater than 300) may be difficult to count or may inhibit the growth of some bacteria, resulting in a low count (Anon, 1990)

2.7.2 MULTIPLE TUBE TECHNIQUE (MTT)

The technique utilizes the ability of coliform to ferment lactose with the production of acid and gas. The MTT has been used for the enumeration of total coliform for over 70 years as an indicator of water quality. The technique is still in use today in many countries for monitoring water supplies and food quality control (Evans *et al*, 1981). MTT involves adding measured volumes of water samples to sets of sterile tubes containing appropriate media and incubation at 37^{0} C for 24 – 48 hours. Positive reactions are confirmed by change of colour of the media and production of gas detected by its appearance in the Durham tube inserted in every tube. The test is called presumptive test because the reaction observed may be occasionally due to the presence of other organisms or combination of organisms (Anon, 1999). An estimate of the Most Probable Number (MPN) of microorganisms present in the original sample is obtained by using McCrady's statistical tables (McCrady, 1915). Hossong *et al* (1981) reported false positive reactions, which are thought to be dependent on the bacterial flora of water and medium used. He concluded that no single specific bacterial group could be identified as being responsible for the false positive reaction of the presumptive coliform test. False negative reactions when using standard most probable number (S_MPN) technique has also been reported (Evans *et al*, 1981). But using a modified most probable number (M_MPN) method, they managed to recover different species of coliform from the false negative tests including *Citrobacter, Enterobacter, Klebsiella and Escherichia.* Even with the problems experienced the technique is still in use until more sensitive and effective methods are put in place.

2.7.3 MEMBRANE FILTRATION TECHNIQUE

Membrane filters were first described by Zsigmondy and Bachman (1918). They are made of cellulose esters. Membrane filtration technique involves filtering a measured volume of water sample through a membrane, which has 0.45μm pore sizes. Usually 100ml.of water sample are used. Microorganisms are retained on the filter surface, which is then incubated face upwards on a suitable selective medium containing lactose. Visible colonies are counted and expressed in terms of numbers per 100mls of sample (Anon, 1985). Since it was first introduced as a tentative method for coliform enumeration, it has gained wide usage for not only total coliform but also for faecal coliform, total bacterial count and a variety of other bacterial tests. The technique has an advantage over other methods as it has the ability to concentrate and localize the bacteria from large sample volumes, thus increasing sensitivity of quantitative bacteriology. Kabler (1954) compared membrane filtration (MF) with most probable number (MPN) in recovery of coliform from water and found 73.8% agreement between the two (Anon, 1999c) while Presnell *et al* (1954) found an 87.1% agreement in detection of coliform in sea water. However Jannasch and Jones (1959) indicated that MPN gave counts twenty times more than MF (Eilers *et al*, 2002)

2.8 ANTIBIOTIC RESISTANCE

Emergence of antimicrobial resistance is of great concern. When resistance develops, a previously effective therapeutic approach may be no longer successful. Waterborne outbreaks of enteric pathogens carrying the resistant factors have led to a number of deaths due to failure of adequate response to antibiotic treatment (Baine *et al,* 1977). Resistance is the ability to survive exposure to an antimicrobial agent. It could be due to the inactivation of drugs by enzymes produced by the organism, altered receptor site or enzyme specificity

or change to an alternative metabolic pathway by the organism. Resistance may be natural or acquired. Natural resistance is an intrinsic property in an organism that confers resistance. Acquired resistance is obtained by an organism, by one mechanism or another. It is acquired by either selection of resistant clones, chromosomal mutation, phage transduction or resistant factor acquisition by conjugation. Bacteria have two types of genetic structures that may confer resistance namely chromosomes and plasmids. Chromosomal resistance depends on the mutation in the bacterial gene leading to resistance to a particular antimicrobial. The antimicrobial acts as a selective agent that allows resistant mutants to emerge, their genesis being dependent on the presence of the agent. Mutated bacteria usually disappear with time in the absence of the antimicrobial (Anon, 2003; WHO, 2003).

There is great concern caused by the presence of antibiotic resistance in members of the Enterobacteriaciae family in the aquatic environment. This has resulted from the extensive use of antibiotics in medicine and agriculture (Armstrong *et al*, 1981, 1982). The use of antimicrobial agents in any environment creates selection pressure that favours the survival of antibiotic resistant organisms. Such organisms are becoming prevalent worldwide (Anon 2000). Increasing antimicrobial resistance presents a major threat to public health since it reduces the efficacy of antimicrobials leading to increased morbidity, mortality and health care expenditure (Coast *et al*, 1996)

Increasing levels of faecal contamination in surface water and the relative ease with which bacteria become resistant is likely to cause a serious public health hazard. In an environment polluted by human and/or animal waste, high frequency of multiple antibiotic resistance is found in coliforms and faecal coliform (Alcaide and Garay, 1984). Presence of organisms with resistance factors in sewage and water and their ability to transfer this resistance to sensitive populations either during conventional sewage treatment or in surface waters has

been a subject of a number of studies and calls for a re-evaluation of water quality standards (Bell *et al,* 1980, Aitherr and Kaswerk, 1982). Significant levels of resistance transfer occur in wastewater treatment plants in the absence of antibiotics to act as selective agents (Mach and Grimes, 1982).

2.9. SALMONELLA

Bacteria in the genus *Salmonella* are of rod-shaped gram-negative enterobacteria that cause typhoid fever, paratyphoid fever and foodborne illness they motile in nature and produce hydrogen sulfide. They are usually isolated on MacConkey agar, Xylose Lysine Desoxycholate agar or Desoxycholate Citrate agar. *Salmonella typhi* was first isolated from human source by Eberth (1880). *Salmonella* was named after Daniel Elmer Salmon, who, together with Theobald Smith first discovered the *Salmonella* bacterium in 1885 from pigs (Salmon and Smith, 1885 as cited by McLaughlin *et al* (2006). There are over 2500 serovars of S*almonella*, which are found in a variety of environments and which are associated with many different diseases (Ryan and Ray, 2004).

Wastewater, especially from slaughterhouses, presents a potential hazard to farm animals and man with respect to salmonellosis. In 32 abattoirs studied by Dixon (1964), as cited by Kayihura, (1982) *Salmonella* was isolated in 930 (21%) of the 4496 swabs of abattoir drains. Variation between abattoirs was noted with the organism being found most frequently in abattoirs slaughtering high numbers of cattle and low proportions of sheep. *Salmonella typhimurium* was the most isolated serotype. Meara (1973) isolated *Salmonella* in 21% of drain swabs in abattoirs. Of 107 samples from abattoir effluent in Kenya, only 2 (1.9%) were positive for *Salmonella* (Kayihura, 1982). Kariuki *et al* (2002) found 4 (1.7%) of 267 samples from slaughterhouse effluent and beef carcasses positive for *Salmonella.*

Muhammed and Morrison (1975) isolated *Salmonella* in one (2.4%) out of 42 sites along the Nairobi River.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 AREA OF STUDY

The Dagoretti slaughter complex (GPS 36.68, -1.28) shown in figure 1 was selected for the study. The Dagoretti complex has four slaughterhouses, namely Thiani, Mumu, Nyongara and Co-operative slaughterhouses. Nyongara slaughterhouse was randomly selected for collection of tap water. Water used in the slaughterhouses is from the City Council. There is no treatment done before the effluent is discharged into The Kabuthi River. The effluent from the four slaughterhouses joins up before it reaches the river.

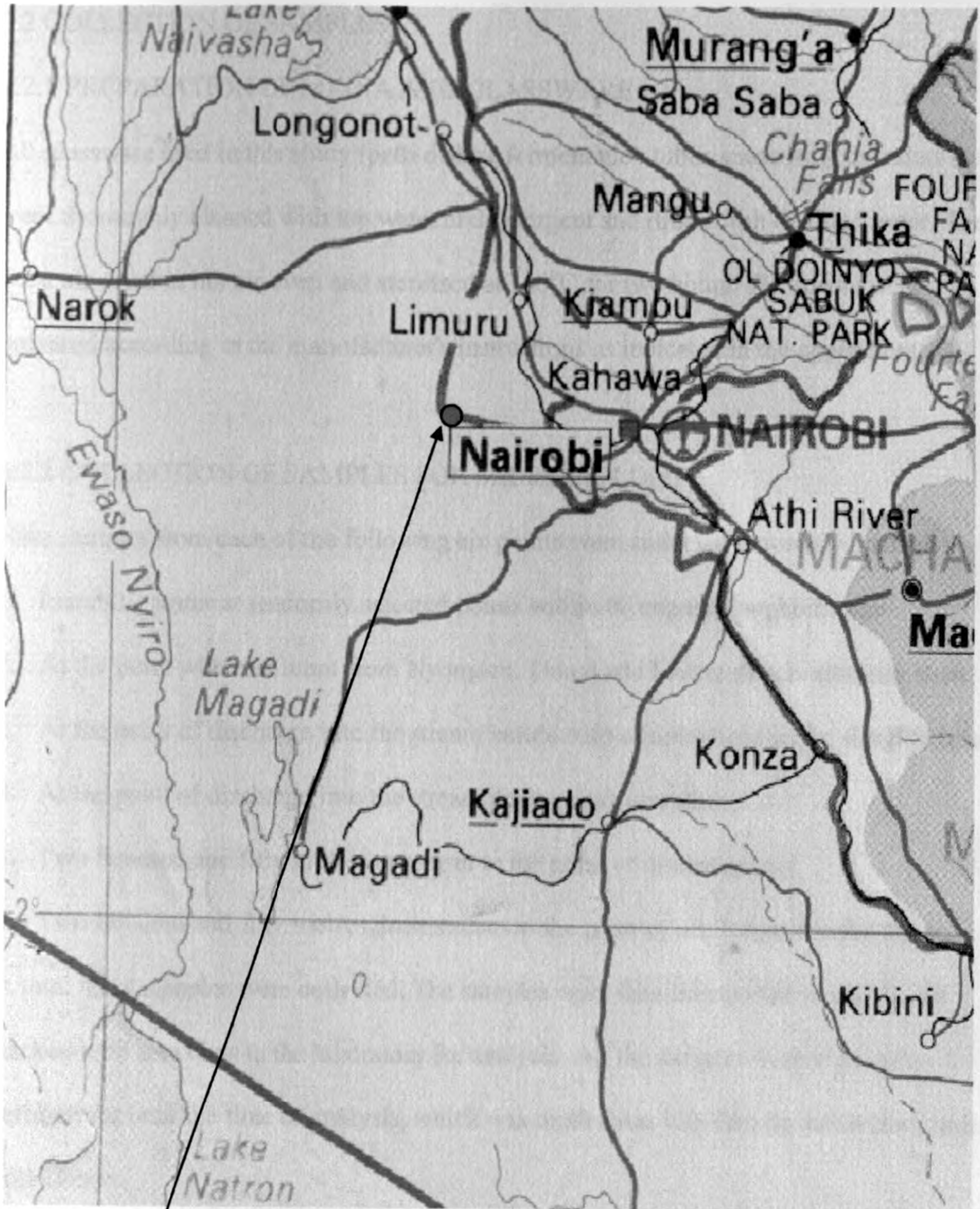

Figure 1 Map showing the location of the slaughterhouses

3.2 COLLECTION OF SAMPLES

3.2.1 PREPARATION OF MEDIA AND GLASSWARE

All glassware used in this study (petri dishes, fermentation tubes, measuring cylinders etc) were thoroughly cleaned with tap water and detergent and rinsed with distilled water. They were air-dried in hot air oven and sterilised at 160°C for two hours. The media were prepared according to the manufacturer's instructions as indicated in the appendices 1.1-1.16

3.2.2 COLLECTION OF SAMPLES FOR MICROBIOLOGY

Nine samples from each of the following six points were collected at weekly intervals:

1. Incoming water at randomly selected points within Nyongara slaughterhouse
2. At the point where effluent from Nyongara, Thiani and Mumu slaughterhouses meet;
3. At the point of discharge into the stream before start of operations in the slaughterhouses
4. At the point of discharge into the stream during peak operation;
5. Two hundred and fifty metres upstream to the point of discharge and
6. Two hundred and fifty metres downstream to the point of discharge into the stream.

A total of 54 samples were collected. The samples were then transported in cool boxes packed with ice cubes to the laboratory for analysis. All the samples were kept in the refrigerator until the time of analysis, which was in all cases less than six hours from time of collection.

3.2.3 COLLECTION OF SAMPLES FOR BIOCHEMICAL OXYGEN DEMAND

Samples were collected weekly for nine weeks at the following four points:

1. Two hundred and fifty metres up-stream to the point of discharge into the stream;
2. At the point of discharge

3. Two hundred and fifty metres downstream; at the point of discharge,
4. At the point where discharge from the three slaughterhouses meet.

These samples were collected in amber coloured bottles and transported in a cool box packed with ice cubes to the laboratory. Analysis commenced immediately.

3.3 DETECTION OF INDICATOR ORGANISMS

3.3.1 TOTAL BACTERIAL COUNT BY POUR PLATE METHOD (Anon 1999c)

Dehydrated standard plate count agar (PCA) (Oxoid) was prepared as per the manufacturer's instructions (Appendix 1.1). The sample collection bottles were shaken and after sterilising the mouth of the sample bottle, one-millilitre portion withdrawn with a sterile pipette to the dilution tubes. Decimal dilutions of the sample were made using sterile physiological saline. One ml of various dilutions was placed in petri dishes and about 15 ml of molten plate count agar medium at a temperature of about 45°C poured to the petri dishes. The agar and the sample were thoroughly mixed by rotating the plate in a figure of eight to uniformly spread the sample over the bottom of the petri dish. The agar was allowed to solidify and the plates were then incubated at 30°C for 24 and 48 hours in an inverted position. Colonies were counted using a colony counter in plates with between 30 to 300 colonies. The mean of each sample was multiplied by the dilution factor and recorded as the total bacterial count per millilitre of water on PCA at 37°C for 48 hours

3.3.2 TOTAL COLIFORM COUNTS USING MULTIPLE TUBE TECHNIQUE

A set of five fermentation tubes all provided with Durham tubes with 10ml of double strength MacConkey broth (Oxoid) were prepared as per the manufacturer's instructions (Appendix 1.2) was sterilised. Ten millilitres of diluted sample was transferred into the

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fermentation tubes. Separately, 1, and 0.1 millilitres of a thoroughly mixed diluted sample were transferred to each of a series of 5 fermentation tubes containing 10 ml single strength MacConkey broth. These were incubated at 37^0C for 24 to 48 hours and observed for acid and gas production. The most probable number of coliform per 100ml of sample was determined from the McGrady's probability tables.

3.3.3 CONFIRMATORY TEST FOR COLIFORM

A loopful from a positive presumptive tube was transferred into a plate containing Eosin Methylene Blue (EMB) agar (Oxoid) (Appendix 1.3) and incubated at 37^0C for 48 hours. Typical coliform colonies indicated presence of coliform, while metallic green colonies were indicative for *Escherichia coli.*

3.3.4. BIOCHEMICAL TESTS FOR COLIFORM

Several different colonies on Eosin Methylene Blue Agar (EMBA) were diluted in 0.2 ml sterile distilled water and each colony subjected to tests to check for indole production, Methyl Red test, Voges Proskeur test and utilisation of citrate as the sole source of carbon, the IMViC tests.

3.3.4.1 TEST FOR INDOLE PRODUCTION

A loopful of the diluted colony was inoculated into 5 ml of Peptone Water (Oxoid) prepared as per the manufacturer's instructions (Appendix 1.4). This was incubated at 37^0C for 24 hours. Indole solution was then added into the 24-hour culture. Formation of a red band at the interface indicated an indole positive reaction.

3.3.4.2. METHYL RED AND VOGES-PROSKEUR TEST

Methyl red and Voges-Proskauer (MR-VP) medium (Oxoid) was prepared as per the manufacturer's instruction (Appendix 1.5). A loopful from the diluted colony was inoculated in two tubes of the medium and incubated at 37^0C for 24 hours. Four drops of methyl red solution were put in one tube while in the other four drops of 40 % potassium hydroxide and two drops of 1% creatine were put, and left to stand for several minutes. For the methyl red test formation of a red ring at the interface was recorded as a positive reaction. For the VP test change of colour to red or brown was considered positive.

3.3.4.3. CITRATE TEST

A loopful from the diluted colony was inoculated in Citrate Agar (Oxoid) slant prepared as per manufacturer's instructions (appendix 1.6). This was incubated at 37^oCfor 24 hours and colour change to blue was recorded as positive.

3.3.5. FAECAL *E. COLI*

One metallic green colony also showing typical *E. coli* IMViC reaction was transferred in two tubes of McConkey broth and incubated at 37^0C and 44.5^0C for 24 hours. Production of gas at both temperatures indicated presence of faecal *Escherichia coli*. Positive cultures were sub-cultured in Nutrient agar (Oxoid) (Appendix 1.7) for further tests.

3.3.6 ANTIBIOTIC SUSEPTIBILITY TESTING FOR FAECAL *E. COLI*

Using an inoculating loop a portion of the growth on nutrient medium was transferred to a tube with physiological saline to make a suspension. All the colonies cultures were about the same age. A sterile cotton swab was dipped into the suspension and excess liquid removed

by pressing the swab firmly against the inside wall of the tube above the level of the suspension. The swab was used to streak uniformly over the entire surface of a plate with Mueller-Hinton agar medium. Antimicrobial disks were placed onto the plates using sterile forceps and gently pressed down onto the agar. The antibiotic disks used were ampicillin (10μg), chloramphenicol (30μg), streptomycin (10 μg), sulphamethoxazole (100μg), nalidixic acid (30μg), trimethoprim (5μg), tetracycline (30μg) ciprofloxacin (5μg), gentamicin (10μg), cefuroxime sodium (30μg), ceftazidime (30μg), and amoxycillin/clavulanic acid (30μg) (AB Biodisk, Sweden). National Committee on Clinical Laboratory Standards (NCCLS) Quality Control Strain *E. coli* ATCC 25922 was used as control

The plates were incubated in an inverted position for 24 hours at $37^{O}C$. After the incubation, the diameter of the inhibition zone was measured and recorded.

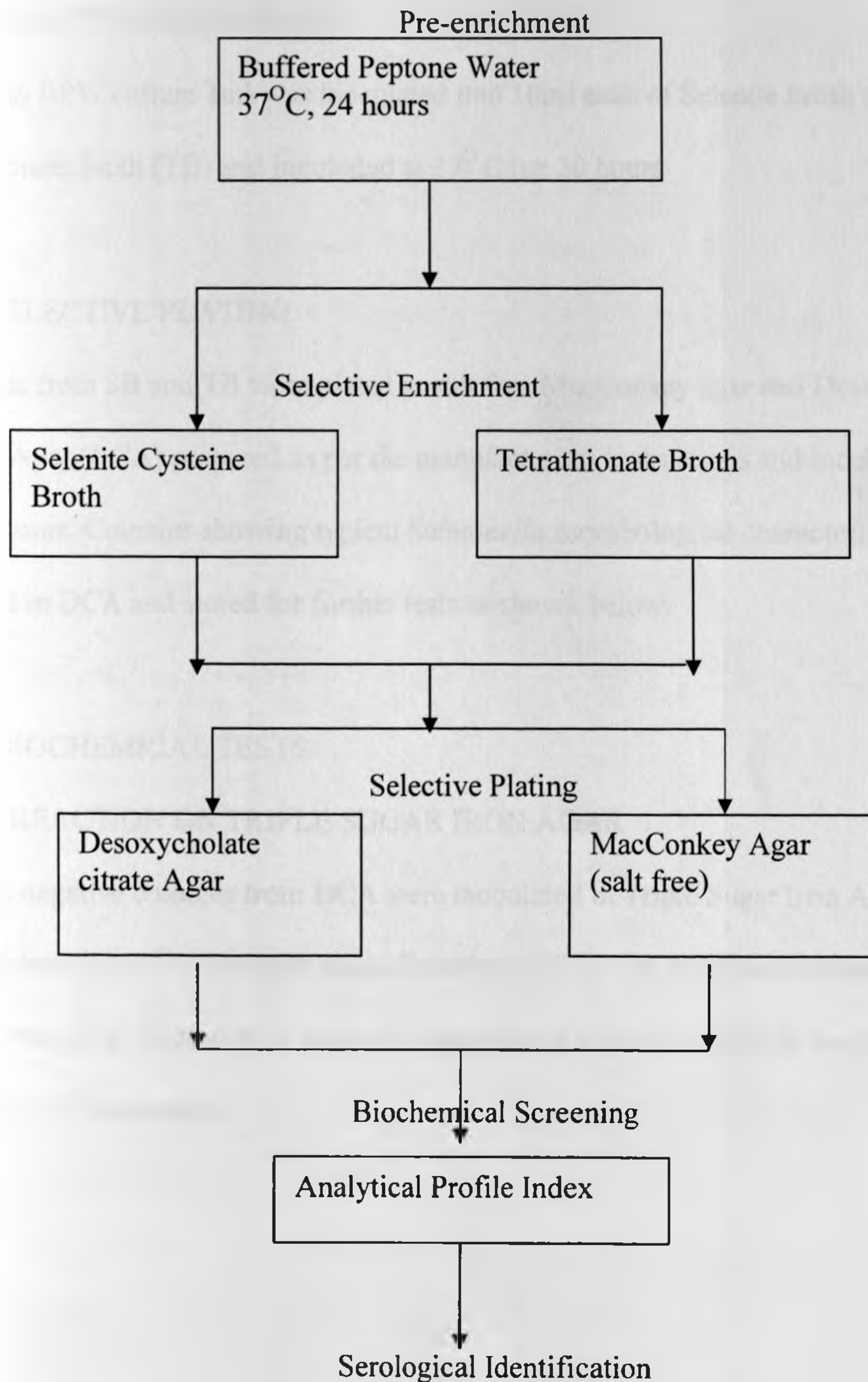
3.3.7. ENUMERATION OF FAECAL STREPTOCOCCI USING THE MEMBRANE FILTRATION TECHNIQUE

One hundred millilitres of diluted sample was filtered through a Sartorious membrane filter of 0.45μm pore diameter and the filter placed aseptically on a petri dish containing Slanetz and Bartley agar (Oxoid) prepared as per the manufacturer's instructions (Appendix 1.9). This was incubated at 37°C for 4 hours and then at 44°C for 24 hours. Pinpoint maroon colonies indicated the presence of faecal streptococci. Number of colonies were counted, multiplied by the dilution factor and recorded as faecal streptococci per 100ml of sample.

3.4 ISOLATION AND IDENTIFICATION OF *SALMONELLA* SPP

A flow chart of isolation and identification of *Salmonella* is shown in fig 2. (Vanderzant, C. and Splittstoesser, D. F. (1992)

Figure 2 Flow Chart for Isolation and Identification of *Salmonella* spp



3.4.1 PRE-ENRICHMENT

Ten millilitres of sample was inoculated in 90 ml buffered peptone water (BPW) prepared according to manufacturer's instructions and incubated at 37^0C for 18-24 hours

3.4.2 SELECTIVE ENRICHMENT

From the BPW culture 1ml was inoculated into 10ml each of Selenite broth (SB) and tetrathionate broth (TB) and incubated at 37° C for 20 hours

3.4.3 SELECTIVE PLATING

Loopfuls from SB and TB were plated in salt free MacConkey agar and Desoxycholate Citrate Agar (DCA) prepared as per the manufacturer's instructions and incubated at 37 ^{O} C for 24 hours. Colonies showing typical *Salmonella* morphological characteristics were sub-cultured in DCA and stored for further tests as shown below.

3.4.4. BIOCHEMICAL TESTS

3.4.4.1 REACTION ON TRIPLE SUGAR IRON AGAR

Lactose negative colonies from DCA were inoculated in Triple Sugar Iron Agar (TSI) slant and incubated at 37^OC for eight hours. Reaction on TSI was noted and cultures showing a yellow butt, pink slant with or without production of hydrogen sulphide were taken to be indicative of *Salmonella*.

3.4.4.2 UREASE TEST

Cultures from TSI showing typical salmonella reactions were inoculated in Urea agar (UA) and incubated at 37°C for eight to 24 hours. Urease negative cultures were subjected to further biochemical tests to identify *Salmonella*.

3.4.4.3 IDENTIFICATION USING ANALYTICAL PROFILE INDEX (API) 20E STRIPS

3.4.4.3.1 PREPARATION OF STRIP

Bacterial suspensions of urease negative cultures were prepared by emulsifying distinct colonies in sterile distilled water. Five ml of sterile distilled water was placed in the honeycombed wells of the API tray to create a humid environment and the API 20E strip (bioMérieux, Marcy-I'Etoile, France) placed in the tray. The tubes of the strip were filled with the bacterial suspension. For the citrate, Voges-Proskeur (VP) and gelatine tests, both the tubes and the cupules were filled. Anaerobiosis was created in the tubes with arginine, lysine, ornithine, Urea and H_2S by overlaying with mineral oil. The lid was placed and the strips incubated at 37°C for 24 hours.

3.4.4.3.2 TEST READING

The tests were read as per the API 20E interpretation table. For the VP test one drop each of 40% KOH, (API reagent 7042) and 6% a-naphthol (API reagent 7043) was added and a pink or red colour within 10 minutes was taken as positive. For the tryptophan test one drop of 10% ferric chloride. (API reagent 7040) was added and change of colour to dark brown was taken as positive. For the indole test one drop of indole reagent was added and formation of a red ring was taken as positive. Organisms were identified from the

identification table in Bergey's Manual. Cultures with the typical salmonella profile were stored serological identification.

3.4.5. SEROTYPING

Using an inoculating loop, a portion of a colony from nutrient agar medium was picked and emulsified in a drop of distilled water on a glass slide. A small drop of O antiserum was added to the suspension and a second suspension in which no antiserum was added prepared as a control to check for auto-agglutination. Any agglutination on the test suspension was recorded as positive.

3.5 BIOCHEMICAL OXYGEN DEMAND (BOD) ANALYSIS

The OXITOP^{R} BOD bottles were thoroughly cleaned with detergent, rinsed with distilled water and flushed with dilute hydrochloric acid to remove any organic matter. The required volume of homogenized sample was taken with the aid of a measuring flask, by means of a funnel, the sample was transferred into the BOD bottle and a magnetic stirrer bar inserted into the bottle. Two sodium hydroxide pellets were placed in the rubber sleeve and the rubber sleeve inserted onto the bottle. The OxiTop® measuring head was tightly screwed on and measurement on the OxiTop® head started. The BOD bottle was placed in the incubator for five days at 20°C and the results read after five days. The results obtained were multiplied by the relevant multiplication factor as indicated on the BOD machine to obtain the actual BOD.

3.6 DATA ANALYSIS

All data were entered using Microsoft® Excel® and analysed using Genstat®

CHAPTER 4

4.0 RESULTS

4.1 OVERVIEW OF THE SLAUGHTERHOUSES COMPLEX AND THE STUDY SITES

Figure 3 shows an elevated view of the Dagoretti slaughterhouses complex; figure 4 shows the sites of sample collection while figure 5 shows other possible sources of pollution along the flow of effluent to the river

Figure 3. An elevated View of Dagoretti slaughterhouses
An elevated view of part of the Dagoretti slaughterhouses complex showing the Mumu and Thiani slaughterhouses

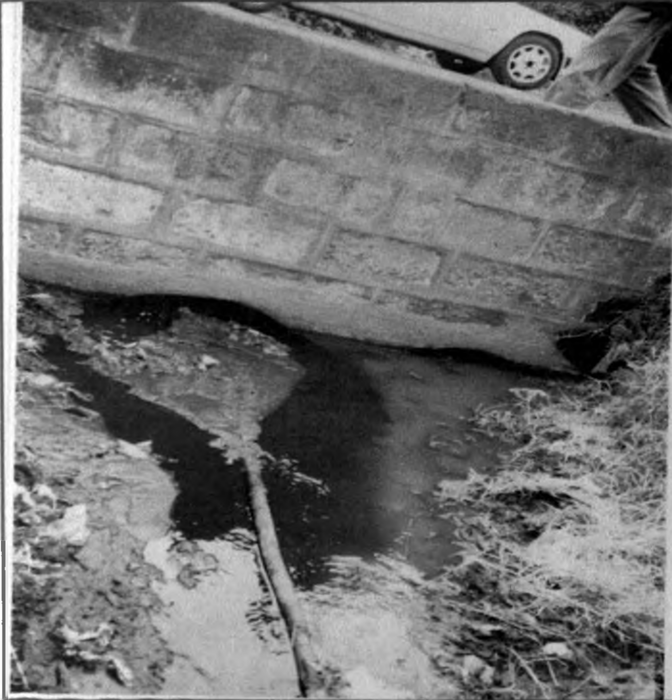
Site 1

Sample collection site 1: 250 metres upstream from the point of effluent discharge into the river showing clear water

Site 2

Sample collection site 2: 250m downstream to the point of effluent discharge into the river showing bloody water.

Site 3/6



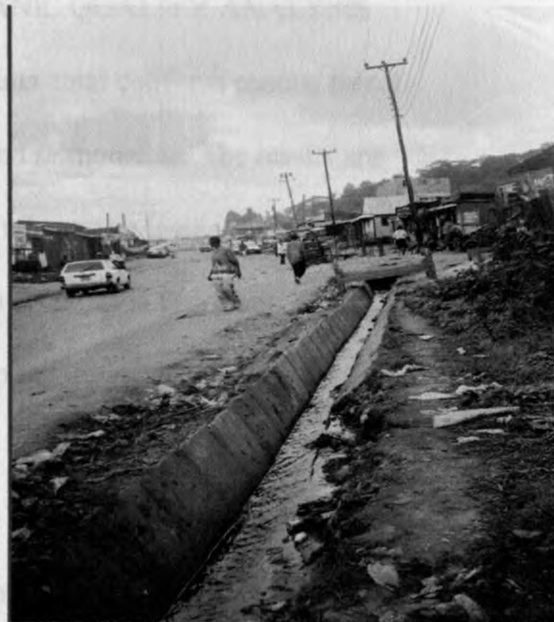
Site 3 and 6: point of effluent discharge into the river

Site 5

Site 5: point at which effluent from all the slaughterhouses meet

Figure 4 Sample Collection Sites

Residential houses and garbage dumps



Road-side kiosks

Petrol station

Figure 5 Other possible sources of pollution to the river

4.2 RESULTS OF MICROBIOLOGICAL AND ORGANIC QUALITY ANALYSIS

Fifty-four samples were analyzed for total bacterial counts, total coliform counts, faecal streptococci and presence or absence of faecal *E. coli* and salmonellae. The results are shown in tables 1to 5 and figure 6. Bacterial counts from tap water inside the slaughterhouse were insignificant. There was a high degree of variation ($p<0.05$) in the counts for each site sampled but there was no specific trend during the sampling period. There was a significant difference ($p<0.05$) between samples taken upstream to the point of effluent discharge into the river and samples taken at the point of discharge for all the parameters. There was also a significant difference between samples taken at the point of discharge before the start of slaughtering and during peak discharge ($p<0.05$). There was no significant difference between samples collected as the discharge left the slaughterhouses and the samples collected at the point it entered the stream for total coliform and faecal streptococci, but the difference was significant for total bacterial count($p<0.05$). *Salmonella* was isolated from site 2 in the first two weeks.

Table 1 Total bacterial counts, total coliform counts, faecal streptococci and presence or absence of faecal *E. coli*, salmonella and BOD in mg/L from the various sites.

Parameter	Site 1	Site 2($x10^6$)	Site 3($x10^6$)	Site 4	Site 5($x10^6$)	Site 6
TVC	140134	272.9	298.8	14	1094.2	69313
Total Coliform	149000	671.9	403.2	4	584.0	1171563
Faecal streptococci	3258	12.8	24.3	19	11.2	135478
Faecal *E. coli*	+	+	+		+	
Salmonella		+				
BOD_5(mg/l)	<1	2150	2944	N/A	4389	N/A

Key

TVC: Total Viable Count

BOD_5: 5-day Biochemical Oxygen Demand

Site 1: Two hundred and fifty metres up stream from the point of effluent discharge

Site 2: Two hundred and fifty metres down stream from the point of effluent discharge

Site 3: Point of discharge at peak slaughter

Site 4: Tap water inside one of the slaughterhouses

Site 5: Effluent as it leaves slaughterhouses

Site 6: Point of discharge before slaughtering commences

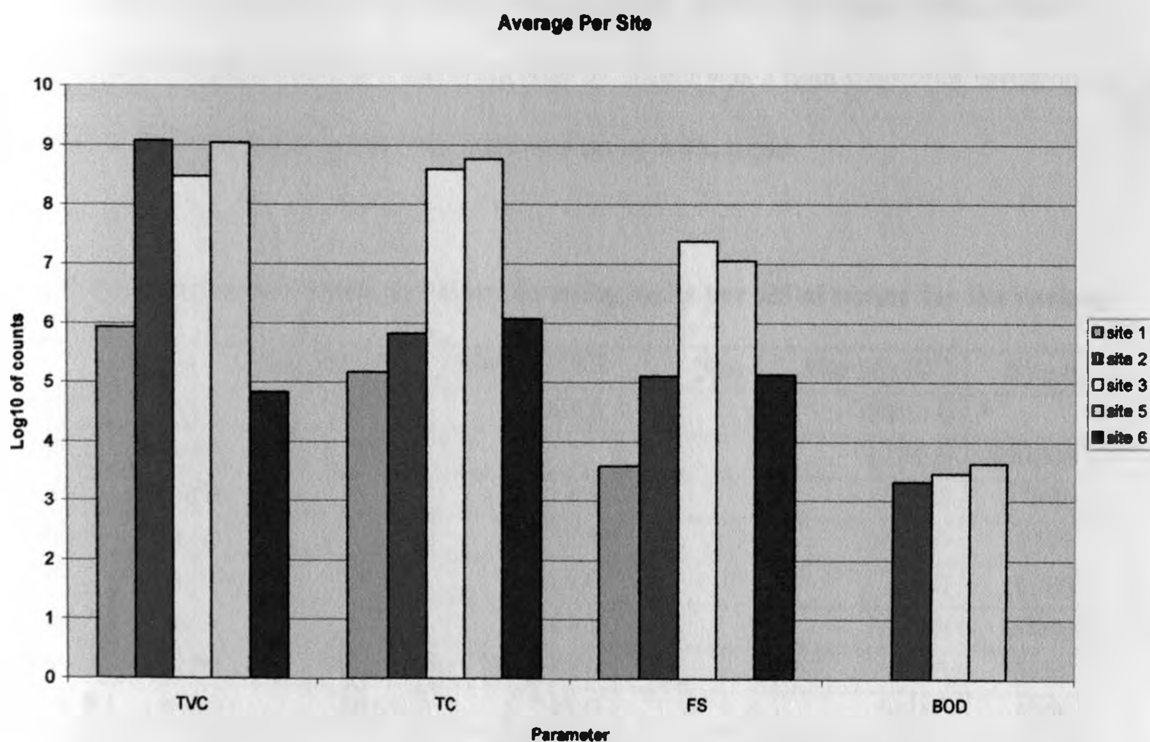


Figure 6 Mean values for the different parameters

4.3 TOTAL BACTERIAL COUNT

Results of the fifty-two samples analysed for total bacterial count are recorded as colony forming units per ml of water in table 2. Samples from the tap water (site 4) did not have significant growth; only two samples had growth of 12 and 113 colony forming units (cfu) per ml. All other samples showed high bacterial growth with large variations between the different sites. Samples collected at the point where effluent from the three slaughterhouses meet (site 5) had the highest bacterial counts with an average of 1.09×10^9 cfu/ml and a standard deviation of 582806767.6. There was a significant difference in bacterial counts between samples collected up-stream to the point of discharge (site 1) and those collected at the point of discharge (site 3) and downstream (site 2). There was also a significant difference ($p<0.05$) between the samples collected at the point of discharge before start of slaughter (site 6) and during peak slaughter (site 3). There was a high degree of variation in the counts at each sampling points but there was no specific trend.

Table 2 Total bacterial counts as colony forming units per ml of water for the various sites.

	Site 1	Site 2($x10^6$)	Site 3($x10^6$)	Site 4	Site 5($x10^6$)	Site 6
Week 1	175000	820.0	64.5	0	1905.0	*
Week 2	130000	10.8	*	0	1150.0	460000
Week 3	725000	*	1.6	0	1150.0	17800
Week 4	190000	2.1	7.9	12	1640.0	13300
Week 5	14000	86.5	219.0	113	1360.0	11300
Week 6	10900	68.5	23.5	0	1430.0	39000
Week 7	5300	1025.0	119.0	0	75.0	5050
Week 8	7153	162.0	215.0	0	970.0	5300
Week 9	3850	8.0	1740.0	0	168.0	2750
Minimum	3850	2.1	1.6	0	75.0	2750
Maximum	725000	1025.0	1740.0	113	1905.0	460000
Average	140134	272.9	298.8	13.9	1094.2	69312.5
STDEV	265740	422.4	668.8	43.3	657.1	179690

Key: * = The organisms were not countable at highest dilution

4.4 TOTAL COLIFORM COUNTS

The most probable number of coliform per 100ml of sample was computed from the 5-tube statistical table (appendix 2.1) and the results obtained presented in table 3. All samples except those collected inside the slaughterhouse had high coliform counts. The highest number of coliform was at site 5. There was no specific trend in the variation of the counts during the sampling period though there was much variation per site.

Table 3 Total coliform counts as the most probable number of coliform per 100 ml of water for the various sites

	Site 1($x10^5$)	Site 2($x10^6$)	Site 3($x10^6$)	Site 4	Site 5($x10^6$)	Site 6($x10^5$)
Week 1	1.2	2530	128	4	1160	*
Week 2	1.5	569	4	0	281	40
Week 3	1.23	*	21	0	890	20
Week 4	1.5	1270	209	15	782	14
Week 5	0.8	166	478	13	80	0.12
Week 6	1.1	46	520	7	588	16.1
Week 7	1.25	478	763	0	1160	0.45
Week 8	3.2	35	588	0	239	2
Week 9	1.95	281	918	0	76	1.05
Minimum	0.79	35	4	0	76	0.13
Maximum	3.22	2530	918	15	1160	40
Average	1.5	671.9	403.2	4.3333	584	11.7
STDEV	0.9	850.6	329.2	6	435.8	14

Key: * = The organisms were not countable at highest dilution

4.5 FAECAL STREPTOCOCCI

Table 4 shows the results from various sites indicated as CFUs/100ml of sample. No attempt was made to differentiate the types of streptococci involved. There was a high degree of variation in the counts at each sampling points but there was no specific trend.

Table 4 Faecal streptococci as colony forming units per 100 ml of water for the various sites

	Site 1	Site 2($x10^6$)	Site 3($x10^6$)	Site 4	Site 5($x10^6$)	Site 6($x10^5$)
Week 1		12.4	10.3	0	8.8	1.5
Week 2	7750	8.6	2.7	0	15.8	0.8
Week 3	1600	9.3	8	15	7.86	0.4
Week 4	1590	5.8	17	43	13	3.9
Week 5	6100	6.7	19.7	30	9.8	0.4
Week 6	3950	16.9	23.7	0	11.5	1
Week 7	623	43.2	57.1	33	16.8	1.7
Week 8	2100	4.0	10.8	0	11	0.5
Week 9	2350	8.4	69	46	5.97	2
Minimum	623	4	2.7	0	5.97	0.4
Maximum	7750	43.2	69	46	16.8	3.9
Average	3257.9	12.8	24.32	18.6	11.2	1.4
STDEV	2489.2	12.8	24	19.6	3.7	1.2

4.6 FAECAL ESCHERICHIA COLI

Results of presence or absence of faecal *E. coli* are shown in Table 5. Fifteen (15) samples (28.8%) were positive for faecal *E. coli.* Six (40%) positive samples were from site 5, while 4 (26.7%), 3 (20%) and 2 (13.3%) were from sites 1, 2, and 3 respectively.

Table 5 Results obtained for presence of faecal *E. coli* for the various sites

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Week 1		+				
Week 2	+				+	
Week 3					+	
Week 4						
Week 5	+					
Week 6		+			+	
Week 7	+	+			+	
Week 8	+		+		+	
Week 9			+		+	

Key: + present,

Blank absent

4.7 ANTIBIOTIC SENSITIVITY OF *E. COLI* ISOLATES

The isolates were resistant to ampicilin (25%), sulphamethoxazole (25%), streptomycin (20%), tetracycline (20%), chloramphenicol (10%), amoxyllin/clavulanic acid (10%) and nalidixic acid (5%). Fifty-five percent and 40% of the isolates showed intermediate sensitivity to streptomycin and ampicilin respectively. Results of antibiotic resistance are shown in Table 6.

Table 6 Antibiotic resistance of faecal *E. coli*

Antibiotic	Specification (μg)	% Resistance
ampicillin	10	25
sulphamethoxazole	100	25
streptomycin	10	20
trimethoprim	5	20
tetracycline	30	20
chloramphenicol	30	10
amoxycillin/clavulanic acid	30	10
nalidixic acid	30	5
ciprofloxacin	5	0
gentamicin	10	0
cefuroxime sodium	30	0
ceftazidime	30	0

Of the twenty isolates tested for antibiotic sensitivity, three (15%) were sensitive to all antibiotics tested, thirteen (65%) showed intermediate sensitivity to one or two antibiotics while two (10%)were resistant to five, one (5%) resistant to six, and one(5%) to eight of the antibiotics used.

4.8 SALMONELLA

Of the 54 samples taken, *Salmonella* was isolated in two samples (3.7%). Six different serotypes were isolated in the samples. The analytical profile index for the isolates indicated that there was an over 98% chance that the isolates were salmonella. Four (75%) of the

isolates tested positive for the 'O' antigen only, one (16%) tested positive for the 'O','6' and '7' antigens and one (16%) for the 'O','4i', '1', and '2' antigens. The four could only be identified as *Salmonella* spp, one of the isolates was of the O, 6, 7, C1 group and one was identified as *Salmonella* Typhimurium.

4.9. BIOCHEMICAL OXYGEN DEMAND

A total of thirty-six samples were analysed for BOD. There was consistently high BOD_5 in the samples taken at the point where the effluents from the three slaughterhouses join (average 4388.9mg/l, with a standard deviation of: 298.72). At the point of discharge, the average BOD_5 was 2944.44mg/L SD= 1042.97 whereas sample down stream average BOD_5 was 2150mg/L SD=970.422. BOD level of samples taken upstream from the point of discharge was below the recognition level of the machine.

There was no significant difference ($p>0.05$) in BOD_5 between samples collected from the different sites. There was however a significant difference between the samples collected upstream to the point of discharge and at the other sites. The results of the biochemical Oxygen demand test are shown in Table 7.

Table 7 Biochemical Oxygen Demand in mg/L. for the various sites

	Site 1	Site 2	Site 3	Site 5
Week 1	0	925	3100	4700
Week 2	0	2700	2200	3900
Week 3	0	925	3600	4600
Week 4	0	2600	2200	4000
Week 5	0	3350	1000	4500
Week 6	0	3200	2700	4500
Week 7	0	2800	3300	4450
Week 8	0	1350	4300	4700
Week 9	0	1500	4100	4150
Minimum		925	1000	3900
Maximum		3350	4300	4700
Average	0	2150	2944.44	4388.89
STDEV		970.422	1042.97	298.724

Key
0= below identifiable range by BOD machine

CHAPTER 5

5.0 DISCUSSIONS

A water quality standard defines the quality goals of a water body or a portion thereof by designating the use or uses to be made of the water and setting criteria necessary to protect the use. Water quality standards are adopted either to protect public health or to enhance water quality (Anon, 1996b). The quality of a water body is affected by the quality of water discharging into it, be it storm water or wastewater.

The quality of wastewater being discharged into water bodies should be such that it does not adversely affect the quality or usability of the water body into which it is discharged. Different countries and states have different requirements for wastewater quality. This is dependent on among other things, the treatment or otherwise of the wastewater, the eventual use of the wastewater or water from the receiving body, and the dilution factor of the receiving water body.

The recommended quality for discharge depends on the intended use of the water. Many of the guidelines given for the quality of wastewater are for wastewater used for irrigation and the quality criteria differ depending on the risk the intended use of the wastewater is likely to pose to human health (Westcott, 1997, Anon, 2005a, Anon, 2006).

Most of the existing standards are for treated or semi treated wastewater. The current study looked at the quality of raw untreated wastewater and its impact on the quality of the receiving river. The results showed very high levels of contamination compared with the existing standards and guidelines. High organic strength of the river water may be an advantage to the farming communities along the river and this may act as fertilizer to crops irrigated but the risk of infection needs to be assessed.

Wastewater is used as a source of irrigation water as well as a source of plant nutrients, allowing farmers to reduce or even eliminate the purchase of chemical fertiliser. Agricultural reuse of wastewater is practised throughout South America and in Mexico and is also widespread in Northern Africa, Southern Europe, Western Asia, on the Arabian Peninsular, in South Asia and in the US. Vegetable, fodder and non-food crops as well as green belt areas and golf courses are irrigated. In a few countries (such as the US, Singapore and Saudi Arabia) wastewater is subjected to advanced treatment (secondary treatment, filtration and disinfection) prior to use (Havelaar *et al*, 1998). Yet, where the wastewater is used untreated and health protection measures are not in place, such practice may contribute to the 'recycling' of excreted pathogens among the urban/peri-urban population. Farmers and their families making use of untreated faecal sludge or wastewater, as well as consumers, are exposed to high risks of disease transmission.

Total bacterial count at site 1 (upstream to the point of discharge into the river) was $1.4x10^5$.compared to $2.7x10^8$ at site 3 (the point of discharge into the river). This marked increase was true for all the other parameters analyzed.

The large difference in all the parameters between samples from the point of discharge before the start of slaughter and during peak slaughter indicates the level of pollution. The NEMA standards are far much lower than what was observed. This is to be expected since there is no wastewater treatment in Dagoretti. This study provides baseline data to be used to assess the efficiency of the treatment process once this is set in place.

All wastewater samples analysed in this study exceeded by far the standards set by the various institutions. Whereas the recommended number of coliform recommended by NEMA is less than 100/100ml of sample, the average number in the samples analysed was $3.3x10^8$ /100ml of sample and $6.7\ x10^8$ /100ml at the point where the effluent is discharged

into the river (site 3). Guidelines in the states of Florida and Washington for wastewater discharges to surface or groundwater that may eventually be used as a source of portable water are 20mg/l and 5mg/ l for BOD, and 0/ml and 5/100ml for total coliform respectively (Anon. 2005b). For Yukon Territory, Canada, the recommended levels are 45mg/l BOD and 20,000/100ml for faecal coliform (Anon 2005a). For agricultural use in Alberta, Canada total coliform allowed is1000/100ml and faecal coliform 100/100ml (Anon 1999b)

There was a significant difference in the faecal streptococci counts in samples collected upstream to the point of discharge and samples collected at the point of discharge and downstream to the point of discharge. There was also a significant difference in the faecal streptococci counts in samples collected at the point of discharge before the start of slaughter and at peak slaughter. This indicates that the effluent from the slaughterhouses contributed significantly to faecal streptococci pollution in the river. The EMCA water quality regulations (Anon, 2006) do not set any standards for faecal streptococci for effluent discharge into the environment. Many studies have indicated that there is a high correlation between faecal streptococci and coliform as faecal pollution indicators (Collin *et al*, 1988, Leclerc *et al*, 1996)

Food poisoning and antimicrobial resistance are two major aspects of the threat *E. coli* poses to public health as important food-borne pathogens. Of the 20 isolates studied, five (25%) demonstrated resistance to two or more of the antibiotics tested. Trends in prevalence of antimicrobial resistance among isolates in this study were similar to those of *E. coli* isolates in food animals in Kenya (Ole Mapenay 2007). Other studies have also found multi-drug resistant *E. coli* in animals, animal products and humans in Kenya (Ombui et al, 1994, Bebora et al, 1994).

Bacteria resistant to antibiotics are present in surface water (Shwartz, *et al*, 2003, Guardabassi *et al*, 1999). Goni-Urizza *et al* (2000) found a correlation between resistant bacteria in rivers and urban water input. Antimicrobial resistance has also been found in marine bacteria and bacteria living in estuaries (Barkay *et al*, 1995). Gentamicin resistance genes were found in bacteria in coastal waters polluted with sewage (Heuer *et al*, 2002) The ability of bacteria to acquire and subsequently spread antimicrobial resistance gene is well known (Hall, 1997). Spread of bacteria with multi-drug resistance through food irrigated with slaughterhouse effluent, or through utilisation of water downstream is of public health importance. The trends in antimicrobial resistance is indicative of antimicrobial consumption in the country as the antimicrobials to which the bacteria showed highest resistance are among those most prescribed in Kenya (Mitema *et al*, 2001). High prevalence of multi-drug resistant *E. coli* in slaughterhouse effluent may reflect a reservoir of resistance genes in animals that can be transmitted through the food chain to humans. This may lead to treatment failure in humans and therefore a national surveillance program to monitor emergence and spread of antimicrobial resistance and antimicrobial consumption in food animals should be established.

Zoonotic non typhoid *Salmonella* species are an important cause of infection in man and animals. Outbreaks in humans have been associated with food-borne transmissions from contaminated animal products (Graham *et al*, 2000). Studies have documented that farm animals are a major reservoir of *Salmonella* (Threlfall, 2000; Ahmed *et al*, 2000), In this study 2 out of 54 (3.7%) samples were positive for *Salmonella.* This is comparable to findings by Kariuki *et al* (2002) who found 4 (1.7%) of 267 samples from slaughterhouse effluent and beef carcasses positive for *Salmonella*. Kayihura (1982) found 1.9% of samples from abattoir effluent in Kenya, positive for *Salmonella.* In this study, one of the isolates

was identified as *S. typhimurium*. This is of significance since *S. typhimurium* especially the multidrug resistant *S. typhimurium* DT 104 strain has been recognised as a major cause of human epidemics and is mainly transmitted through consumption of contaminated animal products (Threlfall, 2000).

There was significant difference in BOD level in samples taken upstream from the point of discharge and those taken at the point of discharge. This implies that the effluent contributes significantly to the organic pollution of the river. There was no significant difference on the BOD level of samples taken at the point of discharge and those taken either at the point where the effluent from the three slaughterhouses meet or samples taken down stream. Biochemical Oxygen Demand (BOD_5) at the point of discharge into the river was 2944.44mg/l compared to the NEMA recommended level of 30mg/l. This is also much higher than the guidelines given for wastewater discharge in Florida State (20mg/l), the State of Washington (5mg/l) (Anon, 2005a), and Yukon County, Canada (45mg/l) (Anon, 2005b)

High BOD can lead to reduced dissolved oxygen while high organic load can favour growth of algae tin the receiving water which impacts negatively on the aquatic environment. High BOD results in anaerobic oxidation which leads to methane production, producing bad odour.

Standards from most countries presume treatment and are thus indicated as standards for treated wastewater and not for raw wastewater. They have been set for wastewater for use in irrigation and not for wastewater being discharged into surface water bodies. Those set for discharge into surface water are for classified water bodies designated for specified use such as recreation, commerce, navigation or fishing (Anon, 1998).

CHAPTER 6

6.1 CONCLUSIONS

- Wastewater from the Dagoretti slaughterhouses complex is of very high organic polluting strength and bacterial counts. This is many times higher than the recommended quality of wastewater for discharge into surface water bodies by NEMA, Kenya and other national standards
- The large difference in quality between the stream water upstream to the point of discharge and at the point of discharge indicated massive pollution of the Kabuthi River.
- Considering that the Kabuthi River is used, just a short distance downstream to the point of discharge for irrigation, there is high risk of infection among people especially the farm workers along the river.
- There is significant level of multi-drug resistance in isolated *E. coli.*
- Zoonotic salmonellae isolated pose a possible risk of infection to people using the river water downstream.

6.2 RECOMMENDATIONS

Treatment of the wastewater from the slaughterhouses should be carried out to reduce the organic and bacterial pollution of the river. This reduction could result from either minimising the amount of water or the amount of organic waste released into the stream.

1. The amount of water could be reduced through the following:

- Use of automatic control system to operate the flow of water in hand washing facilities
- Use of dry-cleaning techniques, such as removal of solid waste from the floors before washing with water
- Use of high pressure rather than high volume of water for cleaning

- Removal of paunch contents that avoids or minimizes release of the contents into the effluent
- Re-use of final rinse water from cleaning operations for initial rinsing the following day

2. The organic waste could be reduced by:
    - Utilizing cleaning procedures that collect and re-use waste material such as blood and paunch content or dispose them through channels other than the effluent
    - Initiate and continually enforce dry-clean up before cleaning the floor
    - Install and maintain properly designed catch-basins and grease traps .
3. Secondary treatment of the slaughterhouse wastewater together with final re-use of the same may ensure that the effluent does not get into the river. The objective of secondary treatment would be to reduce the BOD through the removal of organic matter. The secondary treatment includes both aerobic and anaerobic lagoons and activated sludge processes. The biogas produced from the anaerobic lagoons could be used in the slaughterhouses for lighting and heating.
4. The treatment methods employed should take into consideration the cost effectiveness of the same in the slaughterhouses
5. Spread of antimicrobial resistance may be reduced through:
    - education of human and animal health service providers on proper use of drugs, selection of correct drug, dosage and optimum treatment duration for treatment of specific infection
    - Public education on judicious use of antimicrobial.
    - reduction in use of antimicrobials as growth promoters in livestock
    - Make effective medicine accessible to the poor to avoid failure to complete recommended dosage or use of cheaper generics of questionable quality.

- National monitoring for resistance and of drug quality.

6.2.1 RECOMMENDATIONS FOR FURTHER STUDIES

1. A study to compare the incidence of zoonotic infections in Dagoretti and the national incidence might probably indicate a significant difference correlating to the river pollution.
2. The effects of fats, oils and grease on soil productivity
3. The effect of effluent pollution on ground water.

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APPENDICES

1.1 Preparation of Plate Count Agar (CM325)

Formula

	gm/liter
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar	9.0
pH	7.0(Approx.)

Directions

17.5g of medium was added to 1 liter of distilled water and dissolve by boiling. The medium was sterilised by autoclaving at 121°C for 15 minutes. It was then cooled to 60°Cand distributed into petridishes

1.2 Preparation of MacConkey Broth (Purple) (OxoidCM5a)

Formula

	gm/liter
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Bromocresol purple	0.01
pH	7.4(approx.)

Directions

To prepare single strength broth, 40g of medium was added to 1 liter of distilled water. Distribute to fermentation tubes fitted with Durham tubes. The medium was sterilised by autoclaving at 121°C for 15 minutes.

1.3 Preparation of Eosin Methylene Blue Agar (Modified) Levine (CM69)

Formula

	gm/liter
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.065
Agar	15.0
pH	6.8(approx.)

Directions

37.5g of medium was suspended in 1 liter of distilled water and boiled to dissolve completely. The medium was sterilised by autoclaving at 121°C for 15 minutes and cooled to 60°C. The medium was shaken to oxidise the methylene blue (i.e. restore its blue colour) and to suspend the precipitate which is an essential part of the medium. This was then dispensed into petri dishes and allowed to solidify.

1.4 Preparation of Peptone Water (CM9)

Formula

	gm/liter
Peptone	10.0
Sodium chloride	5.0
pH	7.2(approx.)

Directions

15g of medium was dissolved in 1 liter of distilled water. It was mix well and distributed to 100ml culture bottles. The medium was sterilised by autoclaving at 121°C for 15 minutes.

1.5 Preparation of MRVP medium (CM43)

Formula

	gm/liter
Peptone	5.0
Glucose	5.0
Phosphate buffer	5.0
pH	7.5(approx.)

Directions

15g of medium was added to 1 liter of distilled water, distributed into final containers and sterilised by autoclaving at 121°C for 15 minutes

1.6 Preparation of Simmon's Citrate Agar (Oxoid CM155)

Formula

	gm/liter
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromolthymol blue	0,08
agar	15.0
pH	7.0(Approx.)

Directions

23g of medium was suspended in 1 liter of distilled water and dissolved by boiling. The medium was dispensed into culture tubes and sterilised by autoclaving at 121°C for 15 minutes. The agar was allowed to solidify in a slant

1.7 Preparation of Nutrient Agar code CM3

Formula

	gm/liter
Lab-Lemco powder	1.0
Yeast extract	2.0
peptone	5.0
Sodium chloride	5.0
Agar	15.00
pH	7.4(Approx.)

Directions

28g of the medium was suspended in 1 liter of distilled water and boiled to dissolve completely. The medium was sterilised by autoclaving at 121°C for 15 minutes

1.8 Preparation of Mueller-Hinton agar code cm337

Formula

	gm/liter
Beef, dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH	7.4(approx.)

Directions

38gm of medium was added to 1 liter of distilled water. and boiled to dissolve the medium completely. The medium was sterilised by autoclaving at 121°C for 15 minutes

1.9 Preparation of Slanetz and Bartley medium (code cm 337)

Formula

	gm/liter
Tryptose	20.0
Yeast extract	5.0
Glucose	2.0
Disodium hydrogen phosphate	4.0
Sodium azide	0.4
Tetrazolium chloride	0.1
Agar	10.0
pH	7.2 (Approx.)

Directions

Forty two grams of the medium was suspended in 1 liter of distilled water and brought to the boil to dissolve the agar. The medium was cooled to $60^{O}C$ and dispensed into petri dishes and allowed to solidify.

1.10 Preparation of Buffered Peptone Water Code CM509

Formula

	gm/liter
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1,5
pH	7.2 Approx.

Directions

Twenty grams of medium was added to 1 liter of distilled water. It was mixed well and distributed to final containers. The medium was sterilised by autoclaving at 121°C for 15 minutes.

1.11 Preparation of Selenite broth Base code CM395

Formula

	gm/liter
Peptone	5.0
Lactose	4.0
Sodium phosphate	10.0
pH	7.1(approx.)

Directions

Four grams of sodium biselenite was dissolved in 1 liter of distilled water and 19g of selenite broth base then added. The medium was mixed well and put into the final containers. It was sterilised by boiling in free flowing steam for 10 minutes.

1.12 Preparation of Tetrathionate Broth Base (Oxoid) CM29

Formula

	gm/liter
Lab-Lemco powder (Oxoid L29)	0,9
Peptone (Oxoid L27)	4.5
Yeast extract	1.8
Sodium chloride	4.5
Calcium carbonate	25.0
Sodium thiosulphate	40.7
pH	8.0 (approx.)

Directions

77g of the medium was added to 1 liter of distilled water and brought to the boil. The mixture was cooled to below 45OC and 20ml of iodine solution added. The medium was then dispensed into the final containers.

Iodine solution

Iodine	6 grams
Potassium iodide	5 grams
Distilled water	20ml

1.14 Preparation of Desoxycholate Citrate Agar (Oxoid CM227)

Formula

	gm/liter
Lab-Lemco powder (Oxoid L29)	5.0
Peptone(Oxoid L27)	5.0
Lactose	10.0
Sodium citrate	5.0
Sodium thiosulphate	5.0
Ferric ammonium citrate	1.0
Sodium desoxycholate	2.5
Neutral red	0.025
Agar	15.0
pH	7.0 (approx.)

Directions

48.5g of medium was suspended in 1 liter of distilled water and boiled to dissolve. The medium was mixed well, poured into petri dishes and allowed to solidify.

1.15 Preparation of Triple Sugar Iron Agar (Oxoid CM227)

Formula

	gm/liter
Lab-Lemco powder (Oxoid L29)	3.0
Yeast extract	3.0
Peptone(Oxoid L27)	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
phenol red	q.s
Agar	12.0
pH	7.4 (approx.)

Directions

65g of medium was suspended in 1 liter of distilled water and boiled to dissolve. The medium was distributed into tubes and sterilised by autoclaving at 121°C for 15 minutes. The medium was allowed to solidify in a slant to form a butt of not less than 3cm deep.

1.16 Preparation of Urea Agar Base

Formula

	gm/liter
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0
pH	6.8 (Approx.)

Directions

2.4g of medium was suspended in 1 liter of distilled water and boiled to completely dissolve. The medium was sterilised by autoclaving at $115^{O}C$ for 20 minutes and allowed to cool to $50^{O}C$. Five milliliters of sterile 40% Urea solution (Oxoid SR20) was aseptically introduced into the medium. The mixture was distributed into sterile culture tubes and allowed to solidify in a slant position.

Appendix :McCrady's statistical table

Table for determining the most probable number (MPN) of bacteria present in 100ml. of sample and 95% confidence limit using 5 tubes of 10ml., 5 tubes of 1ml. and 5 tubes of 0.1ml (From Kenya Bureau of Standards, 1985)

Number of tubes giving positive reaction			MPN	95% confidence limit within which MPN can lie	
5 tubes of 10ml each	5 tubes of 1ml each	5 tubes of 0.1ml each		Lower limit	Upper limit
0	0	1	2	0.5	7
0	1	0	2	0.5	7
0	2	0	4	0.5	7
1	0	0	2	0.5	7
1	0	1	4	0.5	11
1	1	0	4	0.5	11
1	1	1	6	0.5	15
1	2	0	6	0.5	15
2	0	0	5	0.5	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	8	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	1	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	11	80
4	3	1	33	11	93
4	4	0	34	12	96
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	114
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	154
5	2	0	49	17	126
5	2	1	70	23	168
5	2	2	94	28	219
5	3	0	79	25	187
5	3	1	109	31	253
5	3	2	141	37	343
5	3	3	175	44	503
5	4	0	130	35	302
5	4	1	172	43	486
5	4	2	221	57	698
5	4	3	278	90	849

5	4	4	345	117	999
5	5	0	240	68	754
5	5	1	348	118	1005
5	5	2	542	180	1405
5	5	3	918	303	3222
5	5	4	1609	635	5805