THE USEFULNESS OF MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEXING TECHNIQUE IN DIFFERENTIATING FECAL COLIFORM BACTERIA FROM DIFFERENT SOURCES

A DISSERTATION FOR A MASTERS DEGREE IN MICROBIOLOGY

BY

MTHEMBU MATHEWS SIMON

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Abstract

Pollution of water sources with human fecal matter and associated intestinal pathogens poses a great risk to public health. Fecal contamination of water is not the only problem to communities that consume untreated water. The extent of the microbial contamination of water sources also needs to be considered when designing treatment regimes for the production of potable water. The more polluted the source of drinking water is, the more extensive and expensive treatment regimes have to be used to produce microbial risk-free water. For decades fecal coliform counts have been used as indicators of fecal contamination and the potential presence of intestinal pathogens in surface waters. However, fecal coliforms fail to provide information about the source of fecal contamination. Knowing the source of fecal contamination is vital in managing this problem in surface waters. This study explored the use of two techniques, multiple antibiotic resistance (MAR) indexing and caffeine detection as means of differentiating *E. coli* isolates from various sources.

A total of 322 *E. coli* were isolated from domestic and wild animals as well as human sewage by using conventional culture methods. Standard chemical and biochemical tests were used to identify these isolates. All isolates were assayed against a battery of 10 antibiotics using the micro-dilution method. The results obtained were used to generate antibiotic resistance profiles which in turn were used to statistically group the isolates into different subsets. Caffeine detection by Thin Layer Chromatography (TLC) was used to differentiate between human and non-human derived *E. coli* isolates.

The correct classification rate was 78% when MAR indexing was used and 50% when using caffeine detection. Sixty percent of *E. coli* from humans were correctly classified and 95.5% of *E. coli* from animals were correctly classified as non-humans sources respectively. The results of this study underscore the validity of MAR indexing as a method of bacterial source tracking. MAR indexing has great discriminatory power without the complexities and the high costs often associated with established genotype-based methods. Caffeine detection indicated an average classification rate (50%). With further research, caffeine detection may give another option for source tracking when genotyping methods are limited by either costs or lack of expertise. The use of combined techniques may provide a much more reliable and cost-effective option for bacterial source tracking when each technique used provide similar results.

DECLARATION

I. M THEMBU MATHEUS Simon declare that this dissertation hereby submitted to the University of Zululand for the degree of Masters of Science has not been previously submitted by me for a degree at this or any other institution or University. This is my work in design and execution. All materials contained herein have been fully acknowledged.

Signature

07/03/2008

Date

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CHAPTER 1

1. INTRODUCTION

1.1 Introduction and Problem Statement

In most developing African countries, the provision of portable water continues to be the major challenge. This challenge results from the pollution of surface waters (Pretorius, 2000). The problem is further perpetuated by the lack of sanitation systems in most rural communities and this causes an increased risk of water-borne related illnesses (Biyela, 2003). It is reflected indeed, that, water scarcity and problems relating to its quality are a worldwide phenomenon. Recent studies put one-quarter of the world's population at risk of water stress or scarcity based on recent developments (Falkenmark, 2006; Loehman and Becker, 2006). In the Eastern Cape Province in South Africa, research on water sustainability, scarcity and poverty were conducted at Quakeni local municipality. It was found that this area was the poorest in water availability and quality (van Vuuren, 2006). It appears then that, the best ways to address water problems are to consider safeguarding the sources of water from contamination and to increase water sustainability through service delivery.

In monitoring water quality, priority should be given to the substances which are known to be important to health and which are known to be present in water sources. In both developed and developing countries, microbial hazard continues to be a threat and a major concern (Boyacioglu, 2006). Methods such as multiple antibiotic resistance (MAR) indexing technique and caffeine detection can be used to monitor microbial contamination by identifying the sources of these contaminants (Sargeant, 1999; Wiggins,

1996).

Multiple antibiotic resistance (MAR) indexing is a method for differentiating between human and non-human sources of fecal contamination in source waters. This method has been used to differentiate bacteria from different sources using antibiotics commonly associated with human and animal therapy as well as animal feeds (Scott *et al.*, 2002). This is based on the premise that bacteria exposed to different kinds and concentrations of antibiotics possess different antibiotic resistance patterns. MAR has the capability of distinguishing between *E. coli* strains from specific point sources such as industrial and municipal effluents and strains from non point sources such as land runoff that are dispersed over wide areas (Guan *et al.*, 2002).

MAR indexing procedure involves the isolation and identification of indicator organisms from known sources and culturing of the isolates on media/broth containing various antibiotics at various concentrations. Plates are then incubated and the organisms are counted according to their susceptibility to various antibiotics to generate antibiotic resistant profiles. The antibiotic resistant profiles are then characterized and analyzed by discriminant analysis (Scott *et al.*, 2002).

In general, studies employing multiple antibiotic resistance indexing have shown not only that MAR indexing is useful in differentiating fecal bacteria of animal and human origin but it can also predict whether bacteria from animal sources are from domestic or wild animals (Wiggins *et al.*, 1999). Because the development of antibiotic resistance is between exposure and use, fecal bacteria from wild animals generally show a lower level

of antibiotic resistance (Carson *et al.*, 2001).

The caffeine detection method has also been proposed as being useful in the detection of the sources of fecal contamination (Sargeant, 1999). This technique differentiates human from non-human sources of fecal contamination. The rational behind the use of the caffeine method is to evaluate the presence of unmetabolized caffeine as a potential indicator of human fecal contamination.

1.2 Aims and objective of the study

1.2.1 Aim:

The overall aim of this study was to determine the reliability of the MAR indexing in discriminating microorganisms from different sources for future application in tracing the sources of fecal contamination in surface water.

1.2.2 The objectives of the study were to:

1. Build a library of *E. coli* isolates from known sources.
2. Determine the antibiotic resistance profiles (ARP) of the isolates
3. Use the MAR index to classify the bacteria according to their hosts or environment
4. Cross-validate the reliability of MAR indexing by comparing classification data obtained using this method with data obtained when using caffeine.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Introduction

Water is the most important natural resource in the world since without it, life cannot exist and most industries cannot operate. Although humans can survive for days without food, the absence of water for few days has fatal consequences. The presence of safe and reliable source of water is thus an essential prerequisite for the establishment of a stable community (Kerr, 1995). In the absence of such a source, a nomadic lifestyle becomes necessary and communities must move from one area to another as demands for water exceeds its availability (Tebbutt, 1998). For this reason, it is important that all water sources are safeguarded to prevent water abuse and water contamination.

The importance of water as a natural resource requires careful management and conservation must be universally recognized. Although nature has the ability to recover from environmental damage, the growing demands on water resources necessitate the professional application of fundamental knowledge of the water cycle to ensure the maintenance of water quality and quantity (Tebbutt, 1998).

2.2 Water management

In rural communities in the republic of South Africa, in Kwa-Zulu Natal (KZN), water is fetched from the streams, rivers, community taps, springs or boreholes and is then carried and stored at home to ensure that it is available when needed (Biyela *et al.*, 2004). In these areas, even if water sources are not contaminated, water can be contaminated during

storage due to common water usage in the household, that is, washing clothes, stock watering, watering garden etcetera, which can result in water related illnesses (Bailey, 2003). Sustainable water resources management can be achieved through the objectives of sustainable water policy which is defined by Tebbutt (1998).

2.3 Water quality and health

Water plays an essential and irreplaceable role in supporting human life and when contaminated, it has a great potential for transmitting a wide variety of diseases and illnesses (Boyacioglu, 2006). In developed countries, water-related diseases are rare due to the presence of efficient water supply and wastewater disposal systems (Tebbutt, 1998). However, in developing countries, as many as 1.3 billion people are without safe water supply and almost 2 billion do not have adequate sanitation and as a result the toll of water-related disease in these areas is frightening in its extent (Tebbutt, 1998). Millions of people die each year as the consequence of unsafe water or inadequate sanitation. Although exact information is difficult to obtain, the World Health Organization (1992) data, gives indication of the magnitude of the problem.

Currently, South Africa is in the brink of crop and water contamination crises (Rand Water, 2007). About 43% of the sources of water managed by the department of water affairs and forestry (DWAF) have microbial and chemical contamination problems, including radioactive contamination (Rand Water, 2007). Recent findings indicate that about R180 billion is needed to build and replace water source services infrastructure in order to combat contamination from the sources of water. Radioactive contamination poses even more serious health impacts. If radioactive contaminated water is used in

farming, radioactive food and meat may be introduced into the food chain, causing serious health implications.

In South Africa, the life threatening consequences of polluted water were brought in mid 2000 with cholera outbreak in KwaZulu Natal (Biyela, 2003). Rural communities were the most affected communities because of their inability to afford tap water. Vulnerability to waterborne pathogens because of inadequate sanitation also perpetuated the problem. Water contamination is an environmental issue threatening the global water supply. In the USA, Colorado river has turned into a salty marshes resulting from river being used to irrigate 3.7 million farm acres, living no protection to the ecosystem downstream from chemical toxic runoff from the fertilizers and pesticides (Rand Water, 2007). Water contamination is a scourge of recent times, with water devastatingly contaminated with agricultural, industrial and municipal waste.

In the whole world, people suffering from gastroenteritis, schistosomiasis, malaria, diarrhea and onchocerciasis are increasing (van Vuuren, 2006). All of these diseases can be water-related although other important environmental factors may play a crucial role.

In developed countries, there is a concern about the possible long-term health hazards which may arise from the presence of trace concentrations of impurities in drinking water, particularly attention being paid to potentially carcinogenic compounds (Tebbutt, 1983; Falkenmark, 2006). It is therefore important that the relationships between water quality and health be fully appreciated by the engineers and scientists concerned with water quality control and management.

2.4 Causes of waterborne disease

Microorganisms are one major cause of illnesses associated with food and water (Boyacioglu, 2006). These illnesses have major economic impacts both in finding treatment and building infrastructures to safeguard the sources of water. In South Africa, a R2.5 billion project was launched in the Western Cape Province to reduce demands, increase storage, desalinate sea water and re-use waste water. In 1990 the worldwide impact of food illnesses associated with microorganisms was about $20 billion and in 1980, about 25000 people died per day from consumption of contaminated water (US Environmental Protection Agency, 1990).

In water, the mere presence of microorganisms does not mean water is unsafe for drinking since not all microorganisms found in water are pathogenic; some are beneficial (Kerr *et al.*, 1995). However, failure to adequately and continuously remove harmful microorganisms is a continuing cause of water-borne diseases. Even in communities that receive treated water and have adequate sanitation infrastructure, water-borne infections may still occur but the incidences are limited (Biyela, 2003). These include infection by pathogens that are generally not destroyed during treatment processes and pathogens that are introduced along distribution systems. To ensure health improvement, an effort is required from all parties concerned, the effort of rapid provision of water supply and sanitation, particularly in rural population (Kerr *et al.*, 1995).

2.5 Importance of safeguarding the sources of water

Rivers and streams are the main sources of water and without water, life is impossible. Water is Africa's scarcest resource. Without sufficient water we cannot grow enough crops and support industrial growth, or develop a growing tourism industry. Our economy is therefore totally dependant on a continual supply of water of sufficient quality and quantity (Loehman and Becker, 2006). The majority of the rural communities do not have access to potable water. They therefore rely on streams, rivers, marshes and other types of wetlands to supply them with enough clean water to satisfy their needs (Falkenmark, 2006). It is therefore important to keep these water sources clean to maintain the water demand by communities (Tebbutt, 1998). To keep these water sources clean, sanitation needs to be practiced which should be accompanied by water research to develop new, easy and cheap technologies to ensure safety and quality of water as well as source water protection (Kerr, 1995).

2.6 Water quality and sanitation

Water and sanitation are essential for a healthy and productive life. In developing countries, water is often remote and unsafe while sanitation is at best primitive. The poor suffers the most. They have neither the knowledge nor the means to improve their conditions. The debilitation effect of endemic diseases together with malnutrition cause untold misery and suffering in developing countries. Poverty is perpetuated by the adverse effect on productivity which is a heart–breaking vicious cycle (Tebbutt, 1998).

Raw water sources consist of surface waters and groundwater. Surface waters include rivers, lakes, and reservoirs fed by direct rainfall and run-off's. Groundwater includes deep and shallow aquifers and springs fed by slow percolation of surface waters through soil and subsoil layers. Deep protected aquifers are the best preferred source of potable water because years of filtration have left water free of turbidity, organic matter and pathogens (Mays, 2004). Rather than chemicals, pathogens have been the major health concern in drinking water.

Pathogenic microorganisms exist in most raw water sources, especially surface waters. To protect the public's health, water must be reduced to safety levels. These are levels that protect the public from infectious outbreaks. Most of the drinking water problems are of microbiological origins and are caused by inadequate or improper water treatment. The general properties of water for example, organic, inorganic and chemical constituents of water also affect the prevalence of microorganisms in water (Pontius, 1990).

Contaminated surface waters tend to purify themselves once the source of contamination is removed while groundwater tend to stay contaminated because the water moves slowly and the source of contamination is difficult to remove (US Environmental Protection Agency, 1990). The public health concern with both the quality and safety of drinking water has stemmed from the following:

- Wide spread publicity concerning the contamination of groundwater with organic chemicals.
- Pesticide infiltration of some drinking water suppliers.
- The presence of chloroform and other by-products in drinking water as a result

of disinfection of surface water by chlorine.

- Elevated levels of sodium in drinking water from natural sources.
- Leaching of lead from old lead pipes and from lead solder in newer pipes.
- And most importantly, microbial contamination from source waters.

The main problem to water quality in third-world countries is fecal contamination. Studies have shown the link between surface water quality and sanitation systems (World Health Organization, 1992). Surface waters in areas without proper sanitation systems tend to be more fecal contaminated. When communities consume these waters without treatment they might be exposed to enteric pathogens and opportunistic pathogens. Exposure of the rural communities to these pathogens explains the high incidences of water-borne disease outbreaks in rural communities. Water-borne disease outbreaks are generally reported during the warm and rainy season (World Health Organization, 1992). These water-borne disease outbreaks are due to the fact that on top of the high average daily temperatures, runoff's is also increased leading to more fecal contamination of source waters. This is due to the drainage or flooding of contaminated surface water into unprotected well shafts (World Health Organization, 1993).

Even in communities that receive treated water and have adequate sanitation infrastructure, water-borne infections may still occur although the incidences may be limited. Some of the facts that may lead to this are pathogens that are not destroyed by the treatment processes for example, *Cryptosporidium* and *Giardia* cyst, pathogens within biofilms and pathogens that are introduced along distribution systems. Water can also be contaminated during storage. The provision of safe drinking water and sanitation systems

is indispensable in the fight against water-borne infections (Howard *et al.*, 1987).

The real measure of the effects of the physical improvement made for communities is the part played by water and sanitation in the improvement of heath and well-being of their inhabitants. To ensure that health is improved to the fullest, an effort is required from all parties concerned for rapid provision of water supplies and sanitation, particularly in rural population (Kerr, 1995).

Dealing with water and sanitation, the following are recommendations:

- Water supply and sanitation should be regarded as an integrated component of primary health care and community development.
- In order to combat water-borne diseases or diseases related to inadequate water supply and sanitation more efficiently, the installation of sanitary excreta disposal facilities should be encouraged, with measures taken to the disposal of waste and improve personal and food hygiene.
- It should be ensured that the communities and individuals are not only made fully aware of the relationship between water, sanitation hygiene and health but also that they are motivated and given the facilities and assistance to participate in stages of improving their own living conditions. Work must be intensified in providing encouragement to the communities to organize for self-help accompanied by necessary education to do this (World Health Organization, 1996).

In the above context, the exchange of ideas for practical, low cost water and sanitation project is of paramount importance. Most of the work can be done using simple methods and local resources.

Many parameters must be taken into consideration in the assessment of water quality such as source protection efficiency and reliable treatment and protection of the distribution networks. The cost associated with water quality surveillance and control must also be carefully evaluated before developing national standards (World Heath Organization, 1996). Some of these parameters are discussed below.

a) Ensuring a safe water supply

Access to safe water is a basic requirement for health. Since contaminated water is the usual source of infectious agents, all efforts must be made to provide drinking water as well as safe water for food preparation and bathing. The supply of water must be of good quality, affordable, and available to all who need it at any time. The World Health Organization (1992) recommends that in urban areas, properly treated drinking water should be made available to the entire population through a piped system. In rural areas where there is no piped water, water from wells and springs should be vigorously boiled before use or treated with chemicals to destroy diarrhea carrying pathogens. A supply of suitable chemicals for treating water and narrow-mouthed pots with covers for storing water, are helpful in secondary transmission of waterborne pathogens. Household filtration of water can also help to eliminate the *Vibrio* species, but should always be followed by disinfection with chlorine or by boiling (World Health organization, 1992).

b) Sanitation

Good sanitation can markedly reduce the risk of transmission of intestinal pathogens. This is especially true where the lack of good sanitation may lead to contamination of clean water sources. The basic principles of sanitary human waste disposal, as well as ensuring the availability of safe water supply, should be given high priority. Appropriate facilities for human waste disposal are basic needs to all communities. In the absence of such facilities, there is a high risk of diarrhoea diseases. The World Health Organization (1993)'s recommendation towards implementation of sanitation systems is that sanitation systems that are appropriate for the local conditions should be constructed with the cooperation of the community.

2.7 Importance of health education in water and sanitation

There are three equal elements for health improvement: safe drinking water, good sanitation and health education. Often, drinking water becomes unsafe because of poor sanitation. An understanding of the source of fecal contamination and transmission of diseases from the origin of contamination can lead to a demand for good sanitation and health education. The greatest need for excreta related health education is concerned with children's fecal matters. The prevalence of diarrhoea and worms among children and their tendency to defecate wherever they happen to be, result in serious health hazards (Kerr, 1995). When large group of people congregate for fairs or funerals, particular care must be taken to ensure safe disposal of human waste and provision of adequate facilities for hand-washing for those people (World Health Organization, 1993).

In Kwa-Zulu Natal in South Africa, the uMhlathuze's City Engineer's Department has undertaken an urgent and immediate education campaign in the Mandlazini area to educate residents about the proper use of toilet pits and keeping water sources safe (Official newsletter of the uMhlathuze municipality, 2006). The Mandlazini community members were taught how to use toilets, about the dangers of defecating on the ground or in/or near drinking water sources, and about the importance of thorough hand-washing after any contact with the excreta. This education campaign was successful. There was a marked reduction in water-borne disease outbreaks which, usually occur in rainy and summer seasons.

2.8 Source water quality

Microorganisms in distributed water mostly originate in the water source. High quality groundwater may be characterized as containing less than one cfu/100ml and heterotrophic bacteria that are often less than 10 cfu/ml (Pontius, 1990). These microbial qualities show little fluctuation because of groundwater aquifer protection from surface protection. Sometimes groundwater, however, is not insulated from surface contamination. Agricultural fertilizer runoff can contain nitrate and improperly protected landfills may introduce a variety of organic matters, many of which are biodegradable (Pontius, 1990). In such situations, bacterial populations in groundwater become excessive. Surface water sources are subject to a variety of bacterial contamination introduced by storm-water runoff over the watershed and the upstream discharges of domestic and industrial wastes. Lake turnovers, decaying algae blooms and bacterial nutrients conditions deteriorate water quality. These lake turnovers introduce a wide range of organisms (some of which may be pathogens) to the water source intake that

may pass through marginal treatment processes or improperly operated treatment systems (Mays, 2004).

Water supplies using a single treatment barrier for surface water treatment will not prevent a variety of organisms from entering the distribution system (Kerr, 1995). Many of these microorganisms are neither immediately killed by disinfectants they are exposed to nor are insufficient contact times allowed, suitable to control the growth of coliforms and kill viruses (Pontius, 1990). Properly operated treatment processes are effective in providing a barrier to coliforms and pathogenic microorganisms reaching the distribution system. This does not, however, preclude the passage of non-pathogenic microorganisms through the treatment systems and processes (Mays, 2004).

2.9 Source water protection

The most critical element necessary for protecting the nation's drinking water supplies is source water protection. Water source protection consists of delineating the sources of water, inventorying the potential sources of water contamination in those areas and identifying the sources of contamination (Mays, 2004). Understanding the conditions of the source and identifying environmental factors such as pH, temperature, potential intermediate host of pathogens may provide a more comprehensive perspective of the source of water.

Pollution of water sources is a problem of increasing concern worldwide. In many countries, especially in developing countries, the number one water quality challenge is contamination by fecal organisms (Biyela, 2003; Official newsletter of the uMhlathuze

Municipality, 2006). The presence of fecal coliform bacteria in water indicates recent contamination by fecal matter (Guan *et al.*, 2002). Human population growth, inadequate sewage disposal systems and inappropriate management of animal waste, especially related to animal feeding operations, are all contributing towards worsening of the problem of fecal contamination (Carson *et al.*, 2001). The use of fecal contaminated water by the communities without adequate treatment poses a high risk of infection by water borne pathogens. Not only is contaminated water unsuitable for drinking, bathing or watering plants and for household use, they all have implications in transmitting fecal pathogens from contaminated waters (Carson *et al.*, 2001).

Traditionally, counts of commensal coliform bacteria have been used to indicate the potential presence of pathogenic microorganisms of intestinal origin as well as to indicate the general microbial quality of waters and its suitability for human consumption. Total and fecal coliform counts are useful for estimating fecal pollution levels but give no indication of the specific sources of microbial pollution, such as human fecal waste, farm animal, pets, or migratory birds (Carson *et al.*, 2001). Hence, all water quality monitoring detection techniques based on the use of indicator organisms have one major shortcoming – failure to identify the source of contamination. It is with further research that studies have been able to develop indicators that are generally source discriminating (Scott *et al.*, 2002). Total coliforms indicating the general quality of water include fecal coliform and other coliform groups which might be endemic to soil and water. Fecal coliforms associated with gastrointestinal tracts of warm-blooded animals when present in water may indicate recent fecal contamination. For decades, the use of pollution indicators saved lives and has proved to be a successful technique (Sargeant, 1999).

Contamination of source waters result in increased health risks to persons exposed to the contaminated water. The increased health risk results from the degradation of recreational and drinking water quality and also nutrient loss from watersheds to surface waters (Hagedorn *et al.,* 1999). Therefore, knowledge of pollution sources could aid in the restoration of the water quality, reduce the amount of nutrients leaving the watersheds, and reduce the danger of infections resulting from exposure to contaminated waters. The Protection Agency's National Database, 305b, (1999) and Hagedorn *et al,* (1999) have reported that fecal coliform bacteria are the widespread problem in rivers and streams, and agriculture and pastureland contributes much of the fecal coliform bacteria in waters.

Identification of the sources of fecal bacterial contamination is an essential first step in seeking control measures for fecal contamination of source waters. In particular, it is important to determine whether the source of fecal contamination is of human, livestock, or wildlife origin. Microorganisms of human origins are regarded as having greater potential to cause disease in humans and therefore waters contaminated with such are regarded as posing a higher risk to human health (Guan *et al.*, 2002).

Multiple antibiotic resistance (MAR) analysis has been used to differentiate bacteria from different sources using antibiotics that are commonly used for human therapy and in animal therapy or animal feeds. In MAR indexing, either *E. coli* or fecal *Streptococci* from different animal sources are analyzed to determine their resistance patterns to different types and concentrations of antibiotics (Sargeant, 1999).

2.10 Rational behind microbial source tracing

Contamination of water by fecal coliform bacteria from human and animal origin may signal the potential presence of human enteric pathogens such as *Salmonella* spp. and *Shigella* spp. The possible sources of fecal contamination include surface runoff's waters from manure-treated agricultural land or from animal feedlots, inadequate septic systems and sewer overflow and from wildlife animal feces (Scott *et al.*, 2002). However, waters contaminated with human feces are mostly likely to have human specific pathogens and therefore can pose a greater health risk. Thus determining the sources of fecal pollution is necessary to develop effective control strategies (Dombek *et al.*, 2000).

Tracing non-point sources of microbial contamination can be achieved through the use of various microbiological, genotypic, phenotypic and chemical methods to characterize groups of microorganisms. These indicator organisms are used for the purpose of detecting the subtle genotypic or phenotypic differences present among different groups of microorganisms. These differences can be subsequently used to identify the host or the environment from which the organisms were derived (Scott *et al.*, 2002). Bacterial source tracing can reliably determine whether fecal bacteria are from human or animal sources. When these are from animal sources, bacterial source tracing can also indicate whether the animal source is livestock or wildlife (Carson *et al.*, 2001; Guan *et al.*, 2002).

Genetic methods can be used to differentiate lineages of bacteria found within different animal hosts. This methodology assumes that if the same species of bacteria from different host environments become adapted to a particular environment and establish residency, the progeny produced by subsequent replications will be genetically identical.

Therefore, over time these microorganisms should possess similar or identical fingerprints which will differ from organisms adapted to a different host environment (Scott *et al.*, 2002).

Microbial source tracing methodologies that focus on phenotypic differences within different lineages of bacteria usually focus on traits that may have been acquired from exposure to different host species or environments. These methods usually target MAR patterns, cell surfaces or flagella antigen or biochemical tests designed to identify variations in the utilization of various substrates that may be found within a particular host environment (Scott *et al.*, 2002).

Direct monitoring for human pathogen has also been used as a means of identifying the presence of human or high risk fecal pollution in water (Sargeant, 1999). Monitoring for pathogens provides direct evidence of their presence and thus circumvents the need to assay for often ambiguous indicator organisms. However, this method is not reliable because many pathogens are not readily detectable in the environment as they are often present in low numbers and their detection does not identify their source (Scott *et al.*, 2002).

Chemical compounds have also been proposed as indicators of human and animal-derived fecal pollution (Wiggins, 1996). Chemical indicators are natural byproducts of human metabolism or activity. Chemical indicators of fecal pollution in water can be broadly divided into those present in feces (direct) and those strongly associated with fecal discharges (indirect) (Sinton *et al*, 1997). The use of chemical indicators of fecal contamination is unique. These methods depend on the metabolism, transportation of the

chemical and persistence of these chemicals to pathogens they are being used to predict (Sargeant, 1999).

2.11 Caffeine as an indicator chemical of fecal pollution

One of the widely accepted chemicals in differentiating between human and non-human contaminated waters is caffeine (Sargeant, 1999). Caffeine is present in feces and may be detected in water polluted with fecal material. It is present as a stimulant in coffee, tea, cocoa, soft drinks and chocolate (Sargeant, 1999). Caffeine is also a component in many prescriptions and over-the-counter drugs, ranging from analgesics to cold medicines. The average human consumes considerable amounts of caffeine. However, caffeine is extensively metabolized with only approximately 3% excreted unchanged in the urine (Sigma catalogue, 2005). Nevertheless, there is far more caffeine introduced to the sewage system by disposal of unconsumed coffee, tea or soft drinks down the sink, and rinsing of coffee pots and cups.

Research suggests that the presence of caffeine in the environment can serve as an indicator of the presence of human sewage (Gardinali and Zhao, 2002). Levels of caffeine in domestic wastewater have been measured to be between 20 and 300g/liter (Sigma catalogue, 2005). Levels in receiving waters can however be much lower due to significant dilution (Gardinali and Zhao, 2002). Caffeine is considered a good, stable, dissolved marker directly related to human activities with no potential biogenic sources because of its high solubility, low octanol-water partition coefficient and negligible volatility (Scott *et al.,* 2002). This is of particular importance in environments where septic tanks contribute large amounts of wastewater discharges in comparison with

treated municipal wastewaters. As such, the detection of caffeine can demonstrate the presence of failing septic systems and other forms of human contamination.

The major problem with this method lies in its cost when it has to be used (Gardinali and Zao, 2002). Caffeine is also easily degraded by soil microbes, so it is not known what proportion of human sources actually contain detectable levels of caffeine. The rational behind the use of the caffeine method is to evaluate the presence of unmetabolized caffeine as a potential indicator of human fecal contamination.

2.12 The use of microbial pollution indicators in bacterial source tracing (BST)

Pollution indicator organisms are used to predict the potential presence of pathogenic microorganisms (Scott *et al.*, 2002). Ideally, they are non-pathogenic, rapidly detected, easily enumerated, have survival characteristics that are similar to those of the pathogen(s) of concern, and can be strongly associated with the presence of pathogenic microorganism(s) (Scott *et al.*, 2002). Total and fecal coliform bacteria have been used extensively for many years as indicators for determining the sanitary quality of surface recreational and shellfish growing waters (Scott *et al.*, 2002). Indicator organisms are useful in that they eliminate the need to assay for every pathogen that might be present in contaminated water.

E. coli and fecal *Streptococci* are the two fecal indicator organisms that are mainly used in multiple antibiotic resistance indexing technique (Krumperham, 1983). *E. coli* is abundant in feces, it is easy to isolate and a data for a number of studies using *E. coli* as an indicator organism are available.

The validity of the use of *E. coli* as an indicator organism for fecal contamination of water has been challenged but the application of water standards in foods has raised questions as well as criticisms (Krumperman, 1983). The native habitat of *E. coli* is the intestinal tract of humans and other warm-blooded animals. Its presence in food or water is generally considered to indicate direct or indirect fecal contamination and the possible presence of enteric pathogens (Krumperham, 1983). *E. coli* has the ability to survive for months externally to the colon and this makes it almost ubiquitous and the significance of its presence in food equivocal (Fearry *et al.*, 1972). *E. coli* might be present in an environment at a concentration much higher than the pathogens it predicts; hence studies suggest that *E. coli* may not be a reliable indicator in tropical and subtropical environments due to its ability to replicate in contaminated soil (Nollet, 2000; Scott *et al.*, 2002).

In the early 20th century, *E. coli* as an indicator organism could not be directly discriminated from *Klebsiella* spp, *Enterobacter* spp and *Citrobacter* spp and even in some *Aeromonas* spp. These groups of microorganisms were designated as and were defined as aerobic or facultative aerobes, gram negative non-sporulating rod shaped bacteria that ferment lactose at 35-37°C with gas formation (Nollet, 2000). Most of these organisms were considered to be of fecal origin. At present time, research indicates that *E. coli* is the only coliform organism that is considered to be primarily of fecal origin (Nollet, 2000), and that makes it an ideal indicator organism of fecal contamination. Although the use of fecal coliform counts as an indicator of water quality is significantly improved, fecal coliforms are not exclusively *E. coli* but also include a few other organisms that are not necessarily of fecal origin for example, thermostable *Klebsiella*

species (Tebbutt, 1998).

2.13 Human feces as reservoir of pathogenic microorganisms

The greatest risk of fecal contamination to humans originates from humans, poultry and swine. Poultry is the permanent reservoir of *Salmonella* species. Swine harbors *Shigella* spp, *Salmonella* spp, enteropathogenic *E. coli* and other pathogenic microorganisms (Krumperman, 1983). A procedure which would distinguish between *E. coli* originating from these high risk sources and *E. coli* originating from other sources could be useful in monitoring and managing the microbial quality of source waters.

2.14 Exposure of microbial communities to antibiotics and the emergence of antibiotic resistance in environmental bacteria

The wide and often indiscriminate use of antibiotics in medical and veterinary practices as well as domestic and agricultural use of pesticides and related compounds has resulted in the establishment of a library of antibiotic resistant organisms in the environment (Champe and Harvey, 1994). Halling-Sorensen *et al.* (1998) have indicated that about 70 to 80% of drug administered in fish farms end up in the environment and some drug metabolites were found in the sediments underneath fish farms. This may provide the opportunity for exposure of bacterial populations to antibiotic resistance genes and the opportunity for the transfer of these genes back into human and animal pathogens. The presence of many different antibiotics in the environment enhances the development of multiple antibiotic resistance among environmental bacteria (Fluit *et al.*, 2001). The development of multiple antibiotic resistance among bacterial community may have insignificant health and economic impacts (Lynn and Solotorovsky, 1981).

The primary aim of the development of antibiotic resistance in microbial communities is to protect themselves from being destroyed by antibiotics. However, antibiotic resistance genes existed before the era of antibiotics and antibiotic-producing organisms are not the only potential source of antibiotic resistance mechanisms (Davies, 1994). It is apparent in antibiotic modification that there is a substantial pool of antibiotic resistance genes in nature. Gene fluxes between bacterial replicas and their hosts are likely to be the rule rather than the exception and appear to respond quickly to environmental changes. This gene pool is readily accessible to bacteria when they are exposed to strong selective pressures of antibiotic usage, that is, hospitals, for veterinary, agricultural purposes, and as growth promoters in animal and poultry husbandry (Davies, 1994).

At the present time it is not possible to conceive ways of avoiding the selection of antibiotic-resistant bacterial mutants that appear during the course of antimicrobial therapy. However, the resistance gene pool would be of no use unless the bacteria had the means to access this collection to their advantages (Davies, 1994).

2.15 The importance of the study

At the present rate of water availability and consumption, it seems as if most of the developing countries will run out of water in the near future. The lack of potable water, especially in rural areas, has serious health implications. At present the HIV/AIDS pandemic has resulted in many people being infected by the virus (Tebbutt, 1998). According to the Millennium Development Goals Review report in 2007, by the end of 2006, about 3.9 million people worldwide were living with HIV (Gumbi, 2007). Most of these people are in undeveloped countries in Sub-Sahara Africa. The number of people

dying from AIDS worldwide increased to 2.9 million in 2006, from 2.2 million in 2001 and it seems as if preventative majors are failing to keep pace with the growth of the epidemic. In 2005 more than 15 million children had lost one or both parents due to AIDS (Gumbi, 2007), a devastating effect to affected community.

Tuberculosis, malaria, cholera and other waterborne diseases are also major health concerns that are associated with inadequate supply of purified water (Pretorius, 2000). Some of the environmental issues include the growing shortage of water for industrial, agricultural and domestic consumption. In the globe of water "stress", source water protection, water quality and adequate water supply are pathways to reduce disparities so to accelerate the process of finding solutions to improve health outcomes for vulnerable populations. Recently, more than half of the world's major rivers are contaminated, thus threatening the health and livelihood of the people who depend on them for drinking, industrial and recreational use (Rand Water, 2007).

Tracing the sources of fecal contamination is the first step in seeking control measures for fecal pollution of surface waters. This study will aid in the development of implementation programs and as well as the development of effective control strategies for fecal contamination control. Several attempts to develop methods to determine the sources of fecal contamination have been made and to date most have proven useful. Compared to methods such as genotypic, the MAR indexing method is cost-effective and easy to perform. It is simple and does not require specialized training and expensive equipment. The MAR indexing method has also been chosen because it is the best method available for rapid source identification on a large number of isolates that are

needed to obtain a statistically valid sample size. However, cross validation with other well-established methods such as genetic fingerprinting could be useful in case one method is more effective than the other is (Carson *et al.*, 2001).

CHAPTER 3

3. METHODOLOGY

3.1 Collection of samples

3.1.1 Humans

For *E. coli* isolates from human origin, sewage samples were used. The samples were obtained from the Kwa-Dlangezwa sewage treatment plant. This plant serves the University of Zululand and the broad Kwa-Dlangezwa community.

The people of the University of Zululand community are exposed to antibiotics and use them for disease control and treatment and the route is either oral or through injection (see Table 3.2).

The samples were collected from three collection points which were:

Collection point 1: This is the nearest and closest point to the University of Zululand community and campus buildings. This point is not contaminated with any other fecal matters except human fecal waste since it is the closest point to the campus.

Collection point 2: This is the sewage receiver. It is the inlet of the sewage treatment plant.

Collection point 3: This is where the waste is mixed and then later filtered. The

possibilities that the latter two points are contaminated with fecal matters other than humans are high since they are open.

Samples were collected between 7.00-8.00 am in the morning when most toilet activities take place. The samples were collected in 500ml sterile bottles and transported to the laboratory using a cooler box with ice and were analyzed within 4 hours.

3.1.2 Animals

E. coli from animals were obtained from domestic as well as wild animals.

3.1.1.1 Domestic animals

To obtain *E. coli* from domestic animal origin, fresh rectal swab were obtained from sheep, swine and cattle from the University of Zululand farm as well as from chicken from a rural family at Kwa-Dlangezwa.

Domestic animal sources used are listed below and the description of each and antibiotic exposure provided.

a) Cattle

Cattle from the University of Zululand, Department of Agriculture were fed their normal diet, which included grass and leaves from trees. In addition to their normal diet, they were also fed commercial food enriched with antibiotics. These antibiotics are for milk production and for growth promotion. When sick, the cattle were treated with tetracycline and berelin (information obtained from farm manager).

b) Swine (pigs)

Swine from the University of Zululand, Department of Agriculture were only fed commercially produced feeds. When these animals were sick, they were treated with different types of antibiotics for disease control and were sometimes vaccinated for diseases prevention. Swine were often treated with medication to remove ticks and for deworming. The table below (Table 3.1) shows the nutritional composition of the Breedmor sawcare with which the cattle and swine were fed. However, this table does not indicate any information about antibiotics added to the breedmor sawcare.

Table 3.1: The nutritional composition of the Breedmor sawcare.

Nutrient	**Quantity (g/kg)**
Protein	135.0
Fat	25
Fibre	80
Moisture	120
Calcium	8-12
Phosphorus	6
Total Lysine	7

c) Sheep

Sheep from the Department of Agriculture farm (University of Zululand) were only fed with their normal diet, which included leaves from trees and grass. No commercial feed enriched with antibiotics were given to them. However, these animals receive, from time to time, antibiotics for disease control and growth promotion. This medication includes deworming medicine and medicine to remove ticks.

d) Chicken

Chicken fecal samples were collected from a rural family near the University of Zululand. These chickens are not fed with any specified or special diet. They are free roaming and find food for themselves. The food they consume include maize, maize meal, leftovers food, some insects, etcetera. These chickens are not receiving any medical treatment or antibiotics even when they are ill. In general, rural families do not subject their domestic animals to treatment even if they are ill.

Rectal swabbing was used to obtain domestic animal samples. From each group of animals, the swabs were collected once from twenty (20) animals. The samples were collected in the morning at about 8.00 am and transported to the laboratory using a cooler box with ice. Samples were analyzed within 4 hours.

3.1.1.2 Wildlife animals

To obtain *E coli* isolates from wild animal origin, fresh fecal samples were collected from wild animals at the Johannesburg Zoo. The animals used included African buffalo, Eland, Gemsbok and Bushbuck.

These animals in the Zoo were fed commercially produced feeds and semi-dried grass. The feeds for these animals contained no antibiotics. The African buffalo received treatment with various medicines when it was ill. Eland, Bushbuck and Gemsbok never received medical treatment or antibiotics.

From the African buffalo and Eland, only fresh fecal samples were used while both rectal swabs and fresh fecal samples were taken from the Bushbuck and gemsbok. The samples were stored on a cooler box with ice. They were transported to the laboratory and were analyzed within 8 hours after collection

3.2 Isolation of *E. coli*

One gram of a fecal sample was added to 10ml nutrient broth. The animal swabs were placed on the mouth of the test tube containing nutrient broth and the applicator stick was cut immediately above the swab with sharp sterile scissors and the applicator stick was used to submerge the freed swab in the broth.

Inoculated test tubes were incubated at 37°C overnight to allow the growth of fecal coliform bacteria. After incubation, a loopful of culture from each test tube was streaked on Levine Ethyl Methylene Blue (EMB) agar plates (Appendix A2). The plates were

incubated for 24 hours at 37°C. Suspected *E. coli* colonies (colonies with gold metallic sheen) were selected and sub cultured on nutrient agar plates (Appendix A1), to obtain pure cultures.

3.3 Identification of the isolates

To confirm whether the isolated colonies were *E. coli* or not, the colonies were diagnosed by Gram reaction (Appendix A3). The colonies were further evaluated with the indole, methyl red, voges-proskauer test and citrate reaction (IMViC test) as well as hydrogen sulphite production (Biyela, 2003) (Appendix A4, A5, A6 and A7).

3.4 Antibiotic selection

Antibiotics used in this study were chosen in order to permit the comparisons with previous similar studies (Wiggins *et al.*, 1999; Carson *et al.*, 2001; Guan *et al.*, 2002; Scott *et al.*, 2002;). Antibiotics were also chosen to reflect antibiotics to which farm animals are exposed and to include the antibiotics commonly used in human therapy. Choosing the antibiotics to which *E. coli* is naturally susceptible (excluding penicillin G), was also considered in this study.

Table 3.2: Antibiotics and their concentrations used in the study as well as their application to humans and animals.

Antibiotic name	**Concentration used (µg/L)**	**Spectrum of use in:**	
		Humans	Animals
Chloramphenicol	16	O, I, DC	
Tetracycline	16	O, DC	
Neomycin	52	O, I, DC	P, C, S, DC
Nalidic acid	25	O, DC	
Kanamycin	8	O, I, DC	
Penicillin G	75	O, I, C	P, DC, GP, EP; C; S, GP
Sulphothiazole	500	O, DC	
Ciprofloxacin	10	O, I, DC	
Gentamycin	8		I, DC
Cephalothin	16		I, DC

Key to the table:

O – Oral

I – Injection

DC – Disease control

P – Used in Poultry

GP – Growth promotion

EP – Egg production

S – Used in swine

C – Used in cattle

The concentrations of antibiotics used reflected previously used concentrations to permit comparisons with the previously conducted studies (Wiggins *et al.*, 1999; Carson *et al.*, 2001; Guan *et al.*, 2002; Scott *et al.*, 2002).

3.5 Antibiotic sensitivity test

Antibiotic sensitivity assay was carried out using 96 well microtiter plates. In a microtiter plate, each isolate was tested for antibiotic resistance against ten antibiotics in different wells. For antibiotic resistance test, 170µl of nutrient both was pipette to each well. Ten microliters of the culture/bacterial suspension was added to the nutrient broth and this was followed by the addition of 20µl of antibiotic to make the total volume of 200µl. The concentrations of the stock solution of antibiotics are shown in Table 3.2. The plates were incubated at 37°C for 18 to 24 hours (Scott *et al,* 2002).

In this procedure, two wells were used as control wells in each test sample in the microtiter plate. The first control was a well containing nutrient broth inoculated with the bacterial suspension only. The purpose of this control was to compare the growth of bacterial suspension in this well with others containing antibiotics. Uninoculated nutrient broth was used as a second control. The purpose of this control was to confirm whether there is growth of bacterial suspension and also whether there is resistance. Since the observations were qualitative, these controls were used to score the various isolates for growth.

Qualitative visual observation was used to make judgments of resistance/susceptibility of *E. coli* isolates to antibiotics. *E. coli* isolates were considered resistant to an antibiotic only if their growth in the presence of antibiotics was as well developed as their growth on the control well. Any sign of inhibition or sensitivity was considered to be indicative of nonresistance/susceptibility (Hagedorn *et al.,* 1999; Carson *et al.,* 2001).

3.6 Data processing and indexing (results analysis)

A stepwise logistic regression model was used for statistical analysis using software SPSS 13.0, 2005 version, to analyze the data. The model used is represented by the equation: prob (event) = $1/1+e^{-(\alpha+B_1X_1+B_2X_2+\ldots\ldots+B_kX_k)}$. Using this model, several tests were done including accuracy prediction (a measure of how well the model performs its ability to accurately classify *E. coli* according to their sources), chi-square test (statistical test of the null hypothesis), degree of freedom (df), Wald test, standard error (SE) and likelihood test (goodness-of-fit test).

The logistic regression model was used to attain the highest predictive accuracy possible using antibiotic resistance patterns obtained from *E. coli.* The data obtained were grouped to provide profiles or indices for a single group of isolates, samples and from the environment where the isolates were obtained for the purpose of evaluation of the health risks.

3.7 Caffeine detection

In this study, the determination of the presence of caffeine in samples was carried out using thin layer chromatography (TLC). A silica gel that has a fluorescent coating was used to visualize caffeine on the TLC. In this procedure, a known quantity of caffeine (standard) was analyzed along with the experimental samples to determine the presence of caffeine.

A pencil dot of about 1 cm from the bottom and about 0.5 cm from the edge was placed on a TLC plate that had been cut to an appropriate size. The dots were spaced evenly across the bottom to match the number of samples to be analyzed.

Five microliters of 1% standard solution was placed into the TLC plate on the first pencil dot and placed on a hot plate to facilitate evaporation. To keep spots small and compact, the solution and the samples were applied in several portions with intermediate drying. Test samples were also added in the same way as the standard solution to the TLC plate. After the addition of all the samples, the TLC plate was placed into the jar containing solvent (ethyl acetate), making sure that the sample dots did not go under the level of the solvent and waited for 10 minutes for the solvent to move up the TLC plate. The TLC plate was investigated under UV light at 254nm to visualize the chromatogram. A digital camera was used to capture the images. Where caffeine was detected, serial dilutions of the standard were made and were read against the test samples for the determination of caffeine.

The R_f value (retention factor value) was used for the qualitative evaluation of caffeine. This value is defined as: R_f = distance starting line – middle spot/distance starting line – solvent front = b/a (Sherma and Fried, 1996). R_f value defines how far up the plate the compound has traveled.

CHAPTER 4

4. RESULTS

4.1 Classification of *E. coli* isolates according to antibiotic resistance patterns

All isolates were Gram negative rod shaped organisms that fermented lactose with gas formation within 48 hours at 37°C. All cultures were isolated using Levine EMB agar and the isolates with metallic sheen were isolated. Cultures with these characteristics were identified as presumptive *E. coli* using biochemical tests (IMViC test)

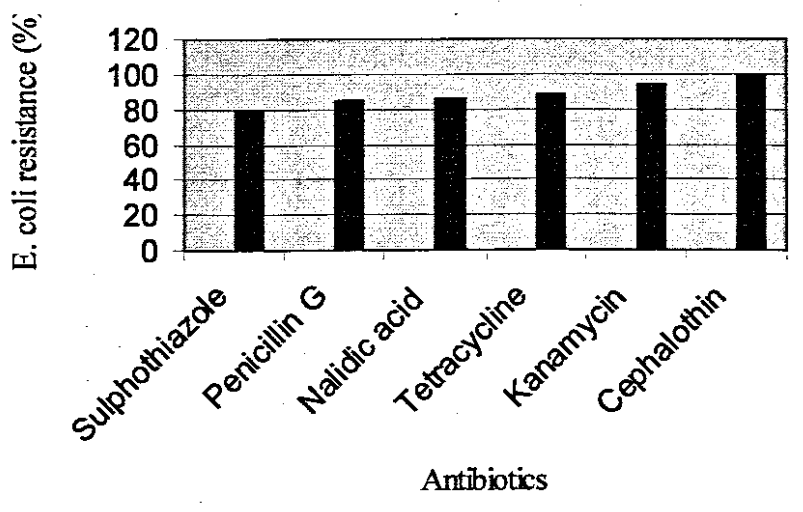
A total of 322 putative *E. coli* isolates were identified from different animal sources and human sewage, as described in Chapter 3. These isolates were tested against 10 antibiotics at the different concentrations shown in Table 3.2. About 30% *E. coli* were isolated from human source, another 30% from domestic animals and 40% from wild animals. This made a total of 30% human *E. coli* and 70% non-humans. More human *E. coli* were isolated compared to other animal sources so that certainty would be gained when humans are compared to non-humans during classification (Table 4.1).

The table below (Table 4.1) shows the sources of isolates, the number of isolates per source and the marginal percentage of the isolates from each source when all isolates from different sources were combined. The data in this table (Table 4.1) were extracted from the antibiotic resistance patterns of *E. coli* (Appendix B). The table also shows the sensitivity of all the isolates used in the study against antibiotics used for example, out of all 322 isolates used in this study, 278(86.3%) were resistant to nalidixic acid while 44

(13.7%) were susceptible.

E. coli has shown between 80-100% resistance to 6 different antibiotics respectively. Figure 4.1 indicates antibiotics to which *E. coli* was more resistant. Excluding Penicillin G, all antibiotics chosen were antibiotics to which *E. coli* is naturally susceptible. There were four respective antibiotics to which *E. coli* was 28-97% susceptible. Forty percent of the isolates were from wild animals. A greater percentage of isolates from humans, compared to wild and domestic animals were resistant to multiple antibiotics. This study did not establish whether the resistance/susceptibility patterns has been affected by their "unnatural" environment or not, since the wild animals were kept in a zoo. The percentage of sensitivity patterns of *E. coli* isolates shown in this table does not reflect the general pattern of sensitivities of *E. coli* isolates in the environment but rather the isolates used in this study.

Figure 4.1 Antibiotics to which *E. coli* was 80-100% resistant



Of the 322 *E. coli* isolates used, all were resistant to two or more antibiotics (Appendix B). A greater percentage of human *E. coli* was resistant to more antibiotic than did animal *E. coli*. All human isolates were resistant to cephalothin while only 1 (1%) was resistant to neomycin (Appendix C). A marked decrease in the percentage of wild animal *E. coli* resistant to antibiotics was observed among wildlife animals. These results are consistent with other reports which reported that multiple antibiotic resistance patterns of fecal *E. coli* from animals were generally lower while humans had higher resistance patterns (Guan *et al.*, 2002; Scott *et al.*, 2002).

Table 4.1: Sources of isolates used and their sensitivity patterns to antibiotics

Antibiotics	Sources of the Isolates		Number of Isolates	Marginal Percentage of the isolates
		Sheep	35	10.9%
		Humans	102	31.7%
		Swine	39	12.1%
		Bushbuck	26	8.1%
		Buffalo	25	7.8%
		Gemsbok	34	10.6%
		Eland	25	7.8%
Chloramphenicol		Resistant	43	13.4%
		Susceptible	279	86.6%
Nalidic acid		Resistant	278	86.3%
		Susceptible	44	13.7%
Neomycin		Resistant	29	9.0%
		Susceptible	293	91.0%
Penicillin G		Resistant	276	85.7%
		Susceptible	46	14.3%
Kanamycin		Resistant	303	94.1%
		Susceptible	19	5.9%
Tetracycline		Resistant	286	88.8%
		Susceptible	36	11.2%
Sulphothiazole		Resistant	258	80.1%
		Susceptible	64	19.9%
Ciprofloxacin		Resistant	9	2.8%
		Susceptible	313	97.2%
Gentamycin		Resistant	91	28.3%
		Susceptible	231	71.7%
Cephalothin		Resistant	322	100.0%
		Susceptible	0	0.0%
Valid			322	100.0%
Total			322	100.0%

4.2 Antibiotic resistance patterns of the isolates against antibiotics

E. coli isolates were resistant to one or more antibiotics and were all resistant to cephalothin. Results of the antibiotic sensitivity patterns are presented in Appendix C. Chicken, sheep and swine isolates were all susceptible to chloramphenicol while all Gemsbok isolates were all resistant. Out of 322 *E. coli* from all the sources used, 279(86.6%) isolates were susceptible to chloramphenicol while 43 (13.4%) were resistant. This shows that most isolates were susceptible to chloramphenicol (Table 4.1; Appendix C).

The 6 antibiotics to which 80-100% of *E. coli* isolates were resistant (Figure 4.1), belong to different groups and have different modes of antimicrobial action. Excluding penicillin G, these antibiotics are effective against gram negative microorganisms (Frankline and Snow, 1981). However, some of these antibiotics are broad spectrum (Margaret and Stucke, 1982). Eighty six percent of *E. coli* were resistant to Penicillin G. Gram negative microorganisms are naturally resistant to penicillin G and this is because of their cell wall characteristics. Penicillin has a Beta-lactam ring. It is an inhibitor of the cell wall synthesis. It acts by binding to the penicillin binding proteins in the final stages of the cross-linking of bacterial cell wall structure, thus result in cell lyses (Madigan et al, 2000). Penicillin also has a low toxicity to microorganisms and is easily inactivated; as a result most *E. coli* are naturally resistant to it (Howard *et al.*, 1987).

Tetracycline is a broad spectrum antibiotic in current use (Champe and Harvey, 1994). Eighty eight (88) percent of *E. coli* was resistant against this antibiotic (Table 4.1). Tetracycline and Nalidic acid have shown similar antibiotic resistance patterns against *E.*

coli from humans and wildlife sources. About 86% *E. coli* were resistant to Nalidic acid (Table 4.1).

All *E. coli* were resistant to Cephalothin. Cephalothin displays good activities against gram positive and in some gram negative microorganisms like *E. coli* (Lynn and Solotorovsky, 1981). The mechanism of action of Cephalothin is similar to that of penicillin (Howard *et al.,* 1987). The high resistance shown by *E. coli* was probably because of the inactivation of cephalosporin by penicillinase.

4.3 Statistical analysis of the results

Two steps were followed in classifying/grouping *E. coli* according to their sources. These steps arise from the statistical model used (section 3.6). The steps are differentiated by the use of different antibiotics. In the antibiotic resistance test, the activities of some antibiotics were similar to all isolates. During classification these antibiotics were computationally excluded because they make no difference in the analysis, for example, all the isolates were resistant to cephalothin, so including or excluding cephalothin in the analysis does not make any difference (Appendix B, see the sensitivity patterns against cephalothin).

Tables 4.2 and 4.3 present information about antibiotics in the model and antibiotics not included respectively. The exclusion of some antibiotics was because, in the analysis, logistic regression model assumes that all relevant predictors are included in the analysis and irrelevant predictors are excluded (Carver, 2005). Hence, only antibiotics which made a difference in classifying the sources of *E. coli* were included in the analysis.

These tables (Tables 4.3 and 4.4) also indicate the tests that were done. In Table 4.2, step 1, p-values for tetracycline and sulphothiazole are 0.000 and in step 2, the same p-values are obtained with tetracycline and gentamycine. These p-values indicate that there is a linear relationship between antibiotic use/exposure and antibiotic resistance profile (Carver, 2005). This relationship can be helpful in classifying *E. coli* according to their sources. In the same table, chloramphenicol ($p = 0.007$ in both steps), neomycin ($p = 0.001$ in step 1; 0.002 in step 2) and sulphothiazole ($p = 0.001$ in step 1) have low p-values. Their low p-values indicate that their overall effects are statistically significant. The estimated B coefficients and standard errors were calculated. These tests were based on Wald statistics (Wald test). The Exponentiated (B) is the exponentiated value of the B coefficient. The results for these tests are given in Tables 4.2 and 4.3. These coefficients estimate the effect of antibiotics in the classification of the sources of *E. coli*. They measure the significance of the statistical results.

Antibiotics that were not included in the classification model (in the analysis) are presented in Table 4.3. Hence, the B coefficient, Wald test and Exponentiated (B) coefficients were not tested. This table (Table 4.3) indicates that gentamycine is statistically significant ($p = 0.001$), in step 1. This means the inclusion and exclusion of gentamycine in the analysis has some effect in the overall classification of *E. coli*. Hence, in step two, in the same table, gentamycine was excluded. It was included for analysis in the same step in Table 4.2, to see its effect. The inclusion of gentamycine led to the increased percentage of the overall classification rates of *E. coli* according to the sources (Table 4.3). Nalidic acid, Penicillin G, Ciprofloxacin and Kanamycin results were not statistically significant. Their p-values were high. This meant these antibiotics had no

effect in the overall classification of *E. coli*; hence they were excluded in the analysis.

Table 4.2: Antibiotics used in data analysis

Antibiotics used in step 1 for analysis						
Antibiotics	B	S.E	Wald	df	Sig.	Exp (B)
Chloramphenicol[a]	2.793	1.041	7.195	1	0.007[c]	16.335
Tetracycline[a]	-2.086	0.500	17.395	1	0.000[c]	0.124
Neomycin[a]	3.375	1.030	10.746	1	0.001[c]	29.232
Sulphothiazole[a]	-2.479	0.622	15.784	1	0.000[c]	0.084
Constant	4.457	0.783	32.442	1	0.000[c]	86.261
Antibiotics used in Step 2 for analysis						
Chloramphenicol[b]	2.823	1.052	7.201	1	0.007[c]	16.820
Tetracycline[b]	2.579	0.538	23.005	1	0.000[c]	0.076
Neomycin[b]	3.138	1.034	9.219	1	0.002[c]	23.061
Gentamycine[b]	-2.448	0.630	15.096	1	0.000[c]	0.086
Sulphothiazole[b]	-1.104	0.372	11.396	1	0.001[c]	0.332
Constant	5.226[d]	0.843	38.431	1	0.000[c]	185.97

Keys to tables 4.2

a. Antibiotics used in the first step.

b. Antibiotics used in the second step, that is, final variables used in the model.

c. P-values very small, thus all variables entered are significant.

B – B-coefficient

S.E. – Standard error

Wald – Wald test

df – Degree of freedom

Sig. - Significant (p)

Exp (B) – Exponentiated value of B-coefficient.

Table 4.3: Antibiotics not used in the analysis

Antibiotics not used in step 1 in the analysis.			
Antibiotics	**S.E**	**df**	**Sig.**
Nalidic acid	2.078	1	0.149[a]
Kanamycine	0.895	1	0.344[a]
Penicillin G	1.086	1	0.297[a]
Ciprofloxacin	0.261	1	0.609[a]
Gentamycine	11.862	1	0.001[a]
Constant	14.998	5	0.01[a]
Antibiotics not used in Step 2 in the analysis.			
Nalidic acid	1.322	1	0.250[a]
Kanamycine	0.758	1	0.348[a]
Penicillin G	1.680	1	0.195[a]
Ciprofloxacin	0.290	1	0.590[a]
Constant	3.346	4	0.502

Keys to tables 4.3

a. P-values high, thus variables are not significant, thatis, parameters are equal to zero.

S.E. – Standard error

df – Degree of freedom

Sig. - Significant (p)

4.4 Classification and differentiation of samples of human and non-human origins

Accuracy prediction is the measure of the ability of the model to accurately classify *E. coli* according to their sources. Table 4.4 presents the accuracy prediction test results for classification of *E. coli* according to human and non-human sources. In step 1, 93/102 (90.3%) *E. coli* from humans were correctly classified. This is a very high percentage likelihood of accuracy, hence, a high percentage of correct classification. Sixty two point seven (62.7) percent *E. coli* from non-humans were correctly classified and 27.3% were incorrectly classified as humans. The overall classification accuracy in step 1 was thus 71.7%.

In stepwise analysis, using forward selection, step 2 has used 5 antibiotics in the equation while 4 antibiotics were used in step1. The inclusion of the 5^{th} antibiotic, gentamycin, in step 2, has improved the classification accuracy and an improved overall classification rate had been attained (Table 4.4). The improvement is due to the fact that gentamycin is significant ($p = 0.001$) (Table 4.3). It had an effect in the classification.

In step 2, 61(59%) human isolates were correctly classified and 41(41%) were classified as non-humans. Two hundred and ten (95.4%) non-humans *E. coli* isolates were correctly classified. The overall percentage of *E. coli* that were correctly classified according to their sources in this step was 78% (Table 4.4). The step in the model where fewer antibiotics were used had a slightly lower percentage of overall accurate classification (71.7%), than the step in which more antibiotics were included in the analysis (78%).

Table 4.4: Classification and differentiation of *E. coli* of humans from non-humans.

Observed	**Predicted**				Percentage (correct classification)
	Sources of the isolates				
	Humans		Non-Humans		
	number	%	number	%	
Step 1 : Humans	93	90.3	9	9.7	91.2
Non-humans	82	27.3	138	62.7	62.7
Overall percentage					71.7
Step2 : Humans	61	59	41	41	60.2[a]
Non-humans	10	4.5	210	95.5	95.5[b]
Overall percentage					78.0[c]

a. Percentage of human *E. coli* predicted correctly

b. Percentage of non-human *E. coli* predicted correctly

c. Overall percentage predicted correctly using the antibiotics: Chloramphenicol, Tetracycline, Neomycin and Gentamycine and Sulphothiazole. Adding more variables does not improve the prediction.

In antibiotic resistance and/or sensitivity test, some antibiotics were found to have the same activities against all the isolates, for example, resistance patterns of penicillin G, sulphothiazole and kanamycin. When only these antibiotics with similar antibiotic activities against the isolates are used for analysis in the study, all the isolates are classified as if they are from the same/one source. These antibiotics did make contribution to the analysis. Table 4.5 shows that all human source isolates were classified as non-humans, making the correct classification percentage to be zero and all non-humans classified correctly as non-humans, making correct classification to be 100 percent. This is only true if different isolates from different sources have the same or similar antibiotic resistance patterns. Table 4.5 is also a proof that only antibiotics with different antibiotic activities should be selected for the analysis because if the antibiotic activities are the same, the sources of the isolates are not differentiated.

Table 4.5: Results obtained when only the antibiotics with same antibiotic resistant patterns are used.

Observed	Predicted		
	Sources of the isolates		Percentage Correct
	Humans	Non-humans	
Humans	0 [a]	102	0
Non-humans	0	220 [b]	100
Overall Percentage			68.3 [c]

a. This is the number of human *E. coli* correctly predicted using antibiotics that were excluded by the model in the analysis.

b. This is the number non-human *E. coli* correctly predicted using antibiotics that were excluded by the model in the analysis.

c. This is the overall percentage of correctly predicted results.

4.5 Model fit and goodness-of-fit statistics

The model fit chi-square and goodness-of-fit tests were used to determine the model fit and goodness-of-fit statistics (SPSS 13.0, 2005 version). This is the statistical test that was used to test if the model used has resulted in accurate classifications or not. It is this statistical test that indicates if the results obtained should be considered or not. Table 4.6 indicates the model fit and goodness-of-fit test results. With the antibiotics used in the model, the goodness-of-fit statistics is 142.501. However, these results are based on the

changes or on the difference of the antibiotics used. The statistics presented in Table 4.6 is significant (p = 0.000), with 63 degrees of freedom. Since the significant test is very low (p = 0.000), this means the antibiotics used (in step 2) has improved the classification of *E. coli* according to their sources.

Table 4.6: Model fitting

Model	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood	Chi-Square	df	Significant (p)
Intercept Only	850.681			
Final	142.501	708.180	63	0.000

4.6 Differentiating between wildlife and domestic animals

MAR technique has been reported to be useful in differentiating between animal and human sources of fecal contamination. If the source is animal, MAR can also indicate if the animal is domestic or not. In this study, differentiating between human and non-human sources of fecal contamination was demonstrated. The study also explored whether MAR could differentiate between domestic and wild animals. Table 4.7 reveals that *E. coli* from domestic animals were incorrectly classified as *E. coli* from wildlife animal. This gives 100% incorrect classification. Wildlife *E. coli* isolates were correctly classified, making the correct classification percentage to be 100%. The overall classification percentage between wildlife and domestic is 50%. These results show poor classification between wildlife and domestic animal sources and may result from various

factors which are discussed in Section 5.5.

Table 4.7: Classification table - differentiate between wildlife and domestic animals

Observed			Predicted		Percentage Correct
			Domestic	Wildlife	
	Animals	Domestic	0	100	0
		Wildlife	0	100	100
	Overall percentage				50

In this study, *E. coli* were also differentiated according to their individual source. Table 4.9 shows how sources of the isolates were classified. The values on the left are the real sources of the isolates while those on top indicate how *E. coli* was classified or predicted. For example, from 36 *E. coli* isolates from chicken, 18 *E. coli* from chickens were correctly classified as originating from chicken, 5 were classified as originating from human source, 9 from swine and 4 were classified as originating from buffalo. Thus only 50% of these isolates were correctly classified. In another example, of the 102 human isolates, 2 were classified as originating from sheep, 90 human isolates were correctly classified, 3 from swine, 1 from bushbuck, 3 from buffalo and another 1 was classified as originating from eland. In this case, 88.2% were correctly classified. The overall classification percentage of *E. coli* according to their individual sources was 70.8%. This

high percentage of correct classification suggests that MAR could be used successfully in differentiating fecal coliform bacteria from human and non-human sources. The classification of chicken *E. coli* had a 50% correct classification percentage. This is a moderate classification. Both Bushbuck and Buffalo *E. coli* were poorly classified. They have a correct classification percentage of 23 and 36% respectively. This poor classification indicates that fecal contamination from these animals can not be traced. Sheep, humans and Gemsbok *E. coli* had very high classification rates (Table 4.8). This is especially important with human *E. coli* because it is from this source that health hazards are experienced.

Table 4.8: Classification of the isolates according to their host sources.

Observed	**Predicted sources**									
Sources	Chicken	Sheep	Human	Swine	Bushbuck	Buffalo	Gemsbok	Eland	Total	Correct classification %
Chicken	18	0	5	9	0	4	0	0	36	50.0%
Sheep	0	31	3	1	0	0	0	0	35	88.6%
Humans	0	2	90	3	1	3	0	3	102	88.2%
Swine	0	0	9	30	0	0	0	0	39	76.9%
Bushbuck	0	1	18	1	6	0	0	0	26	23.1%
Buffalo	0	0	9	0	1	9	1	5	25	36.0%
Gemsbok	0	0	0	0	2	0	32	0	34	94.1%
Eland	0	0	5	1	0	2	0	17	25	68.0%
Overall classification rate									322	70.8%

4.7 Caffeine detection

Ten *E. coli* isolates from each animal source were randomly selected and tested for the presence of caffeine metabolites. From human sources, 20 *E. coli* isolates were selected. In total, 100 *E. coli* isolates were screened for the presence of caffeine, that is, 40 from wildlife, 40 from domestic animals and 20 from human isolates. This ratio of distribution was chosen so that certainty would be gained when human *E. coli* are compared to non-humans.

In screening for the presence of caffeine, quantitative analysis was done. Caffeine was screened from *E. coli* isolates from different sources with an aim of detecting the presence of unmetabolized caffeine in them. In all wildlife and domestic animals, no caffeine was detected. Out of 20 human *E. coli* isolates screened, caffeine was detected from only 10 (50%) isolates. This means caffeine was detected from 10% of *E. coli* that were used in this test. Classification of *E. coli* in this way represented 90% correct classification percentage. The presence of unmetabolized caffeine in these isolates was confirmed by running the experiment in duplicate. The picture of the TLC plate taken using a digital camera is shown in Figure 4.2 below. TLC plates where no caffeine was detected were not taken.

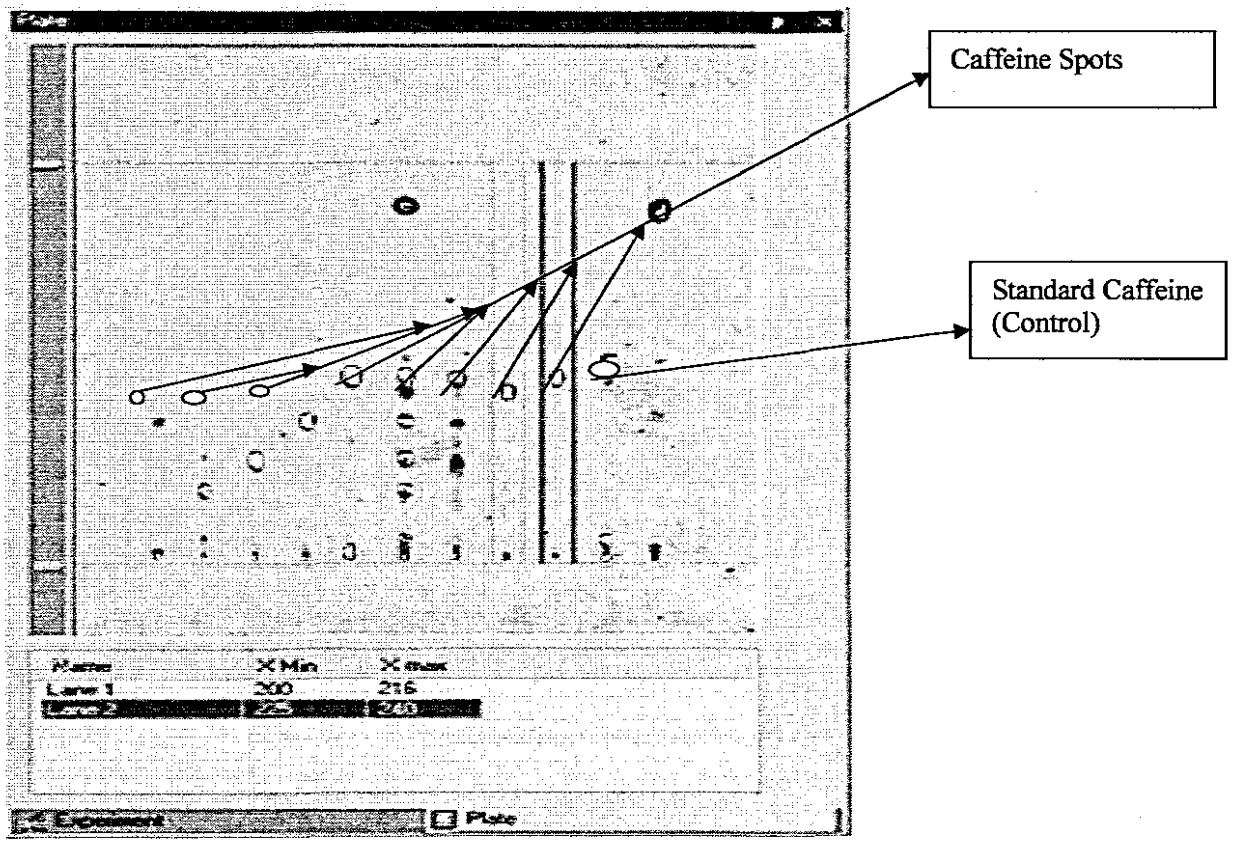


Figure 4.2: TLC plate showing caffeine spots.

The R_f value of the detected caffeine was calculated by dividing the distance traveled by solvent (ethyl acetate) over the distance traveled by caffeine in the TLC plate. The R_f defines how far up the plate the compound has traveled. R_f values are between 0 and 1 and are best between 0.1 and 0.8 (Keuker, 1989). In this study, the average R_f value was found to be 0.28. According to Keuker, (1989), this value is "almost" the best. This represents a good classification of *E. coli*.

CHAPTER 5

DISCUSSION

5.1 Introduction

The procedure used in isolating and screening *E. coli* isolates was found to be simple, quick and reliable. Samples were first cultured on Levine EMB agar and the cultures with gold metallic sheen were randomly selected and then sub-cultured. Pure cultures were screened and confirmed to be presumptive *E. coli* using IMViC test. Sampling from all the sources used was randomized hence all the samples were representative of the sources and environment from which the isolates were collected.

5.2 Determination of antibiotic resistance patterns

Antibiotic resistance patterns of *E. coli* isolated from domestic wastewater (sewage) and animal feces were determined using a battery of antibiotics (chloramphenicol, nalidic acid, neomycin, penicillin G, kanamycin, tetracycline, sulphothiazole, ciprofloxacin, cephalothin and gentamycin). Antibiotic resistance patterns of commensal fecal flora of human and animals are influenced by many factors. These factors include the presence of intrinsically antibiotic resistance bacteria and the fact that antibiotics ingested by the host animal select for the survival antibiotic resistant strain. Selective pressures imposed on the commensal gastrointestinal flora of animals and humans by antibiotic use result in patterns of antibiotic resistance that reflect to some extent the microflora's exposure to antibiotics (Biyela, 2003).

Several classes of antibiotics are approved for both human and animal use (Witte, 1997). This practice certainly contributes to shared patterns of antibiotic resistance in the fecal flora of domestic animals and humans. There are significant differences in the prevalence of antibiotic resistance among isolates from different host species. In some cases there are enough similarities that may prevent the classification of indicator organism according to their sources in the field. This was the case with cephalothin. The use of several concentrations of each antibiotic rather than one, to establish antibiotic resistance patterns coupled with statistical treatment of the data can provide a predictive power necessary to provide useful information about the sources of isolates (Fluit *et al.,* 2001).

5.3 Antibiotics used in the analysis

In antibiotic resistance pattern analysis, there are antibiotics which have shown same/similar activities when tested against the isolates, for example, cephalothin (all isolates are resistant to cephalothin). This antibiotic was computationally excluded from the analysis.

Two steps were used to classify isolates according to their sources. The two steps are differentiated by using different antibiotics in the analysis (Table 4.4). In step 1, the antibiotics used were those that show significant difference in activities when tested against the isolates from different sources but showing same or similar activities from each source. Step two includes antibiotics used in step 1 and one additional antibiotic, Gentamycin, which showed similar activities when tested against isolates from different sources and from each source. This step has resulted in improvements in the classification

of the sources of *E. coli* (Table 4.4). It is the results of this step that will be considered the most. The highest correct classification rates in previous studies were obtained by using a subset of antibiotics tested for analysis (Wiggins, 1996; Hagerdon *et al.,* 1999). However, in this study omission of any of the antibiotic resistance data resulted in a lower correct classification rate. The higher correct classification rates in step two are due to the number of antibiotics used for the analysis. Step two was established using five antibiotics while step 1 used only four (Table 4.2).

When the antibiotics with the same antibiotic resistance patterns are used, all human and non-human isolates are incorrectly classified as if they are from the same source. This is only true if the isolates do not show any difference or have similar antibiotic resistance patterns.

5.4 Classification of humans from non-human isolates

E. coli isolates from humans were correctly classified at the rate of 60.2 % and pooled animal isolates were correctly classified at a rate of 95.5%, with the overall classification percentage of 78% (Table 4.4). When isolates from animal sources were analyzed as separate source categories (Table 4.8), human isolates were correctly classified at a rate of 88.2% and 11.8 % classified as animals with the correct overall classification percentage of 62.8%. In this classification, 2 human isolates were incorrectly classified as sheep, 3 classified as swine, 3 as buffalo, 3 as eland and 1 was classified as bushbuck.

The highest percentage of correct classification between humans and non-humans were obtained when more antibiotics were used in the analysis. This was also accompanied by

high predictive accuracy (Table 4.4, step 2), with the overall correct classification of 78%. The correct classification of humans from non-human source was very crucial in this study. This is the gist and forms the most important part of this study. Some human fecal bacteria are potential pathogens (Franklin and Snow, 1981). They can cause water-borne illnesses and other diseases that they can be associated with (Biyela *et al.*, 2004). It is therefore important to identify/ differentiate between human and non-human sources of fecal contamination.

5.5 Classification of domestic animals from wildlife

One of the aims of this study was to differentiate wildlife from domestic animal sources. This is possible due to variations in antibiotic resistant patterns between wildlife and domestic animals. *E. coli* from domestic animals are expected to be more resistant to antibiotic compared to wildlife animal isolates since domestic animals are expected to be more exposed to antibiotics than wild animals.

MAR could not convincingly classify chicken isolates according to their host source. The correct classification was 50%. This average classification needs further investigation. Bushbuck and African buffalo had a very poor classification of 23.1% and 36.0% respectively. Their poor classification had probably been impacted on by the environment where they were found. These animals were actually not "wildlife" in the true sense of the word. They were not found in their natural environment, but in the Johannesburg Zoo where they were subjected to human control like domesticated animals. This might have an impact in antibiotic use resulting to their antibiotic resistance patterns being similar to domestic animals.

Like domestic animals, most wildlife isolates were obtained from ruminants. In a study conducted by Hagedorn *et al.*, (1999), using fecal *streptococci*, they reported that the antibiotic resistance patterns of beef cattle and wildlife isolates were very similar. This could also be another reason for the similarity of antibiotic resistant patterns between domestic and wildlife animals found in this study.

5.6 Classification rates

The correct classification rates of *E. coli* isolates from known sources obtained in this study are higher than those for Wiggins *et al.* (1999) and Carson *et al.* (2001). However, this study and their studies are both composed of isolates from sources within a limited geographical area. Wiggins *et al.* (1999)'s sampling size was relatively higher (135-285 isolates per source), while in this study, the sampling size were relatively smaller (26-102 isolates per source).

Compared to other studies (Wiggins *et al.*, 1999; Carson *et al.*, 2001), it has been demonstrated that smaller sampling size and fewer sampling sites per source results in higher correct classification rates. These classification rates result from the relative homogeneity of the antibiotic resistant patterns (MAR) of isolates from individual animal population. This was also demonstrated by Wiggins *et al.*, (1999), in his second study, where samples were less homogeneous (more sampling sites) and samples sizes were larger and it therefore resulted in poor classification rates.

A study of MAR using fecal *streptococci* by Hagedorn *et al,* (1999) resulted in higher correct classification rates than those obtained in Florida and were less compared to those found in this study. The sources of isolates designated human in the two studies probably contributed to the difference in the correct classification rates. As in the Florida study, human isolates from our study were obtained from domestic wastewater, which provides a cross-section of human MAR and thus high variability in MAR. When isolates are collected from the sources that are not representative of large source population (as was the case in our study) they yield higher correct classification rates. One possible explanation could be that the sample sources in our study were homogeneous. They were not representative of a large sample sources. Another possible contributory factor to the difference in classification rates might be that Hagedorn *et al.* (1999) used fecal *Streptococcus* in his study rather than *E. coli* and also the difference in antibiotics used.

The sampling constraint applied to animal sources as well. While MAR tends to form a tight cluster within one population, patterns of isolates from different populations of a given source are more heterogeneous. Cattle feces for the Florida study (Hagedorn *et al.,* 1999) were obtained from seven different farms in an attempt to broaden the population sampled. Fecal samples from cattle and chicken were obtained from only two farms in Montgomery County, USA (Hagedorn *et al.,* 1999). However, in our study, the samples were collected from only one farm. This has led to the tight clustering of MAR of isolates, that is, similarity of antibiotic resistant patterns, since they are from one location and has resulted in high correct classification rate. The tight clustering of MAR of isolates from one location could be advantageous in some types of studies, that is, differentiation of septic tank and cattle farm inputs in a particular watershed (Witte, 1997).

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Water quality managers who need to identify the sources of fecal contamination in a watershed are generally primarily interested in discriminating between animal and human contamination. Secondly, they are interested in determining the major sources of animal contamination. MAR and other bacterial source tracing methods can be used to trace the sources of fecal contamination but none achieve perfect discrimination between different sources. It should be noted that a useful technique should have at least a correct classification percentage greater than 50%, a higher discriminatory power.

5.7 Antibiotic use and the emergence of antibiotic resistance

The patterns of antibiotic resistance in bacterial communities may change drastically depending on geographic location, farm management, and levels of use as well as kinds of antibiotics used in the local human population and livestock husbandry. For this reason, the MAR technique might be more suitable for microbial source determination or surveillance in designated geographic locations and self-contained systems, such as estuaries. This is under the provision that a comprehensive and representative criterion database is locally established beforehand (US Environmental Protection Agency, 1990).

As patterns of antibiotic use change, the antibiotic resistance patterns of microorganisms also change. In the United States, the emergence of fluoroquinolone resistant *Campylobacter jejuni* in chicken was linked to the approval of fluoroquinolone use in poultry in 1995 (Smith *et al.*, 1999). Withdrawal of antibiotic use resulted in decreased prevalence of antibiotic resistance. This was also the case when antibiotic use was terminated in swine herds (Langlois *et al.*, 1983; 1988). Hagedorn *et al.* (1999) used the

MAR indexing technique of fecal *streptococci* to identify cattle as the predominant source of fecal contamination in the Page Brook watershed in rural Virginia in USA. This resulted in the implementation of restricted access of the cattle to the stream and a 94% reduction of fecal coliform bacteria in the watershed. There are, however, some cases where withdrawal of antibiotics did not have a profound effect on the levels of antibiotic resistant bacteria, a need for further investigations.

Selective pressure on antibiotic treatment on the animal's commensal microflora is an important determinant of the prevalence of antibiotic resistance in a population (Witte, 1997). This implies that the databases that were developed and used in the differentiation of the sources of fecal contamination will require periodic updating.

5.8 Caffeine detection

Caffeine was determined by TLC with ethyl acetate as a mobile phase. It was not detected in animal *E. coli*. This confirms the claims that animals do not secrete caffeine or the amount of caffeine secreted by animals is so small that it cannot be detected. Unlike human beings who have substantial amount of caffeine they ingest in their diet, all animal sources used do not have caffeine supplement in their diet. This, however, exclude all cattle and sheep sources because their diet was not fully scrutinized. Their diet included grass and leaves from the trees and the nutritional values from those plants were not determined. When plants containing significant amounts of caffeine are consumed by animals, these animals may excrete caffeine metabolites through feces which can be detected from microorganisms isolated in the feces, thereby confusing results. This makes caffeine detection not a reliable method in the determination of the sources of fecal

contamination in these cases because of the excretion of caffeine metabolites by such animals. *E. coli* sources used in this study did not excrete caffeine, hence it was not detected. This implies that fecal bacteria from these sources can be used to differentiate between humans and non-human sources. Differentiation between human and non-human can be achieved by the determination of the presence of caffeine.

The detection of caffeine from human isolates was done because humans consume beverages, coffee and certain foodstuffs which contain substantial amounts of caffeine. This caffeine is sometimes partially metabolized in the body and excreted with fecal bacteria. Caffeine detection is only useful in assessing the impact from human sewage. It only indicates whether the source is human or non-human. The only drawback in using caffeine in bacterial source determination is that after being excreted, it sometimes becomes present in minute quantities in the environment which makes it difficult to detect (Scott *et al.*, 2002). Caffeine was not detected from 10% human *E. coli.* This study could not establish why it was not detected. Some of the reasons might be that it was present in minute quantities to the isolates or after secretion and that it was degraded from *E. coli.* Failure to detect caffeine has resulted in misclassification of human *E. coli.*

The two methods evaluated varied in their ability to differentiate *E. coli* isolates from various sources. MAR provided the greatest discriminatory power, the highest rate of correct classification, ease of standardization and performance. Caffeine detection technique also provided moderate classification but it requires major capital investment especially when it has to be routinely used in tracing sources of fecal contamination. MAR technique is simple and cost effective, which makes it more suitable for

surveillance of local self-contained water or environmental systems. While either technique could have been used in this study alone or in the field, the advantage of using two methods is mainly the additional confidence generated when the methods used provide the same or similar results.

Our results support the data (Carson *et al.,* 2001; Guan *et al.,* 2002; Scott *et al.*, 2002), that reported that antibiotic resistance profiles together with proper statistical analysis of the data can be used in the identification of the sources of fecal contamination. It has also been demonstrated in our study that few sampling sources result in higher classification rates. Wiggins *et al.,* (1996), had more heterogeneous isolates than our study and had low correct classification rates. This study also confirms Gardinali and Zhao (2002)'s report. It has proved that caffeine can be used to differentiate between human and non-human sources of fecal contamination.

CHAPTER 6

6 CONCLUSIONS AND FUTURE STUDIES

6.1 Conclusion

Antibiotic resistance patterns of *E. coli* isolates from human and non-human sources were obtained. These patterns have indicated that there may be a relationship between antibiotic use and antibiotic resistance profiles. This relationship may thus be useful in the classification/identification of the sources of fecal contamination. Determination of the sources of these fecal contaminants can, in turn, lead to the development of effective control strategies for fecal contamination.

Using MAR technique, more antibiotics used in the analysis increase the prediction accuracy/classification rates. The MAR indexing technique has shown that it can be used as a method of choice in tracing the sources of fecal contamination. This method has received significant attention as a viable tool for tracing the sources of fecal contamination. It has shown 70% overall classification rates according to individual source and 78% classification rate when *E. coli* are classified according to human and non-humans. These high classification rates make MAR an ideal method in the classification of fecal contamination according to their sources. The MAR approach used in this study could not differentiate between domestic and wild animals.

Caffeine has indicated a high prediction accuracy which indicates that caffeine could be used in bacterial source discrimination between human and non-human sources of fecal pollution. Caffeine cannot actually be used to classify or discriminate microorganisms according to their source of contamination since it is not normally found in microorganisms. Caffeine detection only indicates whether the source is human or non human, indicating the likelihood of differentiating between human and no-human sources of contamination. However, there are some limitations that are associated with the use of caffeine. Results can be confused if there are animals which are capable of producing caffeine.

In conclusion, this study has indicated that laboratory methods coupled with statistical analysis can provide a very high likelihood that sources of fecal contamination can be identified and classified according to their sources. This remediation and type of data could be used for the improvement of water quality.

6.2 Future studies

1. To investigate whether the antibiotic resistance profiles (ARPs) of isolates from one geographic location can be used to predict the sources of isolates from the other location.
2. Build the database of the ARPs of the isolates that will include wild animals from game reserves for representativeness in the database.
3. The role/use of genetic fingerprinting in tracking the high-risk sources of bacterial contamination.

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APPENDIX A: AGAR COMPOSITION AND PREPARATION

A1. Nutrient agar	g/l
Meat extracts	1.0
Peptone	5.0
Yeast extract	2.0
Sodium chloride	8.0
Agar	15

Preparation

Suspend 31g in 1000 ml distilled water. Boil with frequent steering. Sterilize by autoclaving at 121°C for 15 minutes. Cool and pour into plates.

A2. Levine Eosin Methylene Blue (EMB)	g/l
Peptone	10.0
Lactose	10.0
Di-potassium hydrogen phosphate	2.0
Eosin y	0.04
Methylene blue	0.06
Agar	15.0

Preparation

Suspend 37.5g in 1000 ml distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 60° and shake the medium in order to oxidize the methylene blue i.e. restore its colour, and to suspend the precipitate which is an essential part of this medium.

A3. Gram stain

Prepare the smear of the test organism by heat fixing the organism on a microscopic slide. Stain the smear with crystal violet for 1 minute. Rinse with water. Add iodine and wait for about 3 minutes and rinse with water. Decolorize with ethanol for about 30 seconds. Wash with water. Counter stain with safranin for about 1-2 minutes, wash and blot dry. Examine under light microscope using oil immersion lens.

Result

Gram positive = pink/red

Gram negative= purple, blue/violet

A4. Hydrogen sulphite production

Materials: SIM Medium

Procedure

Inoculate a colony of the test culture into a tube containing SIM agar by making stab inoculation using a transfer needle. Incubate at 37°C for 48 hours.

Result: blackening along the line of inoculation is positive.

A5. Citrate test

Materials: Simon's citrate

Ammonium citrate	1.0
Di potassium sulphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	12

Preparation

Suspend 22g in 1liter distilled water. Bring to boil to dissolve completely. Dispense into final containers and sterilize by autoclaving at 121°C for 15 minutes. Allow to set with a slant.

Results:

Deep blue slants – positive

Remains green - negative

A6. Indole production

Materials:

SIM medium

Kovac's reagent

Procedure

Inoculate a colony of the test culture into a tube containing SIM agar by means of step inoculation. . Incubate at 37°C for 48 hours. Add 5 ml Kovac's reagent and agitate gentle. Observe the result after few minutes.

Results:

A bright red colour appear in the reagent layer: Positive

A7. Methyl red and Vokes-Paskeur test

Materials

MRVP

Methyl red

Solution A reagent

Solution B reagent

Procedure

Inoculate MRVP medium with a 24 hour old culture of the test organism. Incubate at 37°C for 48 hours. Divide into two for MR and VP test.

MR- Add 10 drops of methyl red and waits for few minutes to read the result.
VP- Add 10 drops of solution A and shake well. Add 10 drops of solution B and shake well. Continuously shake at an interval of 3-4 minutes. Read the results after 15 minutes.

Result:

MR: Red (+), Yellow (-)

VP: Deep rose color (+)

APPENDIX B

Raw data– Antibiotic resistance results

B1. Antibiotic resistance results for sheep isolates

Isolates	Chloramp	tetracy	neomycin	nalidic	kanamy	PenG	sulphoth	ciproflox	gentamy	cephaloth
SS/UZ01	s	S	S	R	R	R	R	S	R	R
SS/UZ02	S	S	S	R	R	R	R	S	R	R
SS/UZ03	S	S	S	R	R	R	R	S	R	R
SS/UZ04	S	S	S	R	R	R	R	S	R	R
SS/UZ05	S	S	S	R	R	R	R	S	R	R
SS/UZ06	S	S	S	R	R	R	R	S	R	R
SS/UZ07	S	S	S	R	R	R	R	S	R	R
SS/UZ08	S	S	S	R	R	R	R	S	R	R
SS/UZ09	S	S	S	R	R	R	R	S	R	R
SS/UZ10	S	S	S	R	R	R	R	S	R	R
SS/UZ11	S	R	S	R	R	R	R	S	R	R
SS/UZ12	S	S	S	R	R	R	R	S	R	R
SS/UZ13	S	S	S	R	R	R	R	S	R	R
SS/UZ14	S	S	S	R	R	R	R	S	R	R
SS/UZ15	S	S	S	R	R	R	R	S	R	R
SS/UZ16	S	S	S	R	R	R	R	S	R	R
SS/UZ17	S	S	S	R	R	R	R	S	R	R
SS/UZ18	S	S	R	R	R	R	R	S	R	R
SS/UZ19	S	S	S	R	R	R	R	S	R	R
SS/UZ20	S	S	S	R	R	R	R	S	R	R
SS/UZ21	S	S	S	R	R	R	R	S	R	R
SS/UZ22	S	S	S	R	R	R	R	S	R	R
SS/UZ23	S	S	S	R	R	R	R	S	R	R
SS/UZ24	S	R	R	R	R	R	R	S	R	R
SS/UZ25	S	S	S	S	R	R	R	R	R	R
SS/UZ26	S	S	S	S	R	R	R	R	R	R
SS/UZ27	S	S	S	R	R	R	R	R	R	R
SS/UZ28	S	S	S	S	R	R	R	S	S	R
SS/UZ29	S	S	S	S	R	R	R	S	S	R
SS/UZ30	S	S	S	S	R	R	R	S	S	R
SS/UZ31	S	S	S	S	R	R	R	S	S	R
SS/UZ32	S	S	S	S	R	R	R	S	R	R
SS/UZ33	S	S	S	R	R	R	R	S	S	R
SS/UZ34	S	R	S	S	R	R	R	S	R	R
SS/UZ35	S	R	S	S	R	R	R	S	R	R
Total	S:35 R: 0	S: 31 R: 4	S: 33 R: 2	S: 9 R:26	S: 0 R: 35	S: 0 R:35	S: 0 R:35	S: 32 R: 3	S: 5 R: 30	S: 0 R: 35

B2. Antibiotic resistance for patterns for chickens isolates

Isolates	chloramp.	tetracy.	neomy	nalidic	kanamy	Pen G	sulphoth	ciprofloxa	Gentamy.	Cephalot
CH/UZ/01	S	R	S	R	R	R	R	S	S	R
CH/UZ/02	S	R	S	R	R	R	R	S	S	R
CH/UZ/03	S	R	S	R	R	R	R	S	S	R
CH/UZ/04	S	R	S	R	R	R	R	S	S	R
CH/UZ/05	S	R	S	R	R	R	R	S	S	R
CH/UZ/06	S	R	S	R	R	R	R	S	S	R
CH/UZ/07	S	R	S	R	R	R	R	S	S	R
CH/UZ/08	S	R	S	R	R	R	R	S	S	R
CH/UZ/09	S	R	S	R	R	R	R	S	S	R
CH/UZ/10	S	R	S	R	R	R	R	S	S	R
CH/UZ/11	S	R	S	R	R	R	R	S	S	R
CH/UZ/12	S	R	S	R	R	R	R	S	S	R
CH/UZ/13	S	R	S	R	R	R	R	S	S	R
CH/UZ/14	S	R	S	R	R	R	R	S	S	R
CH/UZ/15	S	R	S	R	R	R	R	S	S	R
CH/UZ/16	S	R	S	R	R	R	R	S	R	R
CH/UZ/17	S	R	S	R	R	R	R	S	S	R
CH/UZ/18	S	R	S	R	R	R	R	S	S	R
CH/UZ/19	S	R	S	R	R	R	R	S	S	R
CH/UZ/20	S	R	S	S	R	R	R	S	S	R
CH/UZ/21	S	R	S	R	R	R	R	S	S	R
CH/UZ/22	S	R	S	S	R	R	R	S	S	R
CH/UZ/23	S	R	S	S	R	R	R	S	S	R
CH/UZ/24	S	R	S	R	R	R	R	S	S	R
CH/UZ/25	S	R	S	R	R	R	R	S	S	R
CH/UZ/26	S	R	S	R	R	R	R	S	S	R
CH/UZ/27	S	R	S	R	R	R	R	S	S	R
CH/UZ/28	S	R	S	R	R	R	R	S	R	R
CH/UZ/29	S	R	S	R	R	R	R	S	S	R
CH/UZ/30	S	R	S	R	R	R	R	S	S	R
CH/UZ/31	S	R	S	R	R	R	R	S	S	R
CH/UZ/32	S	R	S	R	R	R	R	S	S	R
CH/UZ/33	S	R	S	R	R	R	R	S	S	R
CH/UZ/34	S	R	S	R	R	R	R	S	S	R
CH/UZ/35	S	R	S	R	R	R	R	S	S	R
CH/UZ/36	S	R	S	R	R	R	R	S	R	R
Total	S: 36 R: 0	S: 0 R:36	S: 36 R: 0	S: 3 R: 33	S: 0 R: 36	S: R: 36	S: 0 R: 36	S: 36 R: 0	S: 33 R: 3	S: 0 R: 36

B3. Antibiotic resistance results for cattle isolates

Isolates	Chloramp	tetracy	Neomyc	Nalidic	Kanamy	Pen G	Sulphoth	Ciproflox	Gentam.	Cephalt.
CW/UZ/01	S	R	R	S	R	R	R	S	S	R
CW/UZ/02	S	R	S	S	R	R	R	S	S	R
CW/UZ/03	S	R	S	S	R	R	R	S	S	R
CW/UZ/04	S	R	S	R	R	R	R	S	S	R
CW/UZ/05	S	R	S	R	R	R	R	S	S	R
CW/UZ/06	S	R	S	S	R	R	R	S	S	R
CW/UZ/07	S	R	S	S	R	R	R	S	S	R
CW/UZ/08	S	R	S	S	R	R	R	S	S	R
CW/UZ/09	S	R	S	S	R	R	R	S	S	R
CW/UZ/10	S	R	S	S	R	R	R	S	S	R
CW/UZ/11	S	R	S	S	R	R	R	S	S	R
CW/UZ/12	S	R	S	S	R	R	S	S	S	R
CW/UZ/13	S	R	S	S	R	R	R	S	S	R
CW/UZ/14	S	R	S	S	R	R	R	S	S	R
CW/UZ/15	S	R	S	S	R	R	R	S	S	R
CW/UZ/16	S	R	S	S	R	R	R	S	S	R
CW/UZ/17	S	R	R	S	R	R	R	S	S	R
CW/UZ/18	S	R	S	S	R	R	R	S	S	R
CW/UZ/19	S	R	S	S	R	R	R	S	S	R
CW/UZ/20	S	R	S	S	R	R	R	S	S	R
CW/UZ/21	S	R	S	S	R	R	R	S	S	R
CW/UZ/22	S	R	R	R	R	R	R	S	S	R
CW/UZ/23	S	R	S	S	R	R	R	S	S	R
CW/UZ/24	S	R	S	S	R	R	S	S	S	R
CW/UZ/25	S	R	S	S	R	R	S	S	S	R
CW/UZ/26	S	R	S	S	R	R	R	S	S	R
CW/UZ/27	S	R	S	S	R	R	R	S	S	R
CW/UZ/28	S	R	S	S	S	R	R	S	S	R
CW/UZ/29	S	R	S	S	R	R	R	S	S	R
CW/UZ/30	S	R	S	S	R	S	R	S	S	R
CW/UZ/31	S	R	S	S	R	R	R	S	R	R
CW/UZ/32	S	R	S	S	R	R	R	S	S	R
CW/UZ/33	S	R	S	S	R	R	R	S		R
Total	S: 33 R: 0	S: 0 R: 33	S: 30 R: 3	S: 30 R: 3	S: 1 R: 32	S: 1 R: 32	S: 3 R: 30	S: 33 R: 0	S: 32 R: 1	S: 0 R: 33

B4. Antibiotic resistance results for human isolates

Isolates	Chloramp	tetracy	Neomyc	nalidic	kanamyci	Pen G	Sulphoth	Ciproflox	Gentam.	Cephalot
HS/UZ/1/01	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/02	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/03	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/04	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/05	S	R	S	S	R	R	R	S	R	R
HS/UZ/1/06	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/07	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/08	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/09	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/10	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/11	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/12	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/13	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/14	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/15	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/16	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/17	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/18	S	R	S	R	R	R	R	S	S	R
HS/UZ/1/19	S	R	S	R	R	R	R	S	S	R
HS/UZ/1/20	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/21	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/22	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/23	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/24	S	R	S	R	R	R	R	S	S	R
HS/UZ/1/25	S	R	S	S	R	R	R	S	S	R
HS/UZ/1/26	S	R	S	S	R	R	R	S	R	R
HS/UZ/1/27	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/28	S	R	S	S	S	R	R	S	R	R
HS/UZ/1/29	S	R	S	S	S	R	R	S	R	R
HS/UZ/1/30	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/31	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/32	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/33	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/34	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/35	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/36	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/37	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/38	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/39	S	R	S	R	R	R	R	S	R	R
HS/UZ/1/40	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/01	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/02	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/03	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/04	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/05	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/06	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/07	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/08	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/09	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/10	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/11	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/12	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/13	S	R	S	R	R	R	R	S	S	R

HS/UZ/2/14	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/15	S	S	S	S	R	R	R	S	S	R
HS/UZ/2/16	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/17	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/18	R	R	S	R	R	R	R	S	S	R
HS/UZ/2/19	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/20	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/21	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/22	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/23	S	S	S	S	R	R	R	S	S	R
HS/UZ/2/24	S	S	S	S	R	R	R	S	R	R
HS/UZ/2/25	S	R	S	S	R	R	R	S	S	R
HS/UZ/2/26	S	R	S	S	R	S	R	S	R	R
HS/UZ/2/27	S	R	S	R	R	R	R	S	R	R
HS/UZ/2/28	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/29	S	R	S	R	R	R	R	S	S	R
HS/UZ/2/30	S	S	S	R	R	R	R	S	S	R
HS/UZ/3/01	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/02	S	R	S	R	R	R	R	R	R	R
HS/UZ/3/03	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/04	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/05	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/06	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/07	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/08	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/09	S	S	S	R	R	S	S	S	S	R
HS/UZ/3/10	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/11	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/12	S	R	S	R	R	R	S	S	S	R
HS/UZ/3/13	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/14	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/15	S	R	S	R	S	S	R	S	R	R
HS/UZ/3/16	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/17	S	R	S	R	R	R	R	R	S	R
HS/UZ/3/18	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/19	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/20	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/21	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/22	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/23	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/24	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/25	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/26	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/27	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/28	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/29	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/30	S	R	S	R	R	R	R	S	S	R
HS/UZ/3/31	S	R	S	R	R	R	R	S	R	R
HS/UZ/3/31	S	R	S	R	R	R	R	S	R	R
Total	S: 101 R: 1	S: 5 R: 57	S: 101 R: 1	S: 9 R: 93	S: 3 R: 99	S: 2 R: 100	S: 3 R: 99	S: 100 R: 2	S: 70 R: 32	S: 0 R: 102

B5. *Antibiotic resistance results for swine*

Isolates	Chloram	Tetracy.	Neomy.	Nalidic	Kanamy.	Pen G	Sulphoth	Cipro	Gentamy	Cephal.
PS/UZ/01	S	S	S	R	R	R	R	S	S	R
PS/UZ/02	S	S	R	R	R	R	R	S	S	R
PS/UZ/03	S	S	R	R	R	R	R	S	S	R
PS/UZ/04	S	R	R	R	R	R	R	S	S	R
PS/UZ/05	S	R	R	R	R	R	R	S	S	R
PS/UZ/06	S	R	R	R	R	R	R	S	S	R
PS/UZ/07	S	R	R	R	R	R	R	S	S	R
PS/UZ/08	S	R	R	R	R	R	R	S	S	R
PS/UZ/09	S	R	R	R	R	R	R	S	S	R
PS/UZ/10	S	R	R	R	R	R	R	S	S	R
PS/UZ/11	S	R	R	R	R	R	R	S	S	R
PS/UZ/12	S	R	R	R	R	R	R	S	S	R
PS/UZ/13	S	R	S	R	R	R	R	S	S	R
PS/UZ/14	S	R	S	R	R	R	R	S	S	R
PS/UZ/15	S	R	R	R	R	R	R	S	S	R
PS/UZ/16	S	R	R	R	R	R	R	S	S	R
PS/UZ/17	S	R	R	R	R	R	R	S	S	R
PS/UZ/18	S	R	R	R	R	R	R	S	S	R
PS/UZ/19	S	R	R	R	R	R	R	S	S	R
PS/UZ/20	S	R	R	R	R	R	R	S	S	R
PS/UZ/21	S	R	R	R	R	R	R	S	S	R
PS/UZ/22	S	R	R	R	R	R	R	S	S	R
PS/UZ/23	S	R	R	R	R	R	R	S	S	R
PS/UZ/24	S	R	R	R	R	R	R	S	S	R
PS/UZ/25	S	R	R	R	R	R	R	S	S	R
PS/UZ/26	S	R	R	R	R	R	R	S	S	R
PS/UZ/27	S	R	R	R	R	R	R	S	S	R
PS/UZ/28	S	R	S	R	R	R	R	S	S	R
PS/UZ/29	S	R	S	R	R	R	R	S	S	R
PS/UZ/30	S	R	S	R	R	R	R	S	S	R
PS/UZ/31	S	R	S	R	R	R	R	S	S	R
PS/UZ/32	S	R	S	R	R	R	R	S	S	R
PS/UZ/33	S	R	S	R	R	R	R	S	S	R
PS/UZ/34	S	S	S	R	R	R	R	S	S	R
PS/UZ/35	S	S	S	R	R	R	R	S	S	R
PS/UZ/36	S	R	S	R	R	R	R	S	S	R
PS/UZ/37	S	R	R	R	R	R	R	S	S	R
PS/UZ/38	S	R	R	R	R	R	R	S	S	R
PS/UZ/39	S	R	R	R	R	R	R	S	S	R
Total	S: 39 R: 0	S: 5 R: 34	S: 13 R: 26	S: 0 R: 39	S: 0 R: 39	S: 0 R: 39	S: 0 R: 39	S: 39 R: 0	S: 39 R: 0	S: 39 R: 0

B6. *Antibiotic resistance results for Bushbuck isolate*

Isolates	Chloramp	Tetracy.	Neomy	Nalidic	Kanamy	Pen G	Sulphoth.	Cipro.	Gentamy	Cepholoth
BB/JZ/01	R	R	S	R	R	R	R	S	S	R
BB/JZ/02	R	R	S	R	R	R	R	S	S	R
BB/JZ/03	R	R	S	R	R	R	R	S	S	R
BB/JZ/04	S	R	S	R	R	R	R	S	S	R
BB/JZ/05	R	R	S	R	R	R	R	R	S	R
BB/JZ/06	R	R	S	R	R	R	R	S	S	R
BB/JZ/07	R	R	S	R	R	R	R	R	S	R
BB/JZ/08	R	R	S	R	R	R	R	S	S	R
BB/JZ/09	R	R	S	R	R	R	R	S	S	R
BB/JZ/10	R	R	S	R	R	R	R	S	S	R
BB/JZ/11	S	R	S	S	R	R	R	S	S	R
BB/JZ/12	S	R	S	R	R	R	R	S	S	R
BB/JZ/13	S	R	S	R	R	R	R	S	R	R
BB/JZ/14	S	R	S	R	R	R	R	S	S	R
BB/JZ/15	S	R	S	R	R	R	R	S	S	R
BB/JZ/16	S	R	S	R	R	R	R	S	S	R
BB/JZ/17	S	R	S	R	R	R	R	R	S	R
BB/JZ/18	S	S	S	R	R	R	R	S	S	R
BB/JZ/19	S	S	S	S	R	R	R	S	S	R
BB/JZ/20	S	R	S	S	R	R	R	S	S	R
BB/JZ/21	S	R	S	S	R	R	R	S	S	R
BB/JZ/22	S	R	S	R	R	R	R	S	S	R
BB/JZ/23	S	R	S	R	R	R	R	S	S	R
BB/JZ/24	S	R	S	R	R	R	R	S	S	R
BB/JZ/25	S	R	S	R	R	R	R	S	S	R
BB/JZ/26	S	R	S	R	R	R	R	S	S	R
Total	S: 20 R: 6	S: 2 R: 24	S: 26 R: 0	S: 4 R: 22	S: 0 R: 26	S: 0 R: 26	S: 0 R: 26	S: 23 R: 3	S: 25 R: 1	S: 0 R: 26

B7. *Antibiotic resistance results for African buffalo isolates*

Isolates	Chloramp	tetracy	Neomyc	nalidic	kanamy	Pen G	Sulphoth	Ciproflox	Gentamy	Cepholoth
AB/JZ/01	R	R	S	R	R	R	R	S	R	R
AB/JZ/02	S	R	S	R	S	R	R	S	S	R
AB/JZ/03	S	R	S	R	R	R	R	S	S	R
AB/JZ/04	S	R	S	R	S	S	R	S	R	R
AB/JZ/05	S	R	S	R	R	R	R	S	S	R
AB/JZ/06	S	R	S	R	S	R	R	S	S	R
AB/JZ/07	S	R	S	R	R	R	R	S	S	R
AB/JZ/08	S	R	S	S	R	R	R	S	S	R
AB/JZ/09	S	R	S	R	R	R	R	S	R	R
AB/JZ/10	S	R	S	S	S	S	S	S	S	R
AB/JZ/11	S	R	S	S	S	S	S	S	S	R
AB/JZ/12	S	R	S	S	S	S	S	S	S	R
AB/JZ/13	S	R	S	S	S	S	S	S	S	R
AB/JZ/14	S	R	S	S	S	S	S	S	S	R
AB/JZ/15	S	R	S	R	R	S	S	S	R	R
AB/JZ/16	S	R	S	R	R	S	S	S	R	R
AB/JZ/17	S	R	S	R	R	S	S	S	S	R
AB/JZ/18	S	R	S	R	R	R	S	S	R	R
AB/JZ/19	S	R	S	S	R	S	S	S	S	R
AB/JZ/20	S	R	S	R	R	S	S	S	S	R
AB/JZ/21	S	R	S	S	R	R	S	S	R	R
AB/JZ/22	S	R	S	R	S	S	S	S	S	R
AB/JZ/23	S	R	S	R	S	R	R	S	S	R
AB/JZ/24	S	R	S	S	R	R	R	S	S	R
AB/JZ/25	S	R	S	S	R	R	R	R	S	R
Total	S: 24 R: 1	S: 0 R: 25	S: 25 R: 0	S: 10 R: 15	S: 10 R: 15	S: 12 R: 13	S: 13 R: 12	S: 24 R: 1	S: 18 R: 7	S: 0 R: 25

B8. *Antibiotic resistance results for Gemsbok isolates*

Isolates	Chloramp	tetracy	Neomyc	nalidic	kanamy	Pen G	Sulphoth	Ciproflox	Gentam	Cepholoth
GB/JZ/01	R	R	S	R	R	R	S	S	S	R
GB/JZ/02	R	R	S	R	S	S	S	S	S	R
GB/JZ/03	R	R	S	R	S	R	S	S	R	R
GB/JZ/04	R	R	S	S	R	R	S	S	R	R
GB/JZ/05	R	R	S	S	S	S	S	S	S	R
GB/JZ/06	R	R	S	R	R	R	S	S	S	R
GB/JZ/07	R	R	S	R	R	R	S	S	S	R
GB/JZ/08	R	R	S	R	R	R	S	S	S	R
GB/JZ/09	R	R	S	R	R	R	S	S	S	R
GB/JZ/10	R	R	S	R	R	R	S	S	S	R
GB/JZ/11	R	R	S	R	R	R	R	S	R	R
GB/JZ/12	R	R	S	R	R	R	R	S	R	R
GB/JZ/13	R	R	S	R	R	S	S	S	S	R
GB/JZ/14	R	R	S	R	R	S	S	S	S	R
GB/JZ/15	R	R	S	R	R	S	S	S	S	R
GB/JZ/16	R	R	S	R	R	S	S	S	S	R
GB/JZ/17	R	R	S	R	R	R	S	S	S	R
GB/JZ/18	R	R	S	R	R	R	S	S	S	R
GB/JZ/19	R	R	S	R	R	R	S	S	S	R
GB/JZ/20	R	R	S	R	R	S	S	S	S	R
GB/JZ/21	R	R	S	R	R	S	S	S	S	R
GB/JZ/22	R	R	S	R	R	S	S	S	S	R
GB/JZ/23	R	R	S	R	R	R	S	S	S	R
GB/JZ/24	R	R	S	R	R	R	S	S	S	R
GB/JZ/25	R	R	S	R	R	R	S	S	S	R
GB/JZ/26	R	R	S	R	R	R	S	S	S	R
GB/JZ/27	R	R	S	R	R	S	S	S	S	R
GB/JZ/28	R	R	S	R	R	R	R	S	S	R
GB/JZ/29	R	R	S	R	S	R	S	S	S	R
GB/JZ/30	R	R	S	R	R	S	R	S	S	R
GB/JZ/31	R	R	S	R	R	S	S	S	S	R
GB/JZ/32	R	R	S	R	R	S	S	S	S	R
GB/JZ/33	R	R	S	R	R	S	S	S	R	R
GB/JZ/34	R	R	S	R	R	R	R	S	S	R
Total	S: 0 R: 34	S: 0 R: 34	S: 34 R: 0	S: 2 R: 32	S: 4 R: 32	S: 14 R: 20	S: 29 R: 5	S: 34 R: 0	S: 29 R: 5	S: 0 R: 34

B9. Antibiotic resistance results for Eland isolate

Isolates	Chloramp	tetracy	Neomyc	nalidic	kanamy	Pen G	Sulphoth	Ciproflox	Gentam	Cepholoth
EE/JZ/01	S	R	S	R	R	R	R	S	S	R
EE/JZ/02	R	R	S	S	R	R	S	S	S	R
EE/JZ/03	S	R	S	S	R	R	S	S	S	R
EE/JZ/04	S	R	S	S	R	R	S	S	S	R
EE/JZ/05	S	R	S	S	R	R	S	S	S	R
EE/JZ/06	S	R	S	R	R	R	R	S	S	R
EE/JZ/07	S	R	S	S	R	R	S	S	S	R
EE/JZ/08	S	R	S	S	R	R	S	S	S	R
EE/JZ/09	S	R	S	S	R	R	S	S	S	R
EE/JZ/10	S	R	S	S	R	S	S	S	S	R
EE/JZ/11	S	S	S	R	R	R	R	S	S	R
EE/JZ/12	S	R	S	R	R	R	S	S	S	R
EE/JZ/13	S	R	S	R	S	S	S	S	S	R
EE/JZ/14	S	R	S	R	S	R	S	S	S	R
EE/JZ/15	S	R	S	R	R	R	S	S	S	R
EE/JZ/16	S	R	S	R	R	R	S	S	S	R
EE/JZ/17	S	R	S	R	R	R	R	S	R	R
EE/JZ/18	S	R	S	R	R	R	R	S	R	R
EE/JZ/19	S	R	S	R	R	R	S	S	R	R
EE/JZ/20	S	R	S	R	R	S	S	S	S	R
EE/JZ/21	S	R	S	R	R	S	S	S	S	R
EE/JZ/22	S	R	S	R	R	S	S	S	S	R
EE/JZ/23	S	R	S	R	R	S	S	S	R	R
EE/JZ/24	S	R	S	R	R	S	R	S	S	R
EE/JZ/25	S	R	S	R	R	S	S	S	S	R
Total	S: 24 R: 1	S: 1 R: 24	S: 25 R: 0	S: 8 R: 17	S: 2 R: 23	S: 8 R: 17	S: 19 R: 16	S: 25 R: 0	S: 21 R: 4	S: 0 R: 25

Keys to the tables B1-B9:

R – Resistant
S – Susceptible

Chloramp – Chloramphenicol

Tetracy - Tetracycline

Neomyc - neomycine

Kanamy- - Kanamycine

Nalidic- - Nalidixic acid

Pen G – Penicillin G

Sulphoth - Sulphothiazole

Ciproflox - Ciprofloxacine

Gentam - Gentamycin

Cepholoth – Cephalothin

APPENDIX C

Raw data: Sources of isolates used and their sensitivity patterns

C1. Antibiotic resistance patterns of the isolates against tetracycline

			Tetracycline		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	0	36	36
		% within Chickens	0	100	100
	Sheep	Count	4	31	35
		% within sheep	11.7	88.6	100
	Humans	Count	97	5	102
		% within Humans	95.1	4.9	100
	Swine	Count	34	5	39
		% within Swine	87.2	12.8	100
	Bushbuck	Count	24	2	26
		% within Bushbuck	92.3	7.7	100
	Buffalo	Count	25	0	25
		% within Buffalo	100	0	100
	Gemsbok	Count	34	0	34
		% within Gemsbok	100	0	100
	Eland	Count	24	1	25
		% within Eland	96	4	100
Total		Count	278	44	322
		% within all the isolates	86.3	13.7	100

C2. Antibiotic resistance patterns of the isolates against neomycin

			Neomycin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	0	36	36
		% within Chickens	0%	100%	100%
	Sheep	Count	2	33	35
		% within sheep	5.7	94.3	100
	Humans	Count	1	101	102
		% within Humans	1%	99%	100%
	Swine	Count	26	13	39
		% within Swine	66.7	33.3	39
	Bushbuck	Count	0	26	26
		% within Bushbuck	0	100	100
	Buffalo	Count	0	25	25
		% within Buffalo	0	100	100
	Gemsbok	Count	0	34	34
		% within Gemsbok	0	100	100
	Eland	Count	0	25	25
		% within Eland	0	100	100
Total		Count	29	293	322
		% within all the isolates	9	91	100

C3. Antibiotic resistance patterns of the isolates against nalidic acid

			Nalidic acid		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	33	3	36
		% within Chickens	91.7	8.3	100
	Sheep	Count	26	9	35
		% within sheep	74.3	25.7	100
	Humans	Count	93	9	102
		% within Humans	91.2	8.8	100
	Swine	Count	39	0	39
		% within Swine	100	0	100
	Bushbuck	Count	21	4	26
		% within Bushbuck	80.8	19.2	100
	Buffalo	Count	15	10	25
		% within Buffalo	60	40	100
	Gemsbok	Count	32	2	34
		% within Gemsbok	94.1	5.9	100
	Eland	Count	17	8	25
		% within Eland	68	32	100
Total		Count	276	46	322
		% within all the isolates	85.7	14.3	100

C4. Antibiotic resistance patterns of the isolates against Kanamycin

			Kanamycin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	36	0	36
		% within Chickens	100	0	100
	Sheep	Count	35	0	35
		% within Sheep	100	0	100
	Humans	Count	99	3	102
		% within Humans	97.1	2.9	100
	Swine	Count	39	0	39
		% within Swine	100	0	100
	Bushbuck	Count	26	0	26
		% within Bushbuck	100	0	100
	Buffalo	Count	15	10	25
		% within Buffalo	60	40	100
	Gemsbok	Count	30	4	34
		% within Gemsbok	88.2	11.8	100
	Eland	Count	23	2	25
		% within Eland	92	8	100
Total		Count	303	19	322
		% within all the isolates	94.1	5.9	100

C5. Antibiotic resistance patterns of the isolates against penicillin

			Penicillin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	36	0	36
		% within Chickens	100	0	100
	Sheep	Count	35	0	35
		% within Sheep	100	0	100
	Humans	Count	100	2	102
		% within Humans	98	2	100
	Swine	Count	39	0	39
		% within Swine	100	0	100
	Bushbuck	Count	26	0	26
		% within Bushbuck	100	0	100
	Buffalo	Count	13	12	25
		% within Buffalo	52	48	100
	Gemsbok	Count	20	14	34
		% within Gemsbok	58.8	41.2	100
	Eland	Count	17	8	25
		% within Eland	68	32	100
Total		Count	286	36	322
		% within all the isolates	88.8	11.2	100

C6. Antibiotic resistance patterns of the isolates against Sulphothiazole

			Sulphothiazole		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	36	0	36
		% within Chickens	100	0	100
	Sheep	Count	35	0	35
		% within Sheep	100	0	100
	Humans	Count	99	3	102
		% within Humans	97.1	2.9	100
	Swine	Count	39	0	39
		% within Swine	100	0	100
	Bushbuck	Count	26	0	26
		% within Bushbuck	100	0	100
	Buffalo	Count	12	13	25
		% within Buffalo	48	52	100
	Gemsbok	Count	5	29	34
		% within Gemsbok	14.7	85.3	100
	Eland	Count	6	19	25
		% within Eland	24	76	100
Total		Count	258	64	322
		% within all the isolates	80.1	19.9	100

C7. Antibiotic resistance patterns of the isolates against ciprofloxacin

			Ciprofloxacin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	0	36	36
		% within Chickens	0	100	100
	Sheep	Count	3	32	35
		% within sheep	8.6	91.4	100
	Humans	Count	2	100	102
		% within Humans	2	98	100
	Swine	Count	0	39	39
		% within Swine	0	100	100
	Bushbuck	Count	3	23	26
		% within Bushbuck	11.5	88.5	100
	Buffalo	Count	1	24	25
		% within Buffalo	4	96	100
	Gemsbok	Count	0	34	34
		% within Gemsbok	0	100	100
	Eland	Count	0	25	25
		% within Eland	0	100	100
Total		Count	9	313	322
		% within all the isolates	2.8	97.2	100

C8. Antibiotic resistance patterns of the isolates against gentamycin

			Gentamycin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	3	33	36
		% within Chickens	8.3	91.7	100
	Sheep	Count	30	5	35
		% within sheep	85.7	14.3	100
	Humans	Count	32	70	102
		% within Humans	32	68	100
	Swine	Count	0	39	39
		% within Swine	0	100	100
	Bushbuck	Count	1	25	26
		% within Bushbuck	4	96	100
	Buffalo	Count	7	18	25
		% within Buffalo	28	72	100
	Gemsbok	Count	5	29	34
		% within Gemsbok	15	85	100
	Eland	Count	4	21	25
		% within Eland	16	84	100
Total		Count	9	44	322
		% within all the isolates	25.8	74.2	100

C9. Antibiotic resistance patterns of the isolates against cephalothin

			Cephalothin		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	36	0	36
		% within Chickens	0	0	100
	Sheep	Count	35	0	35
		% within sheep	100	0	100
	Humans	Count	102	0	102
		% within Humans	100	0	100
	Swine	Count	39	0	39
		% within Swine	100	0	100
	Bushbuck	Count	26	0	26
		% within Bushbuck	100	0	100
	Buffalo	Count	25	0	25
		% within Buffalo	100	0	100
	Gemsbok	Count	34	0	34
		% within Gemsbok	100	0	100
	Eland	Count	25	0	25
		% within Eland	100	0	100
Total		Count	322	0	322
		% within all the isolates	100	0	100

C10. Antibiotic resistance patterns of the isolates against chloramphenicol

			Chloramphenicol		Total
			Resistant	Susceptible	
Sources of the isolates	Chickens	Count	0	36	36
		% within Chickens	0	100	100
	Sheep	Count	0	35	35
		% within sheep	0	100	100
	Humans	Count	1	101	102
		% within Humans	1	99	100
	Swine	Count	0	39	39
		% within Swine	0	100	100
	Bushbuck	Count	6	20	26
		% within Bushbuck	23.1	76.9	100
	Buffalo	Count	1	24	25
		% within Buffalo	4	96	100
	Gemsbok	Count	34	0	34
		% within Gemsbok	100	0	100
	Eland	Count	1	24	25
		% within Eland	4	96	100
Total		Count	43	279	322
		% within all the isolates	13.4	86.6	100