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A STUDY OF ANTIBIOTIC RESIDUES IN MEAT SOLD IN BUTCHERIES AND SUPERMARKETS AROUND MAFIKENG, NORTH WEST PROVINCE

By

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Dissertation submitted in fulfilment of the requirements for the degree Masters of Agriculture in Animal Health at the North West University, Mafikeng Campus

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I, Tsepo A Ramatla, declare that the dissertation entitled "**A study of antibiotic residues in meat sold in butcheries and supermarkets around Mafikeng, North West Province**", hereby submitted for the degree of Master of Agriculture in Animal Health, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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Student Name	**Signature**	**Date**

ABSTRACT

This study examined antibiotic residue and the frequency of contamination of meat purchased from main butcheries and supermarket around Mafikeng, North West Province, South Africa. The present study was thus designed to estimate residue levels of tetracycline, sulphonamides, ciprofloxacin and streptomycin antimicrobial residues in chicken, pork and beef samples. A total of 150 (50 chicken, 50 pork and 50 beef) samples composed of kidney, liver and muscles were analysed using Enzyme-Linked-Immunosorbent Assay (ELISA), Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

Results obtained from this study reaveled the presence of antibiotic residues in different organs (liver, kidney and muscle) with pork tissues having contamination of 8 (6%), 1 (2%) and 10 (20%) samples respectively of liver, muscles and kidneys contained tetracycline, streptomycin, sulphonamides and ciprofloxacin residues. In chicken, tissues, 22 (44%) and 10 (20%) samples of liver and muscles respectively, contained tetracycline, streptomycin, sulphonamides and quinolones (ciprofloxacin) residues. While beef, tissues, 15 (30%), 5 (10%) and 1 (2%) samples of liver, kidney and muscles respectively contained tetracycline, streptomycin, sulphonamides and quinolones (ciprofloxacin) residues

In addition, the results obtained in this study revealed that 65 (43%) out of 150 samples were simultaneously detected positive for four antibiotics (tetracycline, sulphonamides, ciprofloxacin and streptomycin) residues, however all the samples were bellow MRL. Although the levels were below their MRL given by the European Union, South Africa and the Codex Alimentarius Commission, their presence may still be regarded as a health hazard as they may causes allergic reactions or produce drug-tolerant bacteria in human after long exposure. There were some samples which had more than one antibiotic residue. In this study, significant correlations ($P \geq 0.05$) between different methods (TLC, ELISA HPLC) used as well as among sample types (muscles, liver and kidney), and species (beef, chicken and pork) were observed. This showed the regularity, consistancy repeatability and quality of

techniques used in this study. In addition the relaibility of results obtained in this study is shown by the recovery rate as well as by the calibration curves of each antibiotic and for each method used. The novelity of this work is that three different techniques (TLC, ELISA and HPLC) were used to analyse samples and results obtained are generally in correlation.

Moreover, collected samples which were confirmed positive for residues were cultured for bacterial contamination and identification using the conventional biochemical methods as well as molecular techniques based on 16S rRNA species specific gene amplification by PCR and MALDI-TOF. Results obtained showed relatively high frequency 30 (40%) of occurrence of organisms; *Macrococcus caseolyticus* (60%), *Enterococcus mundtii* (3.3%), *Bacillus spp.*(3.3%), *Bacillus cereus* (3.3%), *Enterococcus spp.*((10%) and *Escherichia spp.*(3.3%) Surveillance of a pathogen over time in a regular manner is necessary to update the distribution level and to estimate its public health importance.

Lastly, all the isolates were evaluated for their antibiotic resistance patterns against some common antibiotics using Kirby-Bauer antibiotic discs diffusion method. The antimicrobial profile of eight isolates showed a high resistance rates for *Macrococcus caseolyticu* 66.6% (20/30), followed by *Enterococcus spp.* 10% (3/30), *Enterococcus faecalis* 10% (3/30), *Enterococcus mundtii* (3.33%), *Escherichia spp.* 3.33% (1/30), *Bacillus spp.* 3.33% (1/30), *Bacillus cereus* 3.33% (1/30). The majority of isolates (83.3%) were resistant to tetracyclines followed by sulphonamides (13.3%) and lastly ciprofloxacin (3.33%). However, these results revealed that only 33.3% of pathogens were resistance. However, 66.6% of isolates were *Macrococcus caseolyticus* which is a non-pathogenic bacterium. Only tetracycline showed strong correlation ($p<0.05$) between residues and resistance from two species; chicken (1.00) and beef (1.00). It was observed that more bacteria were isolated from the butcheries than supermarkets. Some isolates showed resistance to multiple antibiotics.

Although results of antibiotic residues obtained in this study were mostly just below the MRLs, there is a risk for consumers in case of chronic exposure. Hense, there is a need of a continous monitoring to ensure the safety of consumers. In addition, training of and information sessions with farmers to ensure that they adhere to the withdrawal periods of antimicrobial in order to obtain low/absence of antimicrobial residues should be maintained. The presence of microbial contaminants and antibiotic resistance isolated in meat might be explained by several factors such as contamination and transfer of resistance integron genes to animals.

It is therefore recommended that effective food safety education and training of personnel that handle food at retail points will help in reducing the effect of these pathogens on humans.

Key words: ELISA, TLC, HPLC, PCR, 16S-rDNA, antimicrobial resistance, beef, chicken, pork

LIST OF ABBREVIATIONS AND ACRONYMS

3'	Reverse Primer or an oligonucleotide that flanks the 3'end of the Amplicon
5'	Forward Primer or an oligonucleotide that flanks the 5'end of the Amplicon
ADI	Acceptable Daily Intake
AMR	Antimicrobial Resistance
API	Analytical Profile Index
CAC	Codex Alimentary Commission
CLIS	Clinical Laboratory Institute Standards
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EU	European Union
ELISA	Enzyme-Linked-Immunosorbent Assay
et al.	(*et alii*) and others
FAO	Food and Agriculture Organisation
HPLC	High Performance Liquid Chromatography
HPLC-PDA	HPLC method equipped with a photodiode array detector
HPLC-UV	HPLC method equipped with Ultraviolet Detection
LOD	Limit of Detection
LOQ	Limit of Quantification
MIC	Minimal Inhibitory Concentration
MRL	Maximum Residues Level
PCR	Polymerase Chain Reaction
PPM	Parts per million

pH	Logarithm for the reciprocal of hydrogen ion concentration in grams atom per litre, used to express the acidity or alkalinity of a solution on a scale of 0 to14
R	Resistant
RNA	Ribonucleic Acid
S	Susceptible
TLC	Thin Layer Chromatography
USA	United States of America
UK	United Kingdom
WHO	World Health Organisation

LIST OF UNITS

±	Plus or minus
:	Is to
>	Greater than
<	Less than
%	Percentage
/	Per
°C	Degree Celsius
g	Gram
L	Litre
Mg	Milligram
µ/g	Microgram/gram
µ/mL	Microgram/millilitre
mL	Millilitre
mm	Millimetre
Mol	Mole
nm	Nanometre
TM	Trade Mark
µg	Micro gram
µL	Micro litre
µm	Micro metre
v/v	volume/volume

Dedication

I dedicate this study to God and my family.

"Bless the Lord, O my soul and all that is within me, bless his holy name. Bless the Lord, O my soul and forget not all his benefits.

(Psalm 103:1-2)"

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CHAPTER ONE

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Meat is an important source of protein around Mafikeng and can be bought from butcheries and supermarkets. Human beings consume protein-rich foods to meet their nutritional requirements, mainly of animal origin (Adzitey & Huda, 2010; Ahmad *et al.*, 2013). Generally, consumption of meat (especially red meat) in South Africa, has been estimated to be 25.73 kg/person/year while for white meat, it stood at 29.69 kg/person/year in 2007–2008 (DoA, 2009). Beef, chicken and pork are the mostl common types of meat consumed in South Africa (Nel, 2005).

It has been observed that low concentrations of antibiotics given to animals in the form of feed or water may lead to improved feed conversion efficiency and consequently improved growth rates (Adams, 2007; Chanda *et al.,* 2014). According to a study conducted by Martos *et al.* (2010), currently, more than forty thousand types of antibiotics have been identified and eighty of these are consumed in the agricultural sector. Animals are usually given antibiotics in order to promote their growth and cure/prevent diseases. The main aim is usually to improve their health (Chang *et al.*, 2000; Giguere *et al.*, 2006; Mehtabuddin *et al.*, 2012; Er *et al.*, 2013). Regardless of their small amounts, antibiotics can lead to antibiotic residues in animal products and become a major cause for concern for the veterinary sector and consequently bacterial resistance against infectious diseases (Mrema *et al.*, 2006; Nisha, 2008; Wageh *et al.*, 2013; Chanda *et al.,* 2014).

Furthermore, dispite the beneficial effects of antibiotics in the treatment of infectious diseases, antibiotic residues in animal products can have serious side-effects in human beings (Normanno *et al.*, 2007; CDC, 2009; Ekene *et al.*, 2014). In addition to the presence of antibiotic residues in food, there is also toxicological concern as some residues could be

carcinogenic and cause allergic hypersensitivity reactions and therapeutic ineffectiveness in human beings (Nue, 1992; Edder, 1999; Won *et al.*, 2011; Magiorkas *et al.*, 2012). Concentrations of drug residue vary considerably from tissue to tissue and are generally higher in the storage tissues such as body fats, or in organs that actively metabolize and excrete them (such as the liver and the kidney) (Wang *at al.*, 2012; Jabbar *et al.*, 2013). Moreover, their molecules are metabolized into other metabolites in the blood, liver, and muscles and partially eliminated through the kidneys or other routes (Buur *et al.,* 2006; Pikkemaat *et al.*, 2008; Pikkemaat *et al.*, 2009).

There are several factors that lead to the occurrence of antibiotic residues in animal products. These include the following: failure to observe drug withdrawal periods; poor records of treatment; failure to identify treated animals; lack of advice on withdrawal periods; off label use of antibiotics; availability of antibiotics to lay persons as over the counter drugs in developing countries; extended usage or excessive dosages of antibiotics; non existence of restrictive legislation or inadequate enforcement and lack of consumer awareness about the magnitude and human health hazards associated with antibiotic residues in food of animal origin (Muhammad *et al.,* 1997; Paige *et al.,* 1997; Darwish *et al.,* 2013).

1.2 PROBLEM STATEMENT

The use of antimicrobials to treat food animals has the potential to affect human health as follows: increasing the risk of antimicrobial residues and influencing the generation or selection of antimicrobial resistant food-borne pathogens (Yan & Gilbert, 2004; Sarmah *et al.*, 2006; Eagar, 2008).

The use of antibiotics that might result in the deposition of residues in meat must not be permitted in food intended for human consumption. Also, the volume of antimicrobial used in food animal production has led to concerns in public, regulatory, and scientific arenas tregarding antimicrobial use in food animals (Bailar & Travers, 2002; O'Connor *et al.,* 2002). The detection of antimicrobial residues in meat is especially of great interest for their impact on public health. Moreover, the presence of residues could lead to bacterial resistance of different antimicrobials (Adesiyun *et al.,* 2007). Therefore, surveillance of antibiotic resistance in bacteria of animal origin is essential. In Mafikeng, North West Province of South Africa, there is currently no information on the occurrence of antimicrobial residues and post-slaughter contamination.

1.3 AIMS AND OBJECTIVES OF THE STUDY

1.3.1 Main objectives

The main objective of this study was to determine antimicrobial residues and identify antibiotic resistance of selected veterinary drugs in chicken, beef and pork in meat sold, (particularly meat from two butcheries and one supermarket) for human consumption around Mafikeng, North West Province.

1.3.2 Specific objectives

The specific objectives of the study were to:

- Screen the level of antimicrobial residues in raw meat sold in Mafikeng using ELISA;
- Further screening of all antibiotics detected positive from ELISA with TLC;
- Confirm positive samples from the screening test using HPLC;
- Determine the prevalence of different microorganisms from samples confirmed positive for antibiotic residues;
- Identify isolates using preliminary biochemical tests (Voges Proskauer, Urease, catalase, oxidase, indole coagulase test and API 20E, API Staph);
- Confirm the isolates using PCR methods; and
- Determine the antibiotics resistance profile of isolates.

1.4 JUSTIFICATION OF THE STUDY

Food is a vital factor for the transfer of antibiotics resistance. Such transfers can occur through antibiotic residues in food (Abdellah *et al.*, 2013). The consumption of animal products containing residual antibiotics for extended periods may lead to undesirable health effects in humans such as neurological disorders, tissue damage, gastrointestinal disturbances, carcinogenicity and allergic reactions in hypersensitive individuals (Adewuyi *et al.*, 2011). In addition, it may lead to the development of new bacterial strains that are resistant to antibiotics (Moat, 1988).

Unfortunately, exposure to sulphonamides can induce adverse reactions in people including photosensitivity, thyroid toxicity (particularly caused by sulfadimidine), toxic epidermal necrolysis, urinary tract disorders, porphyria, hematopoietic disorders as well as teratogenic effects (Wang *et al.,* 2006). The presence of sulphonamides or higher concentrations may impose health threats due to toxicity and skin allergies (Wang *et al.,* 2006; Shareef *et al.*, 2009). Tetracycline contaminants are considerably important, because of their accumulative toxicity, allergic reactions and the chance that pathogenic organisms could become resistant to these drugs (Andersen *et al.,* 2005). In addition, tetracycline residue in meat can be directly toxic or cause allergic reactions in some hypersensitive individuals (Kebede *et al.*, 2014). Quinolones residues can produce allergic hypersensitivity reactions/toxic effects (Canada-Canada *et al.*, 2009).

It is thus, necessary to determine and monitor antibiotics residues in meat sold around Mafikeng because of increased microbial resistance and the health concern since most people around Mafikeng use meat as a major source of protein.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ANTIBIOTICS

The word *antibiotics* is derived from the Greek words anti (against) and bios (life) and refers to a substance which kills any living organism (Blomberg, 2007). Several antimicrobial agents are used in animals/human beings to treat or prevent certain diseases caused by infectious agents (Donoghue, 2002; Reyes-Herrera, 2005; Raissy & Ansari, 2011). However, the major antibiotics used for human either belong to the same general classes, or have the same mode of action as the one used for animals (Prescott, 2008; Er *et al.*, 2013; Zeina *et al.*, 2013). They are produced by living organisms, which are able to kill/inhibit the growth of other microorganisms (Nita, 2007; Wang *et al.*, 2008). They are products of the earth, more specifically the soil; they are also by-products of cellular metabolisms and most antibiotics are "all natural" (Kenneth, 2002).

Antibiotics work in the following ways:

- *Bactericidal*: killing bacteria by disrupting processes essential to bacterial survival; and
- *Bacteriostatic*: preventing the growth of bacteria.

Bacteria are classified according to their chemical structure, each class is characterised by a typical core structure and the various members of the class are differentiated by the addition/removal of secondary chemical structures from the core structure (Guardabassi & Dalsgaard, 2004).

In general, the types of antibacterial drugs used for the control, prevention and treatment of diseases in animals are similar to those used to treat humans (Hakem *et al.*, 2013). They are also important to lower the transmission of pathogenic agents from animals to humans

(Reyes-Herrera, 2005). Therefore, these drugs (metabolites) are accumulated into body cells and are acknowledged as drug residues and can be detected in meat (Cars *et al.*, 2008).

According to a study conducted in Lebanon by Ibrahim *et al.* (2010), the exact amounts of antibiotics used by farmers in livestock production are not known since they are not regulated. These antibiotics, despite their effectiveness, can leave residues in the treated animal and contaminate edible parts such as the muscles, meat and milk (Pavlov *et al.*, 2008). In addition, resistant bacterial infections are associated with increased mortality, treatment and morbidity compared to their susceptible counterparts (Barza & Travers, 2002).

The major worldwide public concern and health hazard associated with antibiotic residues is the development of an antimicrobial resistance bacterial strain of animal origin and its resultant effect on human health (Zeina *et al.*, 2013). Despite the obvious benefits, improper use of different classes of antibiotics causes bacterial resistance against infectious diseases (Nisha, 2008). Nevertheless, some figures can be used to indicate the orders of magnitude of the consumption of antibiotic agents (Fischer *et al.*, 2003; Companyó *et al.*, 2009).

Some antibiotics (such as tetracycline) undergo minimal metabolism and are mainly excreted in urine and faeces in their microbiologically inactive forms. They are widely distributed in the body with the highest concentrations of residues found in the kidney and liver (Cetinkaya *et al.*, 2012). This tissue distribution pattern is comparable in all food producing animals (Agwuh & MacGowan, 2006; Pavlov *et al.*, 2008; Cetinkaya *et al.*, 2012).

With the global increase in meat consumption in order to meet protein demands, the meat should be clean and free from diseases. Surveillance of emerging infectious diseases in food animals is an important component of food safety systems. Therefore, abattoirs and their regulations play a significant role in food safety and hygiene (Komba et al., 2012). Veterinarians and operators of food business should consider high priority hazards as a major

public health risk in their Hazard Analysis and Critical Control Points (HACCP) plans. Application of HACCP system principles and process hygiene assessments in abattoirs is a legal obligation. Foods of animal origin are considered to be the key sources of food borne diseases. Animal products such as meat and its products are generally regarded as high risk commodities with respect to pathogen contents, natural toxins and other possible contaminants and adulterants. The literature reveals a higher interest for food-borne pathogens (bacterial pathogens have been reported in slaughtered food animals) (Haileselassie *et al.*, 2013).

Table 2.1: Antibiotics commonly used in animal production industries (USDA, 2007)

Antibiotic	**Class industry**
Aminoglycoside	Swine, poultry, beef cattle
β-Lactams	Swine, poultry, beef cattle
Chloramphenicol	Beef cattle
Macrolides	Swine, poultry, beef cattle
Quinolones & Fluoroquinolones	Poultry, beef cattle
Streptogramins	Swine, poultry, beef cattle
Sulphonamidess	Swine, poultry, beef cattle
Tetracyclines	Swine, poultry, beef cattle

2.2 MAJOR GROUPS OF ANTIBIOTICS FOUND AS RESIDUES IN FOOD

The most frequently and most important used group of veterinary drugs are the antibiotics (Fischer *et al.*, 2003). Kaneene & Miller (1997) and Salem (2003) found that the most used antimicrobials in food animals can be grouped into five major classes as follows: the β-lactams (e.g penicillins and cephalosporins), tetracyclines (e.g oxytetracycline, tetracyclines and chlorotetracyclines), aminoglycosides (e.g streptomycin, neomicin and gentamicin), macrolides (e.g erythromycin) and sulphonamidess (e.g sulfamethazine) and

quinolone/fluoroquinolones (eg enrofloxacin, ciprofloxacin). Figure 2.1 shows the groups of antibiotics which mostly presented in animal food in the African continent.

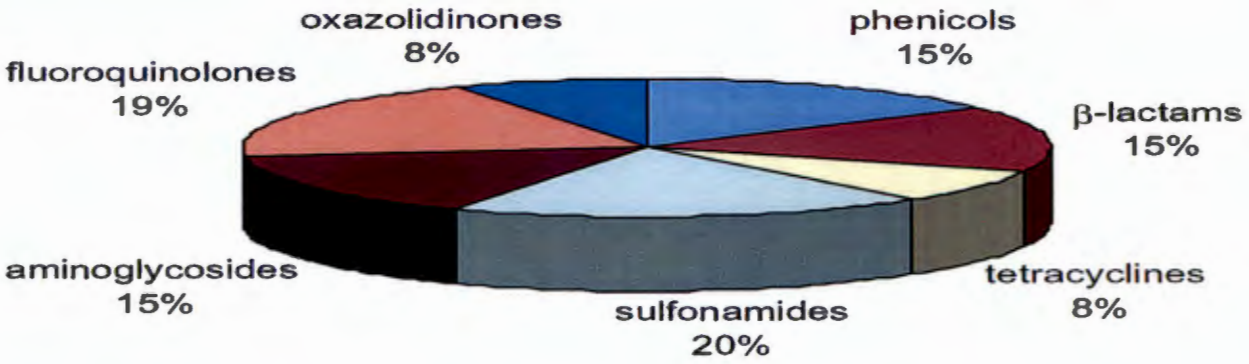


Figure 2.1: Groups of antibiotics mostly present in food (Mungroo & Neethirajan, 2014)

However, a survey conducted by Eagar *et al.* (2008) revealed that out of all antimicrobial usage in animals in South Africa (from 2002 to 2004), macrolides were the most used antibiotic with 42.4%, followed by tetracyclines (16.70%), sulphonamides (12.40%), penicillins (10.70%) and quinolones (0.20%).

2.2.1 Tetracycline

Tetracyclines have served for decades as a vital class of antibiotics in the health of food animals and production (Olatoye & Ehinmowo, 2010). They are used routinely in veterinary medicine for the prevention and control of diseases. They are also used for growth promotion and for the prevention diseases in both chicken and pig production (Dang *et al.*, 2013). They are antibiotics with a broad antibacterial spectrum and have a good activity against acute diseases caused by Gram positive and Gram negative bacteria such as mycoplasma and rickettsia (Rajaian *et al.*, 2008). They have been widely used for the treatment of infectious diseases as well as an additive in animal foodstuffs. They are not relatively easy and inexpensive to produce, but they do not cause severe side-effects and have favourable oral bioavailability and pharmacokinetic parameters (Zakeri *et al.*, 2008). Hence, the purpose of tetracycline as contaminants in propolis is considerably important, because of

their accumulative toxicity, biological adverse effects or allergic reaction and the possibility that pathogenic organisms could become resistant to such drugs (Andersen *et al.*, 2005). Moreover, Tanase *et al.* (1998) maintains that adult dentition is caused by tetracycline.

The use of these drugs against infectious diseases has become a vital problem, as their residues, either in milk or meat, can be directly toxic or cause allergic reactions in some hypersensitive individuals (Zhou *et al.*, 2009; Kebede *et al.*, 2014). Even more vital, consuming food with low levels of Tetracycline for long periods can cause the spread of drug resistant microorganisms (Cinquina *et al.*, 2003). The metabolisms of tetracyclines are known to bind to plasma proteins at varying degrees in different species of animals (Neilsen & Gurd-Hansen, 1996). In addition, tetracycline has a short half-life time (7-10 Hours) (Davis *et al.*, 2006). The acceptable MRLs for tetracyclines as recommended by the regulations governing the maximum limits for veterinary medicine and stock remedy residues that may be present in food stuff, governed under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972) are 100, .300, and 900 µg/kg for liver, muscles, and the kidney, respectively (Mesgari *et al.*, 2009).

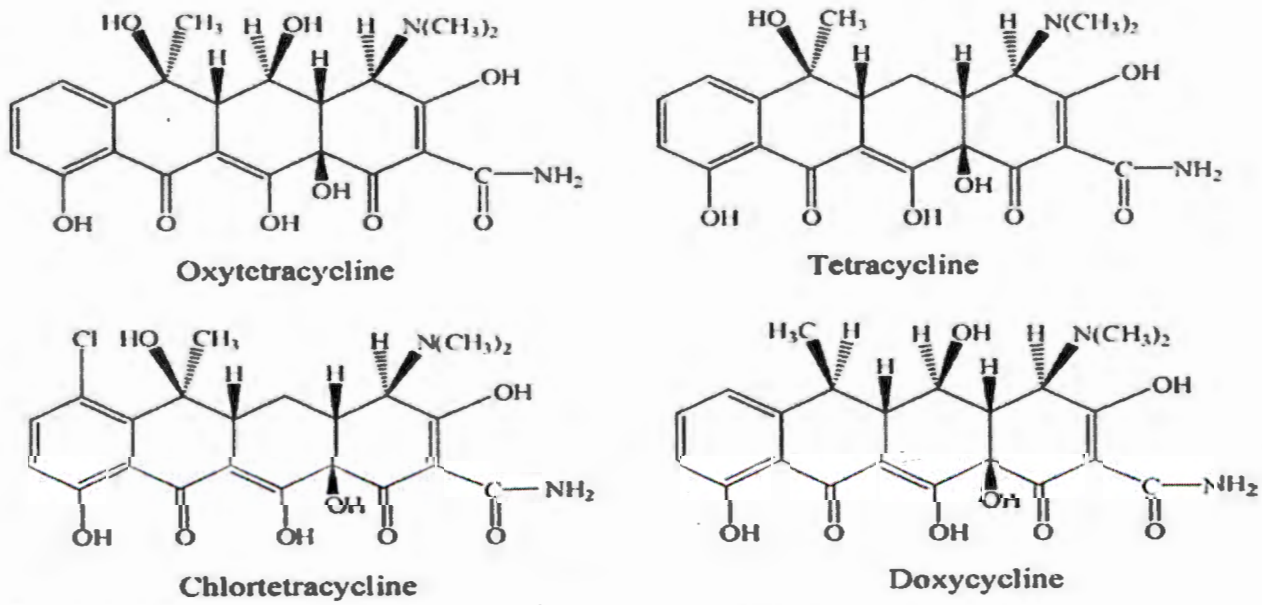


Figure 2.2: Chemical structures of four tetracycline antibiotics (Zhou *et al.*, 2009)

They are rapidly eliminated from edible tissues, thus in most cases, a withdrawal period of only 7-10 hours is generally enough for concentrations to fall below the tolerance level (Lynas *et al.*, 1998). Moreover, most tetracyclines bind almost irreversibly in bone tissues (Zakeri & Wright, 2008). Figure 2.2 shows all the chemical structure of four tetracycline antibiotics (oxytetracycline, tetracyclines, chlortetracyclines and doxycyclines).

Table 2.2: Mechanisms of tetracycline action and resistance (Sköld, 2000; Mathers *et al.*, 2004)

Antibiotic class	Mechanism of antibiotic action	Resistance mechanisms
Tetracyclines (Tetracycline, Chlortetracycline, Oxytetracycline)	Inhibition of protein synthesis (binds 30S ribosomal subunit); interferes with aminacyl-tRNA binding	➢ Ribosomal protection protein ➢ Efflux pumps ➢ Enzymatic (drug alteration)

2.2.2 Streptomycin

Streptomycin is an aminoglycoside produced by some *Streptomyces griseus* strains (Pandey *et al.*, 2014). Streptomycin is well-known for its anti-tuberculosis activity, and has been used in veterinary medicine for the treatment of Gram negative bacterial infections because of its effectiveness and low cost (Ishii *et al.*, 2008; Unusan, 2009). Its action is based on inhibition of ribosomal protein that leads cells to death (Holzgrabe *et al.*, 2011). Most aminoglycoside antibiotics such as streptomycin are widely used in medical and veterinary practices to treat infections from Gram negative bacteria. Susceptible strains include *Escherichia coli*, *Salmonella* spp., *Campylobacter fetus*, *Leptospira spp.* and *Brucella spp.* is also sensitive (Pyun *et al.*, 2008).

Residues of treatment with streptomycin may be found in different food products such as meat, milk and honey (Pyun *et al.*, 2008). The use of streptomycin in animal agriculture has been linked to the increased emergence of resistant strains of pathogenic bacteria that

could potentially impact on human health (Ferguson *et al.*, 2002; Wu *et al.*, 2010). Streptomycin residues can be found in agricultural products such as meat, liver, kidney, milk and honey (Edder *et al.*, 1999; Chang-Won Pyun *et al.*, 2008).

For consumer protection, regulatory authorities have established residue limits for streptomycin in edible tissues and milk (Wu *et al.*, 2010). In Europe, the European Commission, Regulation 2377/90 has established the maximum streptomycin residue limit (MRL): 600 mg/kg in meats, and 1000 mg/kg in kidney (Wu *et al.*, 2010). Figure 2.3 shows the chemical structure of streptomycin.

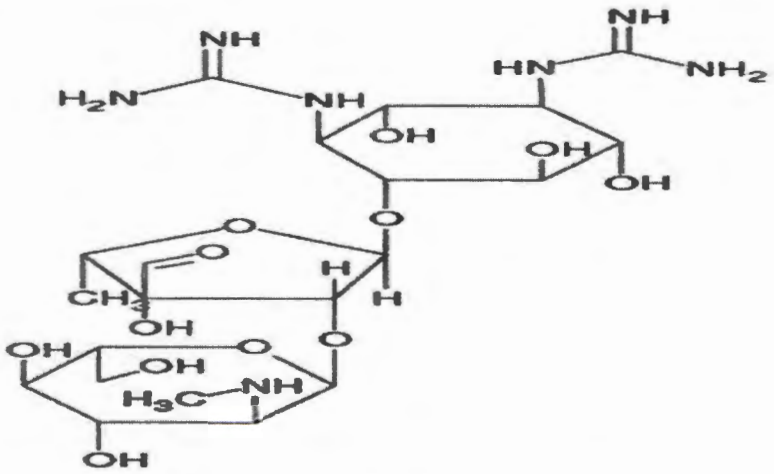


Figure 2.3: Chemical structure of streptomycin (Bogialli *et al.*, 2005)

2.2.3 Sulphonamides

Sulphonamides are a group of synthetic antimicrobial with broad spectrum effects against most Gram positive, Gram negative bacteria and protozoa (Löhren & Cumminutesg, 2009; Mubito *et al.*, 2014). Sulphonamides are widely used for therapeutic and prophylactic purposes in both humans and animals. In addition, they can also be used as additives in animal feed because prolonged ingestion of sulphonamides may have a growth promoting effect (Long *et al.*, 1990; Kim & Park, 1998; Schwarz & Dancla, 2001).

Due to growing demands for meat production, several agents have been employed for animal treatment and for growth promotion. The misuse of this antimicrobial can lead to antimicrobial residues (Table 2.3). These include various types of antibiotics (sulphonamides

and synthesitic as well as natural anabolic agents) (Mahgoub *et al.*, 2006). Sulphonamides (e.g. sulfamethazine, sulfamethiozole, sulfadiazine, sulfamerzine, sulfanilamide, sulfadimethoxine and sulfaquinoxaline) are among the oldest groups of antimicrobial agents as shown in Figure 2.4.

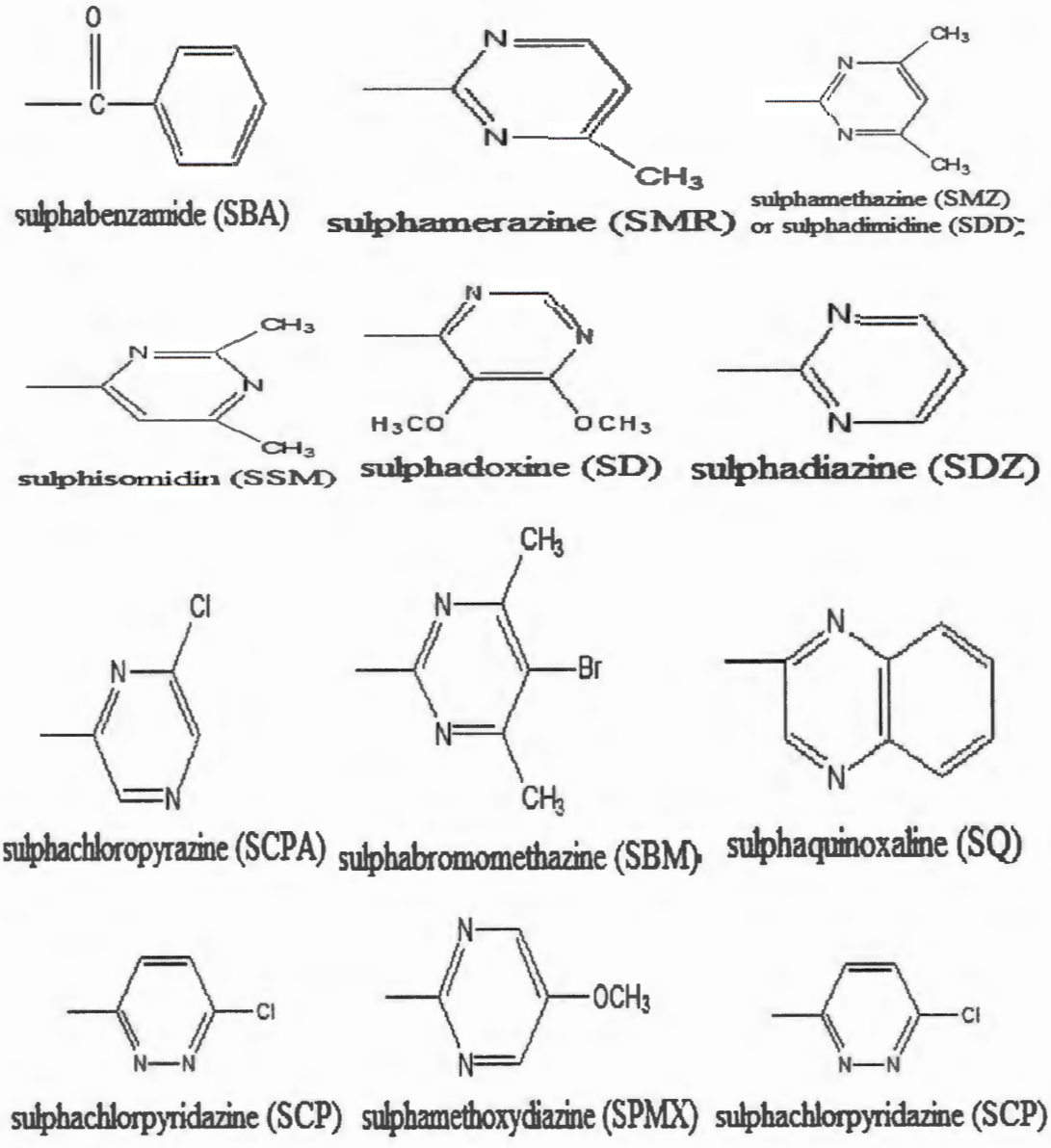


Figure 2.4: Structures of sulphonamides (Wang *et al.*, 2006)

They have a broad spectrum of bacteriostatic action (Richelle, 2007; Sulejmani *et al.*, 2012). Unfortunately, exposure to sulphonamides can induce adverse reactions in people including photosensitivity, thyroid toxicity (particularly caused by sulfadimidine), toxic epidermal

necrolysis, urinary tract disorders, porphyria, hematopoietic disorders, onset of fetal hyperbilirubinemia and kernicterus during late pregnancy, as well as teratogenic effects (Wang *et al.*, 2006). Sulphonamides can persist at injection sites (especially in the muscle) for more than 30 days after application (Reeves, 2005; 2007). Some sulphonamides (e.g sulfadimethoxine, sulfanquinoxaline and sulfadimidine) are used solely in cattle and poultry as soluble powders or solutions for addition to drinking water or as extended-release tablets for cattle. For the second, maintenance of therapeutic concentrations for 2–5 days has been claimed after a single dose. Overall, the potency of sulphonamides is low, for high doses (20–100 mg/kg) to be used therapeutically (Wang *et al.*, 2006). Ninety percent or more of sulphonamides are bound to plasma protein and excreted through urine (Shoaf *et al.*, 1987; Kumar *et al.*, 1998). Furthermore, most reliable samples to test for antibiotic residues can be plasma protein or kidney. Several organisations such as the Food and Agriculture Organisation (FAO) and the European Union (EU) have set tolerance or Maximum Residue Limits (MRL) for antibiotic residues in foodstuff derived from animals. According to the supervision commission report of Codex Alimentarius Commission (CAC), regulations governing the maximum limits for veterinary medicine in South Africa, (Act No. 54 of 1972), the maximum residue levels of all sulphonamides (sulfamethazine, sulfamethiozole, sulfadiazine, sulfamerzine, sulfanilamide, sulfadimethoxine and sulfaquinoxaline) should not exceed 100 µg/kg in chicken, beef and pork.

2.2.4 Quinolones

Quinolones (ciprofloxacin, enrofloxacin, oxolinic acid, flumequine, nalidixic acid, sarafloxacin, danofloxacin, orbifloxacin, marbofloxacin, gatofloxacin, grepafloxacin) have been widely used in animal production and veterinary medicine for the treatment and prevention of diseases (Bregante *et al.*, 1999; Buket *et al.*, 2013) and also in human beings (Canada-Canada *et al.*, 2009; Chafer-Pericas *et al.*, 2010). Sultan (2014) maintains that

ciprofloxacin and enrofloxacin are usually prescribed for the treatment and prevention of infectious diseases in farm animals. Moreover, it is normally measured as a protective asset when raising animals under intensive husbandry production methods. Since the quinolones are the most widely used antibiotics in veterinary medicine, residues of the drug may be observed in foods of animal origin if proper checks and balances are not employed (Bregante *et al.*, 1999; Er *et al.*, 2013). In addition, Jiang *et al.* (2013) posit that, the most popular antibiotic agents used for the promotion of growth and for the treatment of infections are fluoroquinolones due to their broad spectrum against both Gram-positive and Gram-negative microorganisms. The problem with quinolones is that they are effective inhibitors of DNA gyrase enzyme, which is vital for DNA replication and transcription (Suto *et al.*, 1992). For residues, it has been indicated that, because of the occurrence of quinolones residues in human food obtained from animal sources, the use of quinolones in human treatment has decreased (Donkor *et al.*, 2011; Buket *et al.*, 2013).

Er *et al.* (2013) maintain that the maximum residue levels (MRLs) of enrofloxacin and ciprofloxacin in chicken muscles and beef muscles is 100 μg/kg while for marbofloxacin is 150 μg/kg in beef muscles. The major route for the removal of fluoroquinolones is through the kidney. This is done through glomerular filtration and, for some drugs (Bregante *et al.*, 1999; Er *et al.*, 2013) it is achieved through tubular secretion, while smaller amounts are eliminated in the faeces (Bregante *et al.*, 1999).

The FDA (2001) maintain that since the approval of fluoroquinolones for use in food producing animals, reports have recognised a relationship between the authorisation of fluoroquinolones for therapeutic use in food producing animals and the development of fluoroquinolones resistance in campylobacter in animals and humans. However, tghe use enrofloxacin in poultry has been prohibited due to its resistance to campylobacter (FDA, 2001). The authorisation of these drugs in food-producing animals in some developed

countries such as Spain, the Netherlands and the United States of America (USA) preceded increases in resistance in campylobacter isolates from treated animals and sick humans (Smith *et al.,* 1999). However, WHO (1998) maintains that a number of quinolones are accredited for use in food animals in different areas such as Asia, Latin America and South Africa. In the European Union (EU), countries have established a maximum residue level (MRL) of 200, 100 and 300 µg/kg for liver, muscle and kidney tissues respectively for enrofloxacin and ciprofloxacin.

2.3 MECHANISMS OF ACTION OF ANTIBIOTICS

A study conducted by Walsh (2000) revealed that antimicrobial agents act selectively on vital microbial functions with minimal effects or without affecting host functions. In other words dissimilar antimicrobial agents act in different ways (Yousif *et al.*, 2014). However, an understanding of these mechanisms as well as the chemical nature of antimicrobial agents is crucial in understanding the development of their resistance. It has been acknowledged that antimicrobial use is the single most important factor that accounts for increased antimicrobial resistance (Aarestrup *et al.*, 2001).

Generally, antimicrobial agents may be described as either bacteriostatic or bactericidal. Bacteriostatic antimicrobial agents can slow down the growth and multiplication of the bacteria giving the immune system of the host time to clear them from the system. Absolute elimination of the bacteria in this case is dependent on the competence of the immune system (Byarugaba *et al.*, 2010).

Bactericidal agents kill the bacteria and, therefore, with or without a competent immune system of the host, the bacteria will be dead (Byarugaba, 2004). However, the action mechanisms of antimicrobial resistance of antimicrobial agents can be categorised further

based on the structure of the bacteria or the function that is affected by the agents (Byarugaba, 2004; Byarugaba *et al.*, 2010).

The mechanisms of antimicrobials can be grouped into inhibition of nucleic acid function and inhibition of protein synthesis (Yousif *et al.*, 2014). The aim of antimicrobial therapy is to rapidly produce and maintain an effective concentration of drug at the site of infection for sufficient time to allow host specific and nonspecific defences to get rid of the pathogen (Prescott, 2000). Details of the effect and mechanisms of action of antibiotics have been described in the literature and a summary of the mode of action for the major classes is provided in Table 2.3.

Table 2.3: Summary of mechanisms of action of antimicrobial agents (Byarugaba *et al.*, 2010)

Group of antimicrobial agents	Effect on bacteria	Mode of action in general
Penicillins	Bactericidal	Inhibition of cell wall synthesis
Cephalosporins	Bactericidal	Inhibition of cell wall synthesis
Quinolones	Bactericidal	Inhibits DNA synthesis
Lincosamides	Bacteriostatic except in the lungs	Inhibition of protein synthesis
Aminoglycosides	Bactericidal	Inhibition of protein synthesis
Tetracyclines	Bacteriostatic	Inhibition of protein synthesis
Chloramphenicol	Bacteriostatic	Inhibition of protein synthesis
Sulphonamidess	Bacteriostatic	Competitive inhibition of DNA synthesis

2.4 EFFECTS AND PROBLEMS OF ANTIBIOTICS

Abjean (1997) and Nisha (2008) maintain that the pathological effects produced by antibiotic residues in food can have the following effects on human beings: immunopathological effects; autoimmunity; carcinogenicity (sulphamethazine, oxytetracycline and furazolidone) mutagenicity such as nephropathy (gentamicin); alteration

of the intestinal flora (tetracycline); hepatotoxicity such as reproductive disorders; bone marrow toxicity (chloramphenicol) and allergies (penicillin). Benet & Belleemain (2005) and Wassenaar (2005) maintain that behavioural practices such as overuse of drugs and lack of understanding about drug usage also contribute to food contamination.

The presence of antibiotics in human food is associated with several adverse public health effects. Furthermore, Kivaria (2007) and Katerina *et al.* (2013) point out that few reports indicate that sensitive individuals may experience allergic reactions to antibiotic residues, particularly penicillin allergic reactions in humans, and can negatively affect the human immune system. Sulphonamides are widely used in animal husbandry, for therapeutic and prophylactic purposes, particularly for respiratory conditions (Wang *et al.*, 2006). There have been some concerns about carryover of veterinary drugs into eggs, meat, and milk and the possible adverse effects of residues on people consuming such foods (Gehring *et al.*, 2006). In addition, the quantity of antimicrobials used in human health systems is further augmented by drugs prescribed in the treatment of syndromes (e.g. AIDS), where antimicrobials are not necessarily pathogen specific (Powers, 2004). Furthermore, Crewe-Browne *et al.* (2003) maintain that in 2000, there was an increase in the rate of invasive *salmonella* infections in HIV/AIDS patients, increasing antimicrobial resistance of a multi resistant *salmonella typhimurium* DT 104, at Chris Hani Baragwana Hospital, South Africa.

2.5 ANTIBACTERIAL RESIDUES

Maximum Residue Limits (MRLs) means the maximum concentration of residues resulting from the use of a veterinary medicinal products which may be legally permitted or recognised as up to standard in food, allocated to entity food commodities (Donkor *et al.*, 2011). Drugs or their metabolites left over in the body after their administration for a longer period of time are referred to as residues (Kang'ethe *et al.*, 2005). According to studies conducted around the world, several factors lead to the occurrence of antibiotic residues in

animal products as follows failure to observe drug withdrawal period; poor records of treatment; failure to identify treated animals; lack of advice on withdrawal periods; off label use of antibiotics; availability of antibiotics to lay persons as over the counter drugs in developing countries and extended usage or excessive dosage of antibiotics (Muhammad *et al.*, 1997; Lee e*t al.*, 2000; Kang'ethe *et al.*, 2005; Kurwijila *et al.*, 2006).

It is based on the type and amount of residue considered to be without any toxicological and microbiological hazard for human health as expressed by the Acceptable Daily Intake (ADI), or on the basis of a temporary ADI that utilises an additional safety factor (EC, 2001; Jabbar *et al.*, 2013). Concentrations drug residue vary considerably from tissue to tissue and are generally higher in tissues of storage such as body fats, or in organs that actively metabolize and excrete them (for example, residues may be highest in body fats, liver and kidneys) (Jabbar *et al.*, 2013). Antibiotic residues in foods of animal origin is a sources of concern among the public and medical health professionals (Adams, 2007). The most common causes of the presence of antibiotic residues in food of animal origin are violation of withdrawal periods, overdosing of antibiotics and use of antibiotics banned for the treatment of economic animals (Kang'ethe *et al.*, 2005; Kurwijila *et al.*, 2006).

According to Nisha (2012), human exposure to animal products containing significant levels of antibiotic residues may cause immunological response in susceptible individuals, reproductive and intestinal disorders, in addition to the transfer of antibiotic resistance bacterial in humans who constitute a major concern for public health. The evaluation of exposure to antibiotic residues has always been controversial, particularly in the geographical regions where the tracking of restrictive policies on the use of veterinary antibiotics is absent (Maia *et al.*, 2009). Many attempts have thus been made to find out antibiotic residues in food of animal origin (Khaskheli *et al.*, 2008; Maia *et al.*, 2009). Residues of antibiotics were quantified in pork and chicken muscle tissues that had earlier been screened with a

microbiological inhibition test by De Wasch *et al.* (1998). Residues from antibacterial drugs in food products of animal origin can represent a risk for consumers. However, the poisonous effects are not very possible since residues are present in very low concentrations. Some substances must take delivery of particular attention due to allergic reactions (IFT, 2006). This is due to the absence of restrictive legislation or their inadequate enforcement and lack of consumer awareness on the magnitude and human health hazards associated with antibiotic residues in foods of animal origin (Paige *et al.*, 1997; Darwish *et al.*, 2013).

2.6 WITHDRAWAL PERIOD OF ANTIBIOTICS

The withdrawal period is the time between the last dose and the time when the concentration of residues in the tissues (muscle, skin/fat liver, kidney, or products milk, honey and eggs) is lower than or equal to the MRL (Myllyniemi, 2004). To ensure that drug residues have declined to a safe concentration following the utilisation of drugs in animals, a particular period of drug withdrawal must be observed earlier to providing any products for human consumption. Moreover, many drugs are remained in animal bodies for longer periods than indicated (Kivaria, 2007). These conditions favour the selection and spread of antibiotic resistant bacteria among animals, the environment and humans. They cause infections that are more complicated to treat, last longer or are more severe than antibiotic sensitive infections (APUO, 2010; Collignon, 2009).

In addition to drug dose, residue levels also depend on withdrawal times (IFT, 2006; Donkor *et al.,* 2011). Despite the fact that most drugs representing a relatively low risk for the general public, when used correctly and in line with orders approved by the manufacturing companies' unpleasant reactions have been frequently reported for some compounds. These include antibacterial, antihelminthic, anticoccidial and antiprotozoal drugs and growth promoters (Teuber, 2001; IFT, 2006; EC, 2010).

2.7 ANTIBACTERIAL RESIDUES IN ANIMALS

The most common uses of antimicrobial residues in food-producing animals is to promote growth, for the treatment and prophylaxis. It is also essential for a sustainable and economically sustainable animal industry (Eagar, 2008). Antibiotics are used in veterinary medicine and subsequently drug residues may persist in foods derived from animals (Ekene *et al.*, 2014). It has been reported that low concentrations of antibiotics in animal feed or water may lead to enhanced feed conversion efficiency and hence improved growth rate (Chanda *et al.*, 2014). This has consequently led to the increase use in antimicrobial residues in food animals. Antibiotics are usually used for the prevention and treatment of animal diseases and to improve the efficiency of animal production (Omeiza *et al.*, 2012). However, their effectiveness is threatened by wide and unfit use, not only in medicine but also in agriculture (Mrema *et al.*, 2006; Wageh *et al.*, 2013; Chanda *et al.*, 2014).

It is evident that this practice results in the emergence of resistant bacteria (Dallal *et al.*, 2010; Hur *et al.*, 2011). In addition, the extensive use of antibiotics has led to the development of resistance against antibiotics by bacteria (Posyniak *et al.*, 2005; Omeiza *et al.*, 2012). Despite all these, antibiotics meant for the treatment of infectious diseases, antibiotic residues in eggs, milk, meat and other products can have serious side effects on human (CDC, 2009; Ekene *et al.*, 2014). In addition, due to lack of bio-security measures, prevalence of infectious diseases and subsequently, the indiscriminate use of the drug without observing withdrawal periods render poultry and pig products unsafe for human health (Mehtabuddin *et al.*, 2012; Mubito *et al.*, 2014). Misuse and overuse of antimicrobial drugs creates selective evolutionary pressure that enables antimicrobial resistant bacteria to rapidly increase in numbers than antimicrobial susceptible bacteria thus increasing the opportunity for individuals to be infected by resistant bacteria (Adesokan *et al.*, 2013).

2.8 ANTIBACTERIAL RESIDUES IN HUMAN BEINGS

Most consumers are concerned about food production methods and the consequences of such agricultural practices on humans, animals and the environment (Herrera, 2010). One of such practices that have attracted public attention is the use of veterinary drugs in agricultural settings and their potential occurrence as residues in food products (Verbeke *et al.*, 2007; Herrera, 2010). Several studies are currently underway in order to investigate the presence of different veterinary dugs, just as is the case with antibiotic residues in animal products due to the side effects on the health of human consumers (Hind *et al.*, 2014). Despite the beneficial effects of antibiotics in the treatment of infectious diseases, antibiotic residues can have severe side-effects on humans (IFT, 2006). In addition, humans as a non target species for these drugs, receive different amounts of antibiotics as a residue which can cause huge health hazards to consumers such as, bacterial resistance, allergic reactions, toxicity, carcinogenic effects and change of natural micro flora of the intestine in consumers (Katerina *et al.*, 2013; Karmi, 2014; Mohammad *et al.*, 2014). Considering the factors mentioned above, the control of antibiotic residues in food is necessary and important to ensure food safety for consumers.

Antimicrobial residues in foods of animal origin may be problematic for several reasons. In addition to the toxic reactivity, effects on intestinal microbiota and the immune system are important (Perrin-Guyomard *et al.*, 2001). Microbiological endpoints are considered more valid and sensitive in the safety evaluation of antimicrobial residues in production animals than standard toxicological endpoints (Boison, 2001).

Antibiotic residues lead to antibiotic resistant and many resistance antibiotics such as *E. coli* strains that cause urinary tract and bloodstream infections in humans are likely to have originated from contaminated retail meat (Carlet *et al.*, 2012). Moreover, Myllyniemi *et al.* (2000) maintain that disorder of normal human flora in the intestines is another harmful effect

of drug resistance in human food. Consumers and retailers in developing countries are increasingly aware of the health disorders due to drug residues in food and urge public health services to do more in terms of control in the meat and animal products sector (Grunert, 2005; Wang *et al.*, 2008). Therefore, misuse of antimicrobial drugs in farms and residues in animal products becomes a major issue for the veterinary sector and public health services in developing countries (Kang'ethe *et al.*, 2005; Ibrahim *et al.*, 2010).

2.9 ENVIRONMENTAL CONCERNS

Antibiotics used in animal agriculture can enter the environment through a number of routes such as the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drugs (Buchberger, 2007). However, different types of drugs have different anticipated exposure routes to the environment (Jørgensen & Halling-Sørensen, 2000). In addition, Sarmah *et al.* (2006) maintain that, after treatment, most antibiotics are excreted by animals through urine and faeces either unaltered or as metabolites. This makes them potentially hazardous to bacteria and other organisms in the environment (Baguer *et al.*, 2000). The most prevalent antibiotics found in the environment (such as surface waters) belong to the macrolide and the sulphonamides groups. Low concentrations of penicillins, tetracyclines and fluoroquinolones have only been found in several cases (Hur *et al.*, 2011). Furthermore, these drugs tend to persist for long periods of time in the environment and can lead to an underestimation of exposure to risk (Agwuth & MacGowan, 2006; Buur *et al.*, 2006).

Although little is known about the extent of environmental occurrence, transport and ultimate fate and effects of pharmaceuticals in general (Kummerer, 2003), it is known that antibiotics are not bio accumulative, but their continual release into the environment gives them persistent quality (Erickson, 2002; Li *et al.*, 2013). Furthermore, Lee *et al.* (2001) posit that wide spectrum resistance to antibiotics is the chronic effect related with soil microflora

which receives antibiotic residues. The resistant genes transferred from environmental bacteria to humans' constitute a main public health concern (Kruse, 1999; Gebre, 2012; Olusola *et al.*, 2012). Without research and the necessary regulations that follow, multiple resistant genes will continue to be released into the environment as a consequence of the misuse of antimicrobials in large-scale animal production in developing countries (Sarmah *et al.*, 2006; Arriola, 2011).

2.10 ANTIBIOTIC RESISTANCE

Antibiotic resistance in bacterial isolates is a global phenomenon and is becoming a problem particularly in developed countries (Ateba *et al.*, 2008). A bacterial isolate is classified as resistant when it is no longer inhibited by concentration of antibiotics that would otherwise inhibits the growth of susceptible members of such group (WHO, 1999). Resistance to an antimicrobial agent was first recognised by scientists soon after penicillin was used for the first time (Campagnolo *et al.*, 2002; Aarestrup, 2005). Veterinary antimicrobial use is a selective force for the appearance and prevalence of antimicrobial resistant bacteria in animal products (Tamura, 2003). However, antimicrobial resistant bacteria are established in the absence of an antimicrobial selective pressure. Anderson *et al.* (2003) maintain that the emergence of multi drug resistant (*Salmonella typhimurium*) definitive type DT104 in the USA and the United Kingdom (UK) is an example of how a highly resistant clone of salmonella has the ability to effectively spread among animals and later to humans. The DT 104 is resistant to certain antibiotics such as chloramphenicol, sulphonamidess streptomycin, ampicillin and tetracycline. According to Rodloff *et al.* (2008), depending on the minimal inhibitory concentration (MIC), values composed to those of the NCCL organisms can be classified as:

a) Susceptible: bacterial strain is said to be susceptible to a given antibiotic when it is inhibited in vitro by a concentration of this drug associated with a high likelihood of therapeutic success.

b) Intermediate: the sensitivity of a bacterial strain to a given antibiotic is said to be intermediate when it is inhibited in vitro by a concentration of this drug associated with an uncertain therapeutic effect.

c) Resistant: a bacterial strain is said to be resistant to a given antibiotic when it is inhibited in vitro by a concentration of a drug associated with a high likelihood of therapeutic failure.

During the transfer of resistant food-borne pathogens or during the intake of resistant strains of the original food microflora and resistant transfer to be pathogenic microorganisms (Pesavento *et al.*, 2007; Abdellah *et al.*, 2013), the gastrointestinal tract of humans and animals is believed to the most probable site for some species to acquire resistant genes via conjugative plasmids and transposons (Doucet-Populaire *et al.*, 1991). In addition, Saulat (2012) posits that the consequences of food-borne illnesses are normally from consumption of food containing pathogens such as bacteria.

Gray *et al.* (2002) acknowledge that as the resistance to most antibiotics rises, and becomes a public health concern, modulation of virulence factor expression by antibiotic treatment may be of increasing importance. In addition, because 95% of antibiotics given to livestock are excreted unchanged, bacteria living on people who have usual contact with animal waste or in an environment close to animal waste are constantly exposed to antibiotics and may build up resistance (Choi, 2007).

The appearance of resistant bacteria is believed to be linked with all types of antimicrobial uses, as well as the use of antimicrobials in human medicine, veterinary medicine and food animal production (Saulat, 2012). Nevertheless, the magnitude of the

public health collision of antimicrobial use in animals remains unclear (Alicia *et al.*, 2003). Rising resistance to antimicrobial agents that are significant in the treatment of human diseases, such as tetracycline and fluoroquinolones for the treatment of some bacterial infections, have major public health implications (Alicia *et al.*, 2003; Bedada & Zewde, 2012). In addition, antibiotics such as tetracycline, fluoroquinolones and third generation cephalosporins for the treatment of infections of some organisms have significant public health implications, as they increase resistance to antimicrobial agents that are important in the treatment of human diseases (Bedada *et al.*, 2012).

Some studies have been conducted around the region to asses antimicrobial resistance and the by different studies revealed high levels of resistance (Ateba *et al.*, 2008 Moneoang *et al.*, 2009; Ateba *et al.*, 2010; Ateba & Setona, 2011). As the animals compete for food sources, the doses received differ between individuals. The consequence of some individuals receiving higher doses than others introduces another differential in the selective pressure on commensal bacteria 28 and opens the possibility for the transfer of genes encoding for antibiotic resistance and multi-drug resistance (Bester & Essack, 2010).

The results of the surveillance yielded levels of resistance that were high in *E. coli* and *Enterococcus*. *E. coli* showed a 67% resistance to one or more commonly used antimicrobials, especially tetracyclines, fluoroquinolones and sulphonamides, of comparative profiles from European counterparts (Van Vuuren *et al.*, 2007). In addition, systematic review of published literature by Nyasulu *et al*, (2012) on antimicrobial resistance surveillance among nosocomial pathogens in South Africa revealed resistance to commonly used antimicrobial drugs in the population. Moreover, Droumev, (1983) maintain that, antibiotics can penetrate and stay in an animal's tissues, particularly if the antibiotic initial dosage of the antibiotics is high or withdrawal time is not sufficient before slaughtering. Human exposure to animal products containing considerable levels of antibiotic residues may

induce and transfer resistance to human pathogens and also cause several problems including disturbed immunological response and disorder of intestinal flora in susceptible individuals (Sogaard, 1973). Results of the 2007 SANVAD surveillance yielded levels of resistance that *E. coli* and *Enterococcus*. for *E. coli* showed a 67% resistance to one or more commonly used antimicrobials, especially tetracyclines, fluoroquinolones and sulphonamides. A susceptibility study was undertaken of *E. coli* strains collected in poultry, measured against five antimicrobial agents used in the South African poultry industry. All strains showed susceptibility to danofloxacin and colistin, while 96% were resistant to two tetracyclines (Bester & Essack, 2010).

2.11 ANTIMICROBIAL RESIDUES AND PUBLIC HEALTH CONCERNS

Monitoring of antibiotic residues is very important in controlling the safety of foods for human consumption (Koesukwiwat *et al.*, 2007; Shukla *et al.*, 2011). The misuse of antibiotics in food producing animals may possibly leave residues in foodstuffs of animal origin (Tollefson & Miller, 2000). Antibiotics can encourage the spread of antibiotic resistance in bacteria, making treatment of human infection more difficult. However, it has been recommended that antibiotics used in human medicine should not be used in animals (Wageh *et al.*, 2013). In addition, bad practices based on the use of antibiotics can increase and select multi-resistance of pathogenic strains of bacteria, which can be transmitted to humans through food (Hayes *et al.*, 2002; Reig & Toldra, 2008).

Consumers and retailers in developing countries are increasingly aware of the health disorders due to drug residues in food and urge public health services to be more vigilant in the control of the meat and animal products sector (Grunert, 2005; Wang *et al.*, 2008). Therefore, misuse of antimicrobial drugs in farms and residues in animal products become a major issue for the veterinary sector and for public health services in developing countries (Kang'ethe *et al.*, 2005; Ibrahim *et al.*, 2010).

Concerns have been raised regarding the possible presence of residues, some of the drugs in foods of animal origin due to the occurrence of antimicrobial residues such as penicillin hypersensitivity in humans and development and transfer of antibiotic resistance between animals and human (Mccracken *et al.*, 2005). In addition, Mather *et al.* (2012) also maintain that antimicrobial resistance (AMR) and the resulting failure of antimicrobial therapies in humans is a mounting public health problem of global significance and presents a key and rising threat to the effective treatment of bacterial infections. The development of antibiotic resistant bacteria and allergic reactions in humans is known are the consequences of long-term ingestion of antibiotics (Nisha, 2012). To date, there is the ongoing threat of antibiotic resistance which is a global public health concern (Cars *et al.*, 2008; Collignon, 2009). Excessive use of antimicrobials in agriculture, subject microorganisms to the same forces of evolution and resistance occurs when antimicrobials are used in humans (Van den Bogaard & Stobberingh, 2000). Resistance of antibiotics are mainly caused by antibiotic residues.

The issue of antimicrobial residues in foods of animal origin has rarely been a serious concern in developing countries, moreover, to the health risk to local populations, the presence of residues from veterinary medicinal products in foods of animal origin could put at risk international trade in the wake of the World Trade Organization (WTO) concord on the submission of Sanitary and Phytosanitary Measures (the 'SPS Agreement'). Compliance with the Codex Alimentarius Commission rules on veterinary drug residues should serve as a guarantee of quality, enabling livestock producers to access other markets (Paige et al., 1997; Mathew *et al.*, 2007).

2.12 IMPACT OF COOKING ON ANTIMICROBIAL RESIDUES IN MEAT

Chicken, beef and pork are among the most common foods consumed worldwide, particularly in South Africa (Fawzi & William, 2015). Regardless of the everyday intake of

chicken, beef and pork, consumers are exposed to the adverse effects of veterinary residues, including tetracyclines, streptomycin and sulphonamides (Ismail-fitry *et al.*, 2008). Antibiotic residues are primarily found in meat samples due to over dosage in farms and during slaughtering before withdrawal period (Kishida, 2007; Ismail-fitry *et al.*, 2008). Not many studies have addressed the results of cooking treatments on antibiotic residues in different samples and different antibiotics. In 2007 and 2009 the largest amounts of antibiotics were sold in the USA for use in food animals and the major classes were tetracyclines (Salah *et al.*, 2013). Antibiotic residues in edible products can appear from the treatment of diseases and nutritional purposes, including foods derived from all livestock, in spite of species posing residue problems (Gratacós-Cubarsí *et al.*, 2007). A Moat (1988) maintains that, tetracycline in animal tissues is more stable under heat treatment and is not totally inactivated by heat processing or cooking conditions. However, other studies have indicated that, at 100°C for 30 minutes or at higher temperature for shorter times, tetracyclines in animal tissue is more sensitive to heat treatment and is totally destroyed (Fedeniuk *et al.*, 1997; Gratacós-Cubarsí *et al.*, 2007). However, many reviews have indicated that temperature through cooking has major impact on the loss of tetracycline residues (Honikel *et al.*, 1978; O'Brien *et al.*, 1981; Loksuwan, 2002; Hassani *et al.*, 2008; Abou-Raya *et al.*, 2013).

Heshmati (2015) argues that, cooking is not effective in reducing the amounts of quinolones residues such as flumequine in fish. Moreover, according to Botsoglou & Fletouris (2001), a quinolones residue in flatfish has high heating stability. Roca *et al.* (2010) maintain that the maximum losses of concentrations of ciprofloxacin and floxacin were 12.71% and 12.01%, respectively at 120°C for 20 minutes. Since quinolones residues were not tainted during processing, their presence in food threatens human health (Heshmati, 2015).

It was reported in the past that sulphonamidess are heat stable compounds (Heshmati, 2015). However, Smit *et al.* (1994) found that 25% of Sulfadimidine was lost in the brine, during manufacture of raw fermented sausages. Moreover, different times (3, 6 and 9 minutes) and temperatures (170, 180 and 190°C) of deep frying had different effects on sulphonamides residues in chicken meat (Heshmati, 2015). In various cooking procedures, adequate heating temperature and time can lessen several antibacterial residues even though it does not generally provide an extra margin of safety for human population. Mohamed & Samah (2013), found that, reduction of the percentage of enrofloxacin residues were 25.6% and 33.3% for frying and grilling respectively. Rose *et al.* (1996) examined the heat stability of oxytetracycline and found the drug to be unstable. Moreover, the result of frying and grilling obtained by Mohamed & Samah (2013) on enrofloxacin residues was almost similar to the one obtained by Lolo *et al.* (2006).

2.13 ANTIBACTERIAL DETECTION IN MEAT

2.13.1 Introduction

The kidney and liver are the target organs for most antibiotics because the drug concentrations in these organs are typically higher than in other edible tissues (Sárközy, 2001; Wang *at al.*, 2012). The muscle can present analytical difficulties because of variability in residue distribution, particularly in areas around injection sites. There is also the concern of a lower probability of finding non compliant samples in muscle compared to matrices such as the liver and kidney (Antignac *et al.*, 2004).

Samples preparation can affect all the later assay steps and therefore is critical for unequivocal, quantification, confirmation and identification of analytes. It includes both the isolation and/or pre-concentration of compounds of attention from various matrices and also makes the analysis more appropriate for separation and detection. Sample preparation typically takes more than 70% of the total analysis time (Reyes-Herrera *et al.*, 2005). The free

parent and metabolite residues are readily extracted by organic solvents, H_2O, or aqueous buffers, depending on their solubilities and polarities. However, residues of some compounds may be present in the conjugated forms (glucuronides or sulfates) and need liberation in the course of enzymatic or chemical hydrolysis prior to extraction. Hydrolysis conditions (viz, pH, temperature, time) have to be cautiously optimised to ensure efficient deconjugation of residues (Wang *at al.*, 2012).

2.13.2 Detecting antibacterial residues

The analytical methods for the detection of antibiotic residues can be divided into two groups as follows: screening and confirmatory (Cháfer-Pericás *et al.*, 2010). There are different screening methods for the detection of antibiotics in foods of animal origin such as Thin Layer Chromatography (TLC) (Juhel-Gaugain & Abjean, 1998), Enzyme Linked Immunosorbent Assay (ELISA) (Huet *et al.*, 2006) and others (Four-Plate Test (EU4pt), the Nouws Antibiotic Test (NAT), and a commercial ampoule test, the Premi Test) (Chen, 2012).

2.13.2.1 Enzyme-Linked-Immunosorbent Assay (ELISA)

Among the different screening methods, the Enzyme-Linked Immunosorbent Assay (ELISA) has been the most widely used and confirmed as a screening method for antibiotics (Edder *et al.*, 1999; Zeina *et al.*, 2013). This method is highly sensitive and has broad specificity (Galarini *et al.*, 2014) and can be used for the screening of a large number of small volume samples (Wang *et al.*, 2009).

The method is always based on the competition enzyme-linked immunosorbent assay (competition ELISA) (Mahmoud *et al.*, 2014). The ELISA microtiter plate is coated with a bound antibody against the antibiotic and the detecting reagent is a covalent complex of this antibiotic and an enzyme. The reagent is mixed with a sample of the antibiotic extract and the mixture is placed in a well. In the control well (absence of antibiotic in the sample), the antibiotic-enzyme conjugate can saturate the bound antibody, and addition of a chromogenic

substrate which results in the development of colour. In the test well, free antibiotic molecules in the extract compete with the conjugate on the bound antibody (Buket *et al.*, 2013). Cross reactivity might occur with the secondary antibody, resulting in nonspecific signal.

The higher the concentration of antibiotic, the less the conjugate can react with the bound antibody, leading to fainter colour development. Data obtained clearly show that ELISA has been the most widely used analytical method in the past decade to measure the presence of antibiotics. The use of ELISA can be justified by the fact that it is simple and affordable, easy to use and does not need expensive equipment such as liquid chromatography. Nevertheless, several researchers, mostly from developed countries combine Imunoaffinity column, HPLC and Liquid Chromatography for specificity and confirmation of results (Tang *et al.*, 2006; Bogialli & Di Corcia, 2009; Zeina *et al.*, 2013).

2.13.2.2 Thin Layer Chromatography (TLC)

Thin Layer Chromatography has been widely used for the determination of antibiotic residues in foods. TLC can be used as a screening test as it is a sensitive and specific method for monitoring low amounts of different biological and chemicals residues (Bedada & Zewde, 2012). Therefore, it is used for separation, purity assessment and identification of organic compounds. Chromatography is used to separate a mixture into its various components. TLC's are available for a large number of antibiotics, since detection and identification procedures have been specifically developed for each antibiotic making use of chromatography. Since it is one of the screening methods, it is generally not expensive and is less time consuming than the more precise quantitative or confirmatory methods. In a broader sense, they do not require very laborious sample pre-treatment steps (Tajick & Shohreh, 2006). The measurement parameter in any form of chromatography is the distribution

coefficient (k) of a substance between the two phases. k is dependent on the temperature and concentration of the solute (Touchstone & Dobbins, 1983).

2.13.2.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a common method for determining the presence of antibiotics (Gigosos *et al.*, 2000). The use of high performance liquid chromatography (HPLC) expanded during the 1990s and the availability of automation somehow facilitated its use as a screening technique (Wilson & Walker, 2001). High performance liquid chromatography (HPLC) is a specific form of column chromatography commonly used in biochemistry and analysis to separate, recognise and quantify active compounds (Malviya *et al.*, 2010). If some analytes are not detected by absorbance, refractive index or fluorescence may require chemical modifications to render chromophore, fluorescent or UV absorbing compounds (Kirbis *et al.*, 2005). HPLC has become the most widely used method for the purpose of antibiotic residues in meat, milk and poultry, and a number of methods have been designed (Wang *et al.*, 2006). In addition, Wang *et al.* (2006) maintain that HPLC has become the most widely used method for the purpose of antibiotic residues in meat, milk and poultry, and a number of methods have been designed.

High performance liquid chromatography is an important qualitative and quantitative technique, commonly used for the estimation of pharmaceutical and biological samples. It is the most adaptable safest, steady and fastest chromatographic technique for quality control of drug components (Martin *et al.*, 2005). It utilises a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analysed, and the solvent(s) used (Martin *et al.*, 2005; Malviya *et al.*, 2010). It also involves the use of high performance

to drive the analyte in solution through a packed chromatographic column and this causes separation of the analyte under test, which can be detected using ultra violet, diode array detector or mass spectrometry (Wilson & Walker, 2001).

2.14 REGULATION OF VETERINARY DRUGS IN THE WORLD

To ensure consumer safety, worldwide regulatory authorities have set MRL's (Maximum Residual Limit) for several veterinary drugs. These MRL's, are expected to regulate the maximum permitted levels of drug residues for each antibiotic considered safely acceptable in food of animal origin (Woodward, 1993).

The maximum residue limits (MRL) are expressed as μg per kg of food, MRLs are usually given in μg/kg, but in order to compare them to the limits of detection (LODs), they can be expressed in ng/100 mg (Okerman *et al.*, 2004; WHO/FAO, 2009) or ng/g. The Food and Drug Administration (FDA) and other regulatory agencies have tabulated food-specific consumption factors. Number of kilograms consumed per day are 0.3 for muscle, 0.1 for liver, 0.05 for kidney, 0.05 for fat, and 1.5 for milk in the USA (Riviere & Sundlof, 2009). Usually, the Food and Agriculture Organisation (FAO), the Food and Drug Administration (FDA) and the European Union (EU) have launched certain regulations to monitor antibiotic residues in foods of animal origin (Botsoglou & Fletuvris, 2001).

At the international level, the Codex Alimentarius Commission (CAC) is responsible for providing advice on risk management concerning veterinary drug residues. In the EU, the use of veterinary drugs is regulated through Council Regulation 2377/90/EC (1990). This regulation describes the procedure for the establishment of Maximum Residue Limits (MRL) for veterinary medicinal products in foodstuffs of animal origin (Sanders, 2007). In addition, directive 96/23/EC Council Directive 96/23/EC regulates the residue control of pharmacologically active compounds such as environmental contaminants, dyes and chemical elements in products of animal origin and live animals (The European Commission, 2010).

Therefore, a lot of antibiotics were used in veterinary medicine in EU countries and TCs were among the most frequently used (Salah *et al.*, 2013). Moreover, the largest amounts of antibiotics were sold in the USA in 2007 and 2009 for use in food animals and the largest classes were tetracycline (TCs) (AHI, 2008; PMNH, 2010). In addition, the EU had set the MRLs of TCs to be 100 µg/kg and 300 µg/kg for chicken tissues and liver respectively (Pavlov *et al.*, 2008; Salama *et al.*, 2011; Salah *et al.*, 2013).

In South Africa, a regulation governing the maximum limits for veterinary medicine and stock remedy residues that may be present in food stuff is governed under the Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1972. The Fertilizer, Farm feeds, Agriculture Remedies and Stock Remedies Act 36 of 1947 and the Medicines and Related substances Act 101 of 1965 are the two different Veterinary drugs Act registered in South Africa. These Acts separate drugs for animal use into stock remedies (Act 36 of 1947) and for animal use into veterinary drugs (Act 101 of 1965) (Chanda *et al.*, 2014). However, Henton *et al.* (2011) maintain that data on the volume of antibiotics used in livestock production is scarce in South Africa.

Furthermore, information is missing about the patterns of antibiotic consumption in food. Part of the chicken sample displayed a residue of 55% over the legal limit in terms of South African Law (Moyane *et al.*, 2013). Sternesjo *et al.* (1998) argue that, in order prevent unwanted drug residues from entering the human food-chain, both government authorities and industries have established extensive control measures.

A study has been conducted in South Africa on antibiotic residues from different animal products such as eggs, fish meat and mane milk (Bester & Lombard, 1979), but no study has been conducted so far in the North West Province, particularly around Mafikeng area.

CHAPTER THREE

3.0 METHODOLOGY

Three different methods (the Enzyme-Linked-Immunosorbent Assay (ELISA), Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) were used in this study to determine the following four antimicrobial residues: sulphonamides, quinolones, tetracycline and streptomycin in meat. ELISA was used mainly for screening purposes while TLC and HPLC were used for confirmation purposes. Meat samples were cultured for bacterial isolation and tested for antibiotic resistance. Samples were collected from 2 butcheries and 1 supermarket because of their size and turnover around Mafikeng area from May to September 2014 in order to analyse for antimicrobial residues in meat samples collected. The supermarket and butcheries selected for this study are among the major suppliers in the area.

3.1 STUDY SITES/AREA

The North West Province is one of the major producers of meat in South Africa. The province has also several abattoirs which slaughter and supplying meat to the market. No study has been done to evaluate the level of antimicrobial residues in meat slaughter in the area. Mafikeng receives meat from surrounding abattoirs, hence cthe reason to collect samples from butcheries and supermarkets.

The study was conducted in Mafikeng, North West Province (Figure 3.1). The city lies between 25 and 28°C South of the Equator and 22 and 28°C longitude east of the Greenwich meridian. It is between 25 and 28°C South of the Equator. It shares an international border with the Republic of Botswana in the North and 260 km West of Johannesburg. Mafikeng is built on the open veld at an elevation of 1500 m along the banks of the Upper Molopo River (Ngoma *et al.*, 2013). Climatic conditions differ significantly

from West to East. The Western region receives less than 300 mm of rain per annum, the central region around 550 mm per annum, while the Eastern and South Eastern regions receive over 600 mm per annum (De Villiers & Mangold, 2012).

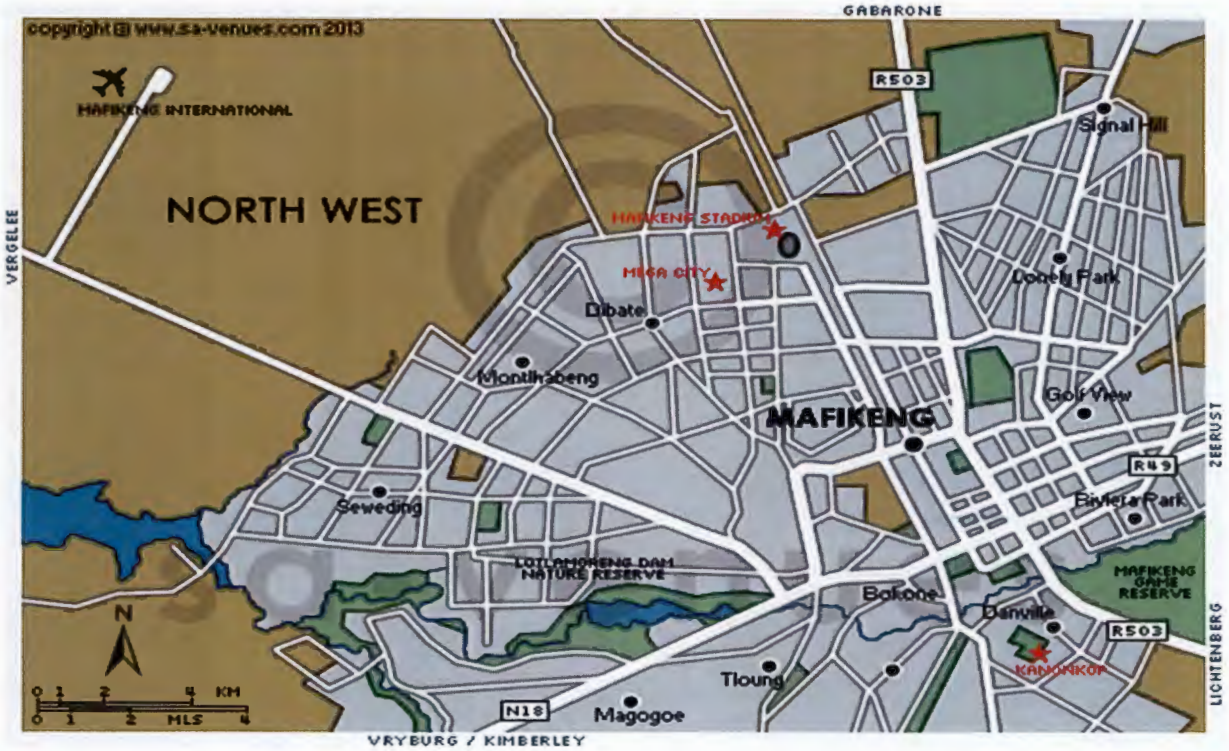


Figure 3.1: Map of sampling areas in Mafikeng (www.sa-venues.com). Accessed 02/11/2014.

3.2 SAMPLING

In order to achieve the objectives of this study, a total of 150 samples (50 chicken, 50 pork and 50 beef) of raw meat muscle, liver or kidney (tissue consumed) were randomly collected between May and September 2014. In order to ensure repeatability of the results, 10 samples were collected per month per species and from the same area (Butchery and Supermarket) as shown in Table 3.1. Collection dates and places were recorded for corresponding codes simultaneously. All samples were collected within the recommended dates for consumption. After collection, samples were packed in properly labelled sterile polyethylene bags and transported under a complete aseptic condition in an icebox to the Animal Health Laboratory

of the North West University, Mafikeng Campus for extraction and cleaning up processes. In order to avoid contamination, when samples were brought to the laboratory, twenty five grams (25 g) of each sample was weighed and packed in properly labelled sterile polyethylene bags and kept at −70°C for not more than 48 hours before analysis. In this study four selected groups antimicrobial were used (tretracyclin, sulphonamide, quinolones and streptomycine).

Table 3.1: Summary of samples collected from butcheries and supermarket around Mafikeng

May				
Area	**Organs**	**Chicken**	**Beef**	**Pork**
Butchery 1	➢ Muscle	1	1	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	2
Butchery 2	➢ Muscle	2	1	1
	➢ Liver	1	1	1
	➢ Kidney	–	1	1
Supermarket	➢ Muscle	2	2	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	1
June				
Area	**Organs**	**Chicken**	**Beef**	**Pork**
Butchery 1	➢ Muscle	2	1	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	1
Butchery 2	➢ Muscle	1	1	1
	➢ Liver	2	2	1
	➢ Kidney	–	1	2
Supermarket	➢ Muscle	2	1	1
	➢ Liver	1	1	1
	➢ Kidney	–	1	1
July				
Area	**Organs**	**Chicken**	**Beef**	**Pork**
Butchery 1	➢ Muscle	2	1	2
	➢ Liver	1	1	1
	➢ Kidney	–	1	1
Butchery 2	➢ Muscle	1	2	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	1
Supermarket	➢ Muscle	2	1	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	1

August				
Area	**Organs**	**Chicken**	**Beef**	**Pork**
Butchery 1	➢ Muscle	1	1	1
	➢ Liver	2	1	1
	➢ Kidney	–	2	1
Butchery 2	➢ Muscle	2	1	1
	➢ Liver	1	1	1
	➢ Kidney	–	1	2
Supermarket	➢ Muscle	2	1	1
	➢ Liver	2	1	1
	➢ Kidney	–	1	1
September				
Area	**Organs**	**Chicken**	**Beef**	**Pork**
Butchery 1	➢ Muscle	2	1	1
	➢ Liver	1	1	1
	➢ Kidney	–	1	1
Butchery 2	➢ Muscle	2	1	2
	➢ Liver	2	1	1
	➢ Kidney	–	1	1
Supermarket	➢ Muscle	2	1	1
	➢ Liver	1	1	1
	➢ Kidney	–	2	1

3.3 EVALUATION OF ANTIMICROBIAL RESIDUES (METHODS)

Screening for the four antibiotics (sulphonamides, quinolones, tetracycline and streptomycin) samples were prepared and analysed using the RIDASCREEN® ELISA test kit (Biopharm AG, Darmstadt, Germany).

3.3.1 Enzyme-Linked-Immunosorbent Assay (ELISA)

An analysis of samples was done in the Animal Health Centre Laboratory using Enzyme-Linked Immunosorbent Assay (ELISA). All samples were analysed in triplicate. All reagents required for the enzyme immunoassay, including standards, were included in the ELISA test kit. Each kit contained sufficient materials for 96 measurements. Each test kit contained 12 strips with 8 removable wells each, conjugate (peroxidase conjugated tetracycline aqueous solution), anti-(sulphonamides, quinolones, tetracycline and streptomycin) antibody, substrate (containing urea peroxide), chromogen (containing tetramethylbenzidine), stop solution (1N sulfuric acid) and buffer (sample and standard dilution buffer).

The ELISA test can detect one substance or a group of related chemicals. In this study, the ELISA kits used for the detection of tetracycline (Art: R3505) was a competitive enzyme immunoassay for the quantitative analysis of tetracycline. According to the manufacturer's instructions, the specificity of ELISA test in detecting (100%), chlotetracycline (70%), rolitetracycline (34%), demeclotetracycline (26%), oxytetracycline (13%), minocycline (3%) and doxycycline (2%). The specificity of the RIDASCREEN®, tetracyclin kit was determinate by analysing the cross-reactivates to corresponding substances in buffer system. The limits of detection (LOD) and recovery rate of this test in meat were approximately 2ppb and 99% respectively.

For sulphonamides, the kit (Art: R3004) used was a competitive enzyme immunoassay for the quantitative analysis of sulfamethoxypyridazine (100%), sulfapyridine (100%), sulfamethoxydiazine (75%), sulfamethaxazole (58%), sulfadimethoxine (41%), sulfadquinoxaline (34%), sulfachroropyridazine (19%), sulfamerazine (15%), sulfadoxine (10%), sulfachloropyrazine (9%), sulfaguanidine (5%), sulfapenazole, sulfamethazin, sulfisoxanzole, sulfanilamide (2%) and sulfacetamide (1%). The specificity of the RIDASCREEN®, sulphonamides kit was determined by analysing the cross-reactivates to corresponding substances in buffer system. The limits of detection (LOD) and recovery rate of this test in meat were 1.5ppb in chicken, 2ppb meat (pork and beef) and chicken 70% and 115% for meat (pork and beef) respectively.

While for quinolones the kit (Art: R3113) was also a competitive enzyme immunoassay for the quantitative analysis of ciprofloxacin (100%), norfloxacin (100%), enrofloxacin (100%), marbofloxacin (100%), danofloxacin (100%), difloxacin (100%), flumequine (100%), ofloxacin (100%), sarafloxacine (43%) and oxalic acid (24%). The specificity of the RIDASCREEN®, quinolones kit was determined by analysing the cross-

reactivates to corresponding substances in the buffer system. The limits of detection (LOD) and recovery rate of this test in meat were 10ppb and 98% respectively.

Moreover the detection of streptomycin ELISA kit (Art: R3103) was a competitive enzyme immunoassay for the quantitative analysis of streptomycin (100%) and dihydrostreptomycine (140%). The specificity of the RIDASCREEN®, streptomycin kit was determined by analysing the cross-reactivates to corresponding substances in the buffer system. The limits of detection (LOD) and recovery rate of this test in meat were 20 ppb for meat, 25ppb for liver and 97% respectively.

3.3.1.1 Samples and extraction

Before analysis, frozen meat was thawed at room temperature and fat removed and all necessary reagents brought to normal room temperature (20–25°C). Extraction of all antibiotics was done according to instructions from the supplier. Approximately five µL of the standard solution of each of the samples were added to 96 well microplates. Then fifty µL of enzyme conjugate and fifty µL of antibody added to the wells and mixed gently, incubated for 1 hour at 23°C. After incubation, the liquid was poured from the wells and washed twice with washing buffer. One hundred µL of the substrate/chromogen was added to the wells and incubated at room temperature for 15 minutes. One hundred µL of the stop solution added to each well and fifty µL per well was used in the assay.

A) Quinolones

The ELISA technique was performed according to manufacturer's instructions. One grams was mixed with 4 ml of methanol/water (70:30 v/v), centrifuged at 4000 rpm for 10 minutes. The supernatant was diluted to 1:2 (1+1) with PBS-Tween-buffer.

B) Tetracycline

The assay was performed according to the procedure suggested by the kit. One grams of solvent was transferred into centrifuge tubes nine ml with 20 mm of PBS buffer, vortexed for

10 minutes and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was transferred into test tubes and mixed with 2 mL, vortexed for 10 seconds and later centrifuged for 10 minutes at a rate of 4000 rpm.

C) Streptomycin

The ELISA kit was used according to the manufacturer's instructions for antimicrobial determination in meat samples. Five grams from each meat sample was vigorously homogenised (using a Blander) with 20 ml of PBS buffer (9.55 g NaH_2PO_4 x H_2O + 2.85 g Na_2HPO_4 x $2H_2O$ + 9 g NaCl + 0.1% Tween 20, fill up to 1000 ml with distilled water) for 30 minutes. Samples were then centrifuged for 10 minutes at a rate of 3000 rpm at room temperature. Aliquots of the supernatant 1:10 were diluted with sample dilution buffer (50 µL supernatant + 450 µL buffer).

D) Sulphonamides

Analysis of sulphonamides was peformed according to the manufactur's instruction. About five grams from each meat sample was vigorously homogenized using blender. One g (1g) of each sample was vigorously homogenised and the meat mixed with 2 mL of methanol, vortexed for 30 minutes and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was transferred to 1.5 mL of methanolic solution and evaporated to dryness by LSE digital dry bath (LSE digital dry bath model D1200-230V, New York, USA) at 40°C. After full drying, deposits were resolved in 0.5 ml of n-hexane, vortexed for 10 seconds, and later centrifuged at 4000 rpm for 10 minutes.

3.3.1.2 Analytical procedures

Fifty µL standards concentrates and 450 µL buffers were prepared in order to produce streptomycin, sulphonamides, quinolones and tetracycline standards which were added to microtiter wells of the ELISA plate. Approximately fifty µL of antibody solution (anti-streptomycin, quinolones, sulphonamides and tetracycline) was then added to each well and

incubated on a plate shaker for 1 hour at room temperature. The solution in the wells was discarded and the microplate tapped several times on blotting paper to ensure complete removal of the solution from wells. The wells were rinsed with washing buffer three times. The washing step was repeated three times. About 100 µL of enzyme conjugate was then added and incubated at room temperature for 15 minutes. The liquid in the wells was discarded and the washing step repeated three times. Approximately 50 µL of substrate and 50 µL of chromogen were added, mixed gently on a plate shaker and incubated for 15 minutes in the dark. Following incubation, 100 µL of stop solution was added to the mixture. Absorbance of the samples was read at 450 nm and the amount of antibiotic calculated based on calibration curve. The reading was done within 30 minutes after adding a stop solution. The percentage (%) of absorbance was calculated using the following formula:

$$\frac{\textbf{Absorbance standard or (sample)}}{\textbf{Absorbance zero standard}} \times 100 = \textbf{\% Absorbance}$$

3.3.2 Detection of antimicrobial using Thin Layer Chromatography (TLC)

All the samples detected positive by ELISA were confirmed by HPLC and TLC. The TLC method was used to compare the results obtained from HPLC. In order to detect antimicrobial residues (sulphonamides, ciprofloxacin, tetracycline and streptomycin) in samples which were positive in ELISA assay, the Thin Layer Chromatography (TLC) technique was performed.

3.3.2.1 Chemicals, reagents and standards

Thin Layer Chromatography method was performed to detect the presence or absence of antibiotics. The following chemicals, reagents and standards were used: Distilled and deioniser water was obtained from the Animal Health Laboratory of the North West University, Citrate buffer, Oxalic acid, Methanol, Acetonitrile, Trichloroacetic acid (TCA), n-hexane, Methanol, hydrochloric acid and ammonia, PBS-Tween-buffer and Streptomycin Extraction Buffer were purchased from Sigma Aldrich (Sigma Chemical Co, St. Louis MO.

USA), HyperSep SAX C_{18} cartridge (Thermo Scientific, United States). For quantitative analysis, stock solutions of antimicrobial sulphonamides (sulphonamides S2151000), ciprofloxacin (ciprofloxacin Y0000198), tetracycline (tetracycline hydrochloride PHR1041-500 µg) and streptomycin (streptomycin sulfate S14000000) were obtained from Sigma Chemical Co., St. Louis MO. USA was prepared. About 1 g of each powder was dissolved in 4 ml of methanol. The stock solutions (sulphonamides) were wrapped with aluminium foil and stored at −4°C.

3.3.2.2 Sample preparation

For each antibiotic a specific method was used according to the literature. Analysis of tetracycline was done according to Thangadu *et al.* (2002) and Tajick & Shohreh (2006) with some modifications. Approximately five grams of homogenised sample was mixed with 25 mL (Citrate buffer) solution vortexed for 15 minutes and centrifuged for 10 minutes at 4000 rpm. The supernatant was transferred to a beaker on a magnetic stirrer and 2.5 mL trichloroacetic acid (TCA) added slowly with constant stirring then centrifuged for 5 minutes at 3000 rpm. Single 110 MM filter paper was used to filter the mixer of the supernatant and trichloroacetic acid. Solid Phase Extraction (SPE) cartridge column was conditioned with 1ml methanol, 1 mL citrate buffer solution and 1 mL of deionised water. The final extract was applied onto the cartridge. Tetracycline was eluted with 1 ml of 0.01 M oxalic acid in methanol followed by 1 ml deionised grade water at a flow of 1 mL/minute.

The method described by Furusawa (2001) and Shareef *et al.* (2009) was used to detect sulphonamides (it was done with minor modifications). About 10 g of minced sample was mixed with 10 mL of acetonitrile. Then, 5 mL n-hexane was added and vortexed for 5 minutes then centrifuged at 3000 rpm for 10 minutes. The upper layer (n-hexane) was discarded and the residue extracted two times with 10 mL acetonitrile. The middle (acetonitrile) was filtered and the filtrate washed with 10 mL 1 M hydrochloric acid and the

flask was filled with deionised water. The supernatant was then passed through a SPE column pre-conditioned with 5 mL of Methanol and 5 mL of deionised water. After sample loading, the column was washed with 5 ml 0.1N hydrochloric acid and 5 mL of methanol. In order to collect residue, sulphonamides was eluted with 5 mL of ammonia/acetonitrile (v/v 1/19) at a flow rate of 1 mL/minutes. The extract was stored in a fridge for further analysis.

Analysis of quinolones (ciprofloxacin) was done according to Tajick & Shohreh (2006) and Canada-Canada *et al.* (2009) with some modifications. A 10 g of homogenized sample was adjusted at pH 7.4 and mixed with 5 mL of acetonitrile, Ultrasound extraction was performed for 15 minutes and vortexed for 1 minute, centrifuged for 10 minutes at 3300 rpm. The organic solvent was evaporated using LSE digital dry bath (LSE digital dry bath model D1200-230V, New York, USA) at 50°C to a volume of 2.5 mL. To extract, <2.5 mL were top up with buffer solution (pH 7.4) to obtain the level required. About 1.5 mL of n-hexane was added, and then centrifuged for 30 minutes at 3300 rpm; the upper layer of n-hexane was then discarded. The samples were kept and stored in a fridge until further analysis.

Analysis of streptomycin was done according to Chang-Won Pyun *et al.* (2008) and Shafqat *et al.* (2012) with some modifications. About 5 g of meat sample was vigorously homogenized (using Blander) with PBS-Tween-buffer for 30 minutes and then centrifuged for 10 minutes at 4000 rpm. The supernatant was diluted with buffer (50 µL supernatant + 450 µL buffer). The sample was filtered (a single 110MM filter paper), and evaporated to dryness using LSE digital dry bath (LSE digital dry bath model D1200-230V, New York, USA) at 40°C. The residue was dissolved with 200 µL streptomycin extraction buffer and stored in the fridge until further analysis.

3.3.2.3 Antibiotic detection

Antibiotic detection was done according to Shafqat *et al.* (2012). A volume of 20 µL of standard solution and the extract were spotted on silica plates with the help of microsyringe automatic TLC spotter. The chromatographic chambers were saturated with the mobile phase for 30 minutes. The plates were transferred to a TLC tank containing acetone/methanol (1:1) as a mobile phase and the TLC plate (Silica gel on TLC Al foil, St Louis, Germany) inserted with the original at the bottom. After running the solvent to the end of the plates, they were removed immediately from the tank because over running can cause the spot to diffuse. The plates were dried using a drier and viewed under ultraviolet (UV wavelength) light (Spectroline model CM-10A Westbury, New York, USA). The plates were viewed under both long and short wave (UV wavelength) light at 254 nm and 365 nm. A "O" code was drawn around any fluorescing or absorbing spots and marked with a pencil and compared with standards.

3.3.3 Determination of antimicrobial residues using HPLC

3.3.3.1 Equipment

In order to run the HPLC analysis for all samples and for all antibiotics, the following equipments were used: centrifugation was performed to separate two immiscible liquids model Z 200 A (Lasec). Vortex obtained from White Sci was used for mixing and thermo scientific apparatus used for the extraction of solid phase (HyperSep SAX C_{18} cartridge column) (Thermo Scientific, United States). LSE digital dry bath, High Pressure Liquid Chromatography (HPLC) (model Shimadzu Class-VP Series, Kyoto, Japan) equipped with SIL-20 auto injector with sample cooler and LC-20 on-line vacuum degassing solvent delivery unit was also used. Separation was done on Nucleosil C_{18} (5µm, 150 x 4.6 mm Shimadzu). RF-20 A XL florescence detector and SPD-M 20 A Deode Array Detector (DAD) were also used. pH meter (No p11/BNC/BANTE made in the USA), high speed

laboratory blender and different glass wares were also used. All chemicals were of HPLC or analytical grades.

3.3.3.2 Chemicals and reagents

In order to run the HPLC analysis for all samples and antimicrobials, the following chemicals and reagents were used: Formaldehyde (CH_2O), Triethylamine, Methanol (CH_3OH), Hydrochloric Acid (HCl); Perchloric acid (HC_lO_4); Orthophosphoric acid (H_3PO_4); Citric Acid ($C_6H_8O_7$); Citric Acid (C_3H_4OH); n-hexane ($CH_3(CH_2)_4CH_4$) and Oxalic Acid obtained from Merck (Merck, Germany); Trichloroacetic Acid ($CC_{l3}COOH$)(TCA) (Merck, Germany); Acetonitrile (CH_3CN) and Acetone (C_3H_6O) obtained from Sigma Aldrich (Sigma Chemical Co, St. Louis MO. USA). Phosphate buffers PBS were prepared by dissolving 8.94 g sodium chloride, 0.77 g disodium hydrogen phosphate and 0.18 g potassium dihydrogen phosphate in 1000 mL deioniser water and adjusted to the proper pH using 1 M hydrochloric acid. Distilled (Labcon WS02-08, South Africa) and deioniser (Shanghai Cerex Analytical Instrument, Pudong Shangai, China) water was obtained from the Animal Health Laboratory of the North West University. Solutions prepared for HPLC were passed throughout a 0.45 µm nylon membrane filter (a single 110MM filter paper, Germany) prior to usage. Stock solutions of sulphonamides, ciprofloxacin, tetracycline and streptomycin were obtained from Sigma Chemical Co., St. Louis MO. USA. All solvents were HPLC standards.

3.3.3.3 Preparation of standard solutions and validation of HPLC

The standards which were used for HPLC were for individual compounds. Stock standard solutions of sulphonamide (sulphonamide S2151000), ciprofloxacin (ciprofloxacin Y0000198), tetracycline (tetracycline hydrochloride PHR1041-500) and streptomycin (streptomycin sulfate S14000000) obtained from Sigma Chemical Co., St. Louis MO. USA, were prepared by dissolving 10 mg of each compound into 10 ml of methanol in order to

obtain a final concentration. Each stock solution was serially diluted to make different concentrations; sulphonamides was diluted to 1, 0.5, 0.25, 0.125 and 0.0625 µg/ml, 0.003, 0.006, 0.012, 0.024 and 0.048 µg/ml for ciprofloxacin, 0.2654, 0.5308, 1.0616, 2.1232 and 04.2464 µg/ml for tetracycline and 0.488, 0.976, 1.953, 2.485 and 3.102 µg/ml for streptomycin. Data obtained was used to test the linearity of the method.

Valitation of HPLC validation was done by injecting five different known concentrations of standards and also by repeating the injection of the same standard at least 3 times in the HPLC, recording the areas calculating the R^2 and checking the repeatability of the retention time and the area. The method was validated if the R^2 was between 0.9-1 and if the retention time variation was $\leq 2\%$.

3.3.3.4 Fortification of samples

Repeatability of the recovery assay was determined by analysis in triplicate of each of the three matrices (liver, kidney and muscle) spiked at three concentration levels. Fortified samples were allowed to stand for 20 minutes before analysis. The solutions were used in the preparation of the calibration curve. For ciprofloxacin and tetracyclines the spiking concentration levels were 3, 6 and 18 µg/kg for sulphonamides, the levels were 10, 30 and 100 µg/kg while for streptomycin, the levels were 4.5, 13.5 and 40.5 µg/kg. Data from these analyses was used to test linearity.

3.3.3.5 Stability

The stability of ciprofloxacin, tetracyclines, sulphonamide and streptomycin were first measured at ambient temperature for 48 hours using both standard and meat samples. The stability of all four antimicrobials during the freeze-thaw procedure needed for sample analyses was further assessed at two different sample concentrations (9.20 µM and 33.6 µM). The samples were removed from the freezer and allowed to thaw at ambient temperature,

then frozen again overnight. This process was repeated three times prior to the final stability determination performed in this study.

3.3.3.6 Sample preparation

The sample preparation as explained in 3.4.1.2 was adopted for both Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) method.

3.3.3.7 High Performance Liquid Chromatography (HPLC) analysis

A summary of the method employed for the detection of antibiotics from meat using HPLC is shown in Table 3.2. Analysis of sulphonamides was done using fluorometric detection according to Shareef *et al.* (2009) with some minor modifications. The mobile phase consisted of 0.02 M phosphoric acid/acetonitrile (60.5/39.5). The flow rate was 1.0 mL/minutes with a temperature set at 40°C. The injection volume of analyte and standard was 20 µL. Sulphonamides was detected at 405 nm excitation and 495 nm emission wavelengths, HPLC analysis was run for 10 minutes, the antibiotic detected at approximately 2.0 to 2.8 minutes.

Analysis of ciprofloxacin was performed using the fluorometric detection method according to Tajick & Shohreh (2006) and Canada-Canada *et al.* (2009) with some minor modifications. The excitation and emission wavelengths were optimised at 280 nm and 450 nm respectively. The mobile phase used was 0.02 M Orthophosphoric acids, acetonitrile (85:15). The injection volume per analyte and standard used was 40 µL. The flow rate was 1.0 mL/minutes with a temperature set at 40°C. HPLC analysis was run for 20 minutes and the antibiotic detected at approximately 14.1 to 15.3 minutes.

Analysis tetracycline was done by photodiode detector (PDA) according to Thangadu *et al.* (2002); Tajick & Shohreh (2006) and Abbasi *et al.* (2012) with some modifications. The separation was done on Nucleosil C_{18} (5 µm, 150 mm x 4.6 mm Shimadzu) column with acetonitrile and 0.01 M aqueous oxalic acid solution by gradient mode as the mobile phase at

a flow rate of 0.8 mL/minutes with a temperature set at 40°C. Moreover, 355 nm was used to for analyse the data. HPLC analysis was run for 10 minutes and the antibiotic detected at approximately 04.1 to 05.1 minutes.

Analysis of streptomycin residues was done using the Photodiode Array Detector (PDA) according to Chang-Won Pyun *et al.* (2008) with some minor modifications. Separation was done on Nucleosil C_{18} (5 µm, 150 mm x 4.6 mm Shimadzu) column with methanol/buffer (40/60) as the mobile phase flow rate of 1.0 mL/minutes with temperatures at 40°C. About 40 µL of the sample was injected and detection done at 250 nm excitation and 450 nm emission wavelengths, the sensitivity range was 4.5 µg/kg. The analysis was run for 10 minutes and antibiotic detected at approximately 05.1 to 06.4 minutes.

Table 3.2: A resume of methods used for the detection of antibiotics in meat through Performance Liquid Chromatography (HPLC)

Antibiotics	**Sulphonamides**	**Ciprofloxacin**	**Tetracycline**	**Streptomycin**
Method of extraction	Solid phase Extraction (SPE)	–	Solid phase Extraction (SPE)	–
Sample amount	10g/15 mL	10g/5 mL	5g/25 mL	5g/20 mL
Standards	1, 0.5, 0.25, 0.125 and 0.0625µg/ml	.003, 0.006, 0.012 0.024 and 0.048 µg/ml	0.5, 1.5, 3, 6 and 18 µg/kg	0.488, 0.976, 1.953, 2.485 and 3.102 µg/ml
Injection volume	40 µL	100 µL	40 µL	40 µL
Mobile phase	0.02 M Phosphoric acid/acetonitrile (60.5/39.5)	0.02 M Orthophosphoric acid, acetonitrile (85:15)	Acetonitrile and 0.01 M aqueous oxalic acid	Methanol/buffer (40/60)
HPLC detection	fluorescence	fluorescence	diode detector	diode detector
Time analysis	10 minutes	20 minutes	10 minutes	10 minutes

3.3.3.8 Method of verification by determining the amount of antimicrobial recoveries

Recoveries were obtained by using samples with known concentrations to which antimicrobial standards in triplicates were inoculated (100mL) with sulphonamides, ciprofloxacin, tetracycline and streptomycin and recoveries obtained using the identical

extraction method as for the samples and the same techniques on HPLC, as summarized in Table 3.3. The mean recoveries obtained were 76.67% 78.3% and 78.69% for muscles, liver and kidney for streptomycin, 73%, 73.67% and 73.33% for muscles, liver and kidney respectively for sulphonamide, 80.67%, 78.67% and 80.67% for muscles, liver and kidney respectively for ciprofloxacin and 64.67% , 64.67% and 64.33% for muscles, liver and kidney respectively for tetracycline. The results obtained in each case were deducted from the ones obtained from the spiked ones in order to obtain the recovery. Quantification of antimicrobial residues in the samples were obtained and calculated from the peak heights extrapolated from the calibration curves of the standards using the following formula:

$$\frac{\textbf{Amount of residue obtained after spiking sample}}{\textbf{Spiked concentration}} \times 100 = \textbf{\% Recovery}$$

Table 3.3: A resume of mean of recoveries obtained from meat spiked with antimicrobial standards on Performance Liquid Chromatography (HPLC)

Antibiotic and type of sample	Fortification level (µg/kg)	Recovery (%)	Mean recovery (%)
STREPTOMYCIN			
➢ Muscle	4.5, 13.5 and 40.5	74, 77 and 79	76.67
➢ Liver	4.5, 13.5 and 40.5	76, 78 and 81	78.3
➢ Kidney	4.5, 13.5 and 40.5	77, 79 and 80	78.67
SULPHONAMIDES			
➢ Muscle	10, 30 and 100	71, 73 and 75	73
➢ Liver	10, 30 and 100	72, 73 and 76	73.67
➢ Kidney	10, 30 and 100	71, 74 and 75	73.33
CIPROFLOXACIN			
➢ Muscle	3, 6 and 18	77, 81 and 84	80.67
➢ Liver	3, 6 and 18	75, 79 and 82	78.67
➢ Kidney	3, 6 and 18	78, 81 and 83	80.67
TETRACYCLINE			
➢ Muscle	3, 6 and 18	62, 65 and 67	64.67
➢ Liver	3, 6 and 18	61, 65 and 68	64.67
➢ Kidney	3, 6 and 18	62, 64 and 67	64.33

Despite the fact that the recovery rate for tetracycline was 64%, the results were considered because it was the best method after several trials.

3.4 CULTURE, ISOLATION AND IDENTIFICATION OF BACTERIA

3.4.1 Bacterial culturing

Twenty five grams (25 g) of each sample were chopped using a sterile knive on a cutting board and transferred to 225 mL of Nutrient Broth (NB) and properly mixed for 5 minutes. The samples were enriched overnight and incubated at 37°C (Ruhe *et al.*, 2006). After 24 hours, a sterile wire loop full of broth was streaked onto different media such as Manitol Salt Agar, MacConkey and Nutrient Agar and then incubated for 24 hours at 37°C. Isolates obtained were purified by further sub-culturing and observed for presumptive identification based on their morphological characteristics and various biochemical tests. Bacterial colonies with dissimilar morphology were selected and purified on nutrient agar for MALDI-TOF and biochemical confirmation. The routine laboratory (Biological) method of Cruickshank *et al.* (1975) was used to characterise different isolates.

3.4.2 MALDI-TOF for identification of bacteria

After purification of the samples on nutrient agar (after 24 hours), for MALDI-TOF identification, 5−10 mg of colony was harvested from nutrient agar and homogeneous in 300 µL sterile distilled water in an eppendorf tube 1.5 mL by pipetting up and down in the tube. About 900 µL of 100% of ethanol (HPLC grade) was added and mixed by pipetting up and down in the tube. The tubes were labelled with the date and isolates coded and packaged in a storage box, stored at −20°C. Finally they were sent for analysis and identification to the University of Pretoria, South Africa ().

3.4.3 Primary biochemical test

3.4.3.1 Cellular morphology

For cellular morphology, Gram staining was used according to Cruikshank *et al.* (1975) and Purkayastha *et al.* (2010) as follows: a small colony was picked up using a sterile

wire loop, smeared on a glass slide and fixed by softly heating. A crystal violet solution was then applied on the smear to stain for 2 minutes and later washed with running water. Lugol's iodine was then added to act as mordant for 1 minute and washed with running water. Acetone alcohol was then added to act as a decolouriser for 5 seconds. After washing with distilled water, safranine was added as a counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted, dried in air and examined under a microscope with high power objectives (100X) using immersion oil.

3.4.3.2 Catalase test

The catalase method was done according to Montso & Ateba (2014). A pure colony was transferred onto the surface of a microscopic slide using a sterile inoculating needle. A drop of 3% hydrogen peroxide was added and the slide observed for the presence of bubbles. All the results were recorded on the data sheet. A sample was deemed catalase positive if any bubbling was observed in the liquid after 10 seconds.

3.4.3.3 Coagulase tube method

The coagulase method was done according to Hassan Ali *et al.* (2010). About 0.5 ml of rabbit plasma was added to a 12 mm x 75 mm test tube. Two drops of overnight broth culture of test organism were added to the test tube mixed gently and incubated in water bath at 37°C for 4 hours. After all the tubes were incubated at 37°C, they were observed for any clot formation. However, if the test tubes remained negative after 4 hours of exposure to 37°C, they were kept at room temperature for overnight incubation to assess if the results would change. The formation of a clot was an indication of coagulase production.

3.4.3.4 Oxidase test

An oxidase test was done according to Ateba & Setona (2011). Pure isolated colonies were singled out using a sterile wire loop and positioned on a Whatmans filter paper and a

drop of the Oxidase™ reagent added to make a smear. After 30 seconds the formation of a purple or blue colour indicated an oxidase positive result and vice versa.

3.4.3.5 Voges proskauer test

The Voges proskauer test was done according to Oyeleke & Manga (2008). Bacteria cultures were inoculated in nutrient broth using a sterile wire loop and incubated at 37°C overnight. After incubation 20 drops of Barritt's reagent A and Barritt's reagent B were added to the broths and shaked gently. The presence of the red colour within 15–20 minutes was an indication that the test was positive.

3.4.3.6 Indole test

The indole test was done according to Hassan Ali *et al.* (2010). This test was performed using Kovaco's reagent. Nutrient broths were inoculated with the test organism and incubated at 37°C for 24–28 hours. After 24 hours, 3–5 drops of Kovac's reagents were added and the red colour ring at the upper layer of the liquid was an indication of indole positive while yellow/no colour was an indication of indole negative.

3.4.3.7 Urease test

Urease Test Broth was performed according to WHO (2003). One ml of the concentrate was aseptically added to 9 mL of cold sterile purified water and mixed thoroughly. About 3 ml was dispensed in small sterile test tubes. Using a sterile wire loop, 1–2 colonies were inoculated in Trypticase™ Soy Agar Slant cultures and the entire surface of the agar slant in a zig-zag manner, incubated with loosened caps at 37°C for 24–48 hours in an aerobic atmosphere. The production of urease was indicated by an intense pink-red (red-violet) colour throughout the broth while a negative reaction was no colour change (remained yellowish to orange).

3.4.4. Confirmatory biochemical tests

Isolates that satisfied morphological structures and primary biochemical tests were confirmed using the Analytical Profile Index (API) API-Staph-Ident, API-20E test and Polymerase Chain Reaction (PCR) analysis.

3.4.4.1 Analytical Profile Index (API) 20E and API Staph-Ident

Bacterial isolates suspected according to conventional biochemical tests results were re-examined using API-20E and API-Staph-Ident system (BioMérieux, France). API-20E was used to classify species of Enterobacteriaceae such as *Proteus spp.*, *Escherichia spp.*, *Klebsiella spp.*, *Enterobacter spp.*, *Citrobacter spp.* and other Gram negative rods (Forbes *et al.*, 2002). API-Staph was used to identify Gram positive *Listeria spp.*, *Staphylococcus spp.* and *Micrococcus spp.* (Downes & Ito, 2001). The test was performed according to the manufacturer's instructions (BioMérieux, France). Briefly, fresh colonies from Nutrient Agar were used to make bacterial suspensions and mixed with API 20E and API Staph-Ident medium provided. The microtubules were inoculated with the suspensions as instructed. The strips were placed into trays hydrated with 5 ml distilled water to create a humid atmosphere. The strips were incubated using an anaerobic incubator for 24 hours. Results were read with or without the addition of reagents. Indices were generated for the different isolates and used to determine their identities using the API web^{TM} identification software.

3.4.5 Molecular characterisation of isolates

3.4.5.1 Extraction of Genomic DNA

For the extraction of genomic DNA, the procedure was done according to Ngoma *et al.* (2013). Pure isolates from the Nutrient agar were inoculated into 5 mL of nutrient broth and incubated aerobically at 37°C for 24 hours while shaking the mixture. Total genomic DNA of cultivated isolates was purified following the manufacture's recommendations (Zymo-Research Fungal/Bacterial Soil Microbe DNA Mini Prep kit, Catalog No. D6005 USA supplied by Biolab, South Africa). Briefly, pellets were suspended in 750 µL lysis

solution, disrupted with a disruptor gene (Inqaba Biotech mode No, SI D258, USA) and vortexed at 14.000 rpm for 5 minutes followed by centrifugation at 10 000 rpm for 1 minute. 400 µL of the upper aqueous phase was aliquoted into a new eppendoff tube and centrifuged at 7000 rpm for 1 minute. 1200 µL of buffer was added to the filtrate and 800 µL of the mixture transferred to the new collection tube and centrifuged at 10.000 rpm for 1 minute. The filtered DNA was pre-washed by adding 200 µL DNA pre-wash buffer and centrifuged at 10.000 rpm for 1 minute. 500 µL of DNA wash buffer was added to the new collection tube and centrifuged at 10.000 rpm for 1minute. Finally, 100 µL of DNA elution buffer was added to elute the DNA in a clean 1.5 mL micro-centrifuge tube.

3.4.5.2 Amplification of 16S rDNA Gene

Amplification of 16S rDNA gene was carried out using a polymerase chain reaction and with the help of an Engine DYAD Peltier thermal cycler (Bio Rad, USA). A reaction volume of 25 µL, containing: 12 µL PCR Master Mix, 1 µL template DNA, 10 µL nuclease free water and 1 µL of each oligonucleotide primer were prepared and mixed in PCR tubes (Ngoma *et al.*, 2013). For the amplification of 16S rDNA Gene, the procedure was done according to Ngoma *et al.* (2013). The PCR of endophytic bacteria were conducted using universal primers: forward 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse 1492R (5'-TGA CTG ACT GAG GCT ACC TTG CGA-3'). These primers were commercially synthesised by Inqaba Biotechnical Industrial (Pty) Ltd. (Pretoria, South Africa). The thermo cycling (Bio-Rad T100™ Thermal Cycler, Singapore) conditions consisted of an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 30seconds and extension at 72°C for 5 minutes, followed by a single and final extension step at 72°C for 7 minutes and incubated at 4°C forever. Amplified PCR products were resolved in 1% agarose gel

discoloured with ethidium bromide (10 $\mu g/ml^{-1}$) and visualised with Syngene Ingenius Bioimager (UK) to confirm the expected size of the product.

3.4.5.3 Electrophoresis of PCR Products

For the electrophoresis of PCR products the procedure was done according to Ngoma *et al.* (2013). The success of the extraction of genomic DNA was determined by testing for the presence of DNA using 1% of agarose gel. The agarose gel was prepared as follows; 100 g was measured and mixed with 100 ml of TE buffer (40 mM Tris, 1mM EDTA and 40 mM glacial acetic acid (pH 8.0). Agarose was then dissolved by using a microwave; the gel was allowed to cool to about 40°C; and ethidium bromide (0.5 mg/ml) was added for staining, gel was cast and allowed to set. After the gel had set inside the electrophoresis chamber, 5µl DNA and 5 µl of loading buffer were mixed and transferred to one of the wells in the gel electrophoresis tank. Electrophoresis conditions were set for 90 minutes at 80 Voltage and 250 MA. Amplicons were visualised under UV light at 420 nm wavelength. A Chemi Doc Imaging System (Bio-Rad Chemi Doc TM MP Imaging System, UK) was used to capture the presence of DNA bands (version 6.00.22) software.

3.4.5.4 DNA sequencing

Through the method described by Ngoma *et al.* (2013), purified PCR fragments of the 16S rDNA of the strains were analysed for nucleotide sequence determination using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd. (Pretoria, South Africa). The acquired sequences were aligned against Gen-Bank data base using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) from the National Centre for Biotechnology Information (NCBI) to identify sequences with high similarities.

3.5.5.5. Phylogenetic analysis

All confirmed sequencing results obtained in this study were analysed and edited using Bio-Edit Sequence Alignment Editor (Ngoma *et al.*, 2013). Multiple alignments of the sequences were carried out using the Mafft programme 6.864 against corresponding nucleotide sequences retrieved from Gen-Bank. Evolutionary distance matrices were generated as described by Tamura *et al.* (2011). The aligned 16S rDNA gene sequences were used to construct a phylogenetic tree as implemented in the MEGA 5.10 package (Fitch, 1986) and the Neighbour Joining (NJ) method (Page, 1996); minimum evolution; maximum likelihood (Hendrickson *et al.*, 2002), UPGMA and maximum parsimony (Tamura *et al.*, 2011). The methods were used in order to expound on the phylogeny and for better comparison. Bootstrap analyses were performed using 1000 replications for neighbour joining, maximum-parsimony and maximum likelihood. The sequences were checked for putative chimeric artifacts using the Chimera-Buster programme. No chimeric sequences were compared to the closest relatives in the NCBI Gen- Bank database by BLAST programme. Manipulation and tree editing were carried out using Tree View (Page, 1996).

3.5 ANTIMICROBIAL SUSCEPTIBILITY

Sensitivity of the isolates to antibiotics was examined against some common antibiotics, including, sulphonamides, quinolones (ciprofloxacin), tetracycline and streptomycin. This test was performed using the Kirby Bauer disc diffusion technique (Bauer *et al.*, 1966). A pure colony of isolates from a fresh culture was used to prepare a bacterial suspension. Aliquots of 100 µL from the suspensions were spread plated on Mueller Hinton agar (MH) using a sterile cotton swab through the entire surface of the Muller Hinton agar plates. After the inoculum was dried for about 5 minutes, four standard antibiotic disks each containing a specific concentration of antibiotics was applied per plate. The antibiotics used in this study were selected because of their usage in veterinary practices. Antibiotic discs

were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates incubated aerobically at 37°C for 18–24 hours. All the isolates were subjected to streptomycin (300 μg), tetracycline (30 μg), sulphonamides (300 μg) and ciprofloxacin (5 μg). The diameter of the inhibition zone (clear area around discs) indicates the sensitivity of bacteria to that antibiotic. After incubation at 37°C for 24 hours, the diameters of the inhibition zone were measured in millimetres to interpret sensitive, intermediate or resistance in accordance with the guidelines of the Clinical Laboratory Institute Standards (CLIS, 2012; 2013).

3.6 STATISTICAL ANALYSIS

The Statistical Analysis System (SAS® software, 1994) (general linear models programme) was used to detect the presence of antimicrobial residues in different species and tissues detected by ELISA and HPLC. A probability of $P \leq 0.05$ was required for statistical significance. Moreover, ELISA, TLC and HPLC were compared using chi-square and Cramer's V tests. One way analysis of variance (ANOVA) and Dunnett's T3 (as the post hoc analysis) tests were used to correlate methods in different antimicrobial. Data analysis was implemented using the SPSS software (version 16; SPSS Inc, USA) and values of $p < 0.05$ considered statistically significant. Correlations between the antimicrobial residue and antimicrobial resistance were also analysed statistically using the SPSS software (version 16; SPSS Inc, USA) and values of $p < 0.05$ were considered correlated.

CHAPTER FOUR

4.0 RESULTS

The main aim of this study was to identify antimicrobial residues in meat sold in butcheries and supermarkets around Mafikeng, North West Province, South Africa. Results obtained from the analysis of samples are presented below.

4.1. DETECTION OF ANTIBIOTIC RESIDUES

4.1.1 Screening of antimicrobial residues using Enzyme-Linked-Immunosorbent Assay (ELISA)

In this study, the overall prevalence of antimicrobial detection in meat sample was 65%. The rate of prevalence of drug residues in the various animal meats by species, organs and antimicrobial residues are presented in Table 4.1, 4.2, 4.3 and 4.4. The results show the presence of quinolones and streptomycin at 42% and 23% respectively above MRL, while the other two antimicrobial (tetracycline and sulphonamides) were detected but not above MRL.

TABLE 4.1: Summary of the detection of quinolones in chicken, beef and pork using ELISA in comparison with Maximum Residue Limits (MRLs) in µg/kg according to EU

		Quinolones					
Samples	(n)	Positive (%)	Mean (µg/kg)	STD dev	Range (µg/kg)	Detection > MRL (%)	EU- MRL (µg/kg)
Chicken							
➢ **muscle**	25	14 (56)	135.8	±9.88	89.6–175.9	11 (44)	100
➢ **liver**	25	18 (72)	178.69	±9.57	152.2–289.1	12 (48)	200
Beef							
➢ **muscle**	15	14 (93)	110.35	±9.49	89.6–146.1	5 (33)	100
➢ **liver**	17	10 (59)	168.28	±9.91	145.2–316.5	9 (53)	200
➢ **kidney**	18	7 (39)	120.63	±12.78	98.2–197.03	Nil	300
Pork							
➢ **muscle**	16	8 (50)	59.3.67	±15.76	42.6–95.85	Nil	100
➢ **kidney**	11	5 (45)	102.51	±10.00	72.5–140.2	Nil	200
➢ **liver**	23	8 (35)	298.63	±16.78	220–355.6	3 (13)	300

n=number of samples, **STD dev**=standard deviation, **MRL**= maximum residue limit, **EU**= European Union

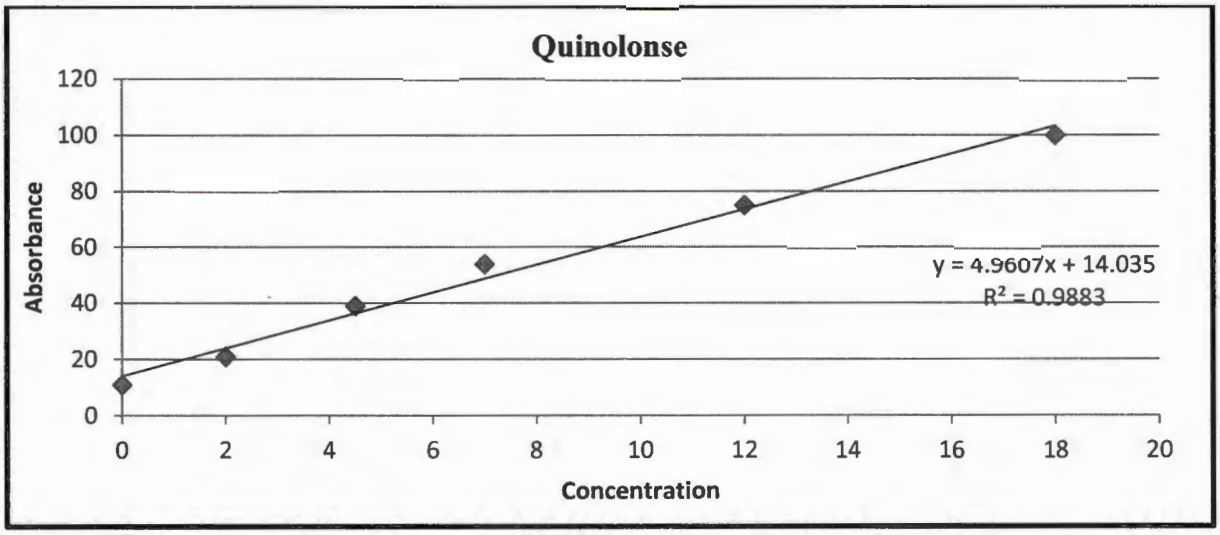


Figure 4.1: Calibration curve for quinolones standards at 0.5, 1.5, 3, 6 and 18 µg/kg run under the UV detector

The range of quinolones in the current study was 159–175 µg/kg with a mean of 168.02, 168.63 and 169.95 µg/kg for beef, chicken and pork respectively above the international level. Figure 4.1 show the calibration curve, used to determine the concentration of quinolones from three different organs (liver, kidney and muscle samples).

TABLE 4.2: Summary of the detection of streptomycin in chicken, beef and pork using the ELISA

		Streptomycin					
Samples	(n)	Positive (%)	Mean (µg/kg)	STD dev	Range (µg/kg)	Detection > MRL (%)	Codex/SA MRL (µg/kg)
Chicken							
➢ **muscle**	25	9 (36)	186.5	±19.28	98.44–452.9	Nil	600
➢ **liver**	25	12 (48)	596.58	±374.61	368.8–986.4	9 (36)	600
Beef							
➢ **muscle**	15	5 (33)	770.67	±325.62	625.9–989.2	5 (33)	600
➢ **liver**	17	8 (47)	614.57	±322.24	498.2–920.1	7 (41)	600
➢ **kidney**	18	8 (44)	956.2	±322.19	614.2–1280.8	7 (39)	1000
Pork							
➢ **muscle**	16	3 (19)	777.33	±313.06	620.3–875.8	3 (19)	600
➢ **kidney**	11	7 (64)	852.36	±322.20	14.15–1052.6	1 (9)	1000
➢ **liver**	23	4 (17)	420.51	±16.07	196.5–535.9	Nil	600

n=number, **STD dev**=standard deviation, **MRL**= maximum residue limit, **SA**= South Africa

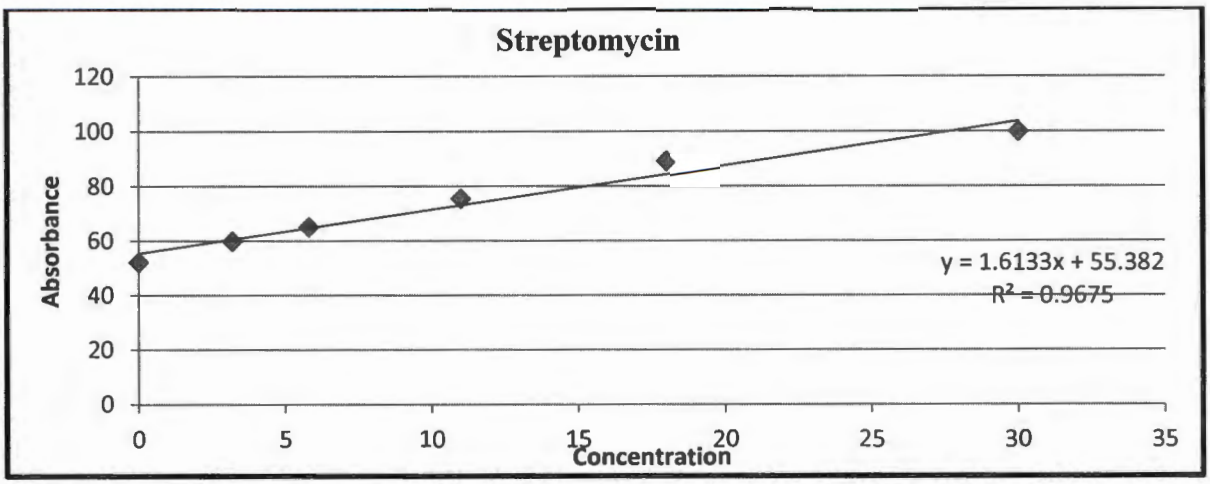


Figure 4.2: Calibration curve for streptomycin standards at 0.5, 4.5, 13.5, 6 and 40.5 µg/kg run under the UV detector

Streptomycin levels in the current study ranged between 186 and 1534µg/kg with a mean of 980.81, 686.42 and 752.61 µg/kg respectively for beef, chicken and pork and were above the EU MRL. As shown in Table 4.2 liver was the most prevalent organ than beef 41% with a mean of 614.57 ±322.24 µg/kg. Figure 4.2 shows the calibration curve for streptomycin.

TABLE 4.3: Summary of the detection of sulphonamides in chicken, beef and pork using the ELISA

		Sulphonamides					
Samples	(n)	Positive (%)	Mean (µg/kg)	STD dev	Range (µg/kg)	Detection > MRL (%)	Codex/SA MRL (µg/kg)
Chicken							
➢ **muscle**	25	3 (12)	47.50	±6.50	32.5–65.9	Nil	100
➢ **liver**	25	7(28)	73.43	±12.59	45.8–81.6	Nil	100
Beef							
➢ **muscle**	15	1 (7)	65.39	–	–	Nil	100
➢ **liver**	17	5 (29)	51.60	±14.88	19.8–87.9	Nil	100
➢ **kidney**	18	5 (20)	58.6	±14.86	37.6–73.9	Nil	100
Pork							
➢ **muscle**	16	–	–	–	–	Nil	100
➢ **kidney**	11	4 (36)	72.07	±16.07	52.8–92.8	Nil	100
➢ **liver**	23	2 (9)	58.50	±4.95	48.2–69.9	Nil	100

n=number, **STD dev**=standard deviation, **MRL**= maximum residue level, **SA**= South Africa

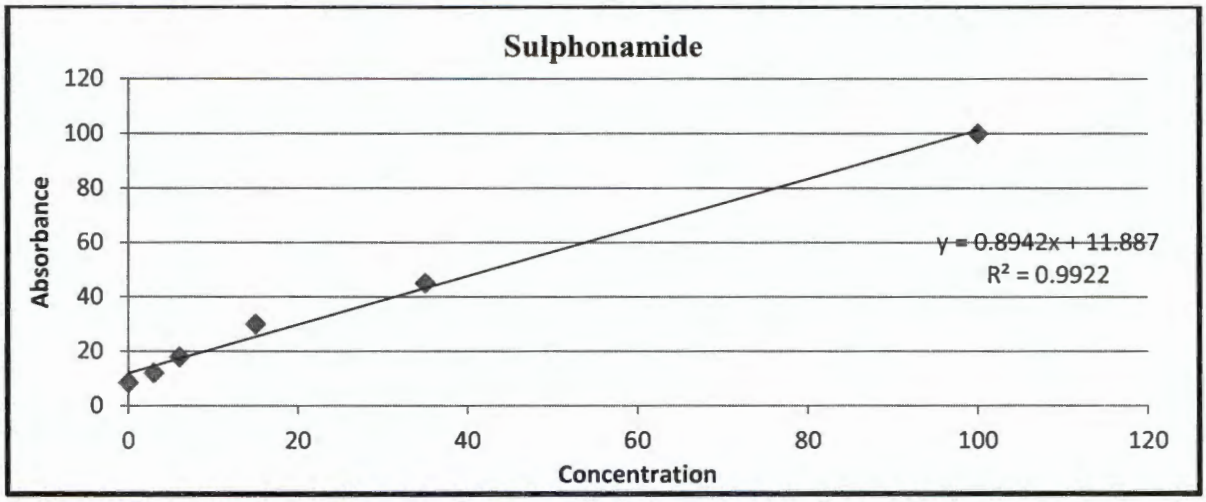


Figure 4.3: Calibration curve for sulphonamides standards at 1, 3, 10, 30 and 100 µg/kg run under the UV detector

Sulphonamides levels in the present study were low compared to the 100 µg/kg specified as Maximum Residues Limit. Kidney from pigs showed the highest concentrators with a mean of 86.07 µg/kg. The calibration curve (as shown in Figure 4.3) was used to determine the concentration of sulphonamides from meat samples.

TABLE 4.4: Summary of the detection of tetracycline in chicken, beef and pork using ELISA

		Tetracyclin					
Samples	(n)	Positive (%)	Mean (µg/kg)	STD dev	Range (µg/kg)	Detection > MRL (%)	Codex/SA MRL (µg/kg)
Chicken							
➢ **muscle**	25	5 (20)	62.5	±23.16	41.2–82.1	Nil	100
➢ **liver**	25	9 (36)	125.5	±22.58	42.56–286.2	Nil	300
Beef							
➢ **muscle**	15	3 (20)	48.61	±30.24	26.6–61.5	Nil	100
➢ **liver**	17	5 (29)	92.3	±23.17	41.2–221.6	Nil	300
➢ **kidney**	18	5 (28)	192.2	±23.15	41.2–359.2	Nil	600
Pork							
➢ **muscle**	16	3 (19)	58.3	±30.26	46.67–86.9	Nil	100
➢ **kidney**	11	4 (36)	198.3	±25.91	101.3–489.1	Nil	600
➢ **liver**	23	4 (17)	98.2	±25.93	43.7–255.9	Nil	300

n=number, **STD dev**=standard deviation, **MRL**= maximum residue level, **SA**= South Africa

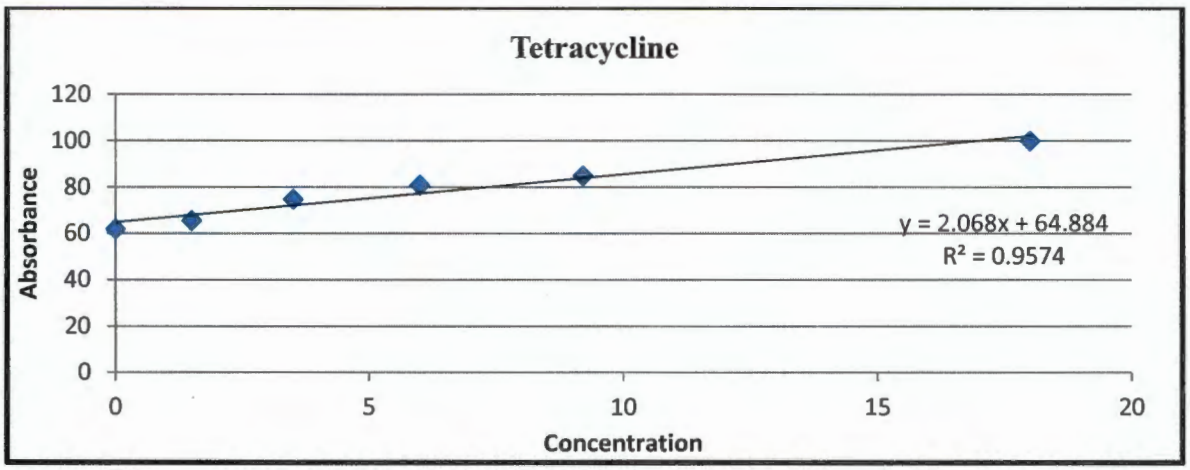


Figure 4.4: Calibration curve for tetracycline standards at 0.5, 1.5, 3, 6 and 18 µg/kg run under the UV detector

Tetracycline levels in the present study were low compared to the 100 for muscle, 300 for liver and 600 µg/kg for kidney specified as Maximum Residues Limit level. As shown in Figure 4.4, the calibration curve for tetracycline with standards at 0.5, 1.5, 3, 6 and 18 µg/kg was used to determine the concentration of tetracycline from meat samples.

4.2.2 Screening of antimicrobial residues using Thin Layer Chromatography (TLC)

Samples that were positive for Enzyme-Linked-Immunosorbent Assay (ELISA) were further analysed by Thin Layer Chromatography (TLC). Chromatograms of samples detected are illustrated in Figure 4.7. The solvent were allowed to go about 90% of the way up the plate. After receiving the solvent fronts to end of plates from the TLC tank (Figure 4.5), they were immediately removed from the tank, since over running can cause the spot to diffuse.

Figure 4.5: TLC tank (chromatographic chamber) containing a mobile phase and silica plates

The result illustrated in Table 4.5 shows a summary of; sulphonamides with the highest (92.5%) of positive samples, followed by quinolones (ciprofloxacin) with 57.8% of positive samples, streptomycin with 49.1% of samples and tetracycline with 26.2% detected for residues.

Table 4.5: The detection of tetracycline, sulphonamides, ciprofloxacin and streptomycin residues from different samples using TLC

	Tetracycline		**Sulphonamides**		**Ciprofloxacin**		**Streptomycin**	
Samples	**n**	**+ve (%)**	**n**	**+ve (%)**	**n**	**+ve (%)**	**n**	**+ve (%)**
Chicken								
➢ **muscle**	5	3 (60)	3	3 (100)	14	3 (21)	9	5 (56)
➢ **liver**	9	8 (89)	7	7 (100)	18	7 (39)	12	8 (67)
Beef								
➢ **muscle**	3	Nil	1	1 (100)	14	Nil	5	Nil
➢ **liver**	5	4 (80)	5	5 (100)	10	4 (40)	7	6 (86)
➢ **kidney**	5	2 (40)	5	4 (80)	7	Nil	7	3 (43)
Pork								
➢ **muscle**	3	1 (33)	Nil	Nil	8	Nil	3	1 (33)
➢ **kidney**	4	2 (50)	4	3 (75)	5	1 (20)	7	4 (57)
➢ **liver**	4	2 (50)	2	2 (100)	8	3 (38)	4	1 (25)

n= number of samples, **+ve**= positive

Beef showed the highest prevalence rate of drug residues compared to chicken and pork. However, from all three organs (liver, kidney and muscle), Table 4.5 shows that the liver was the organs with highest prevalence (with 76%) followed by the kidney with 69% and the muscle with 58%.

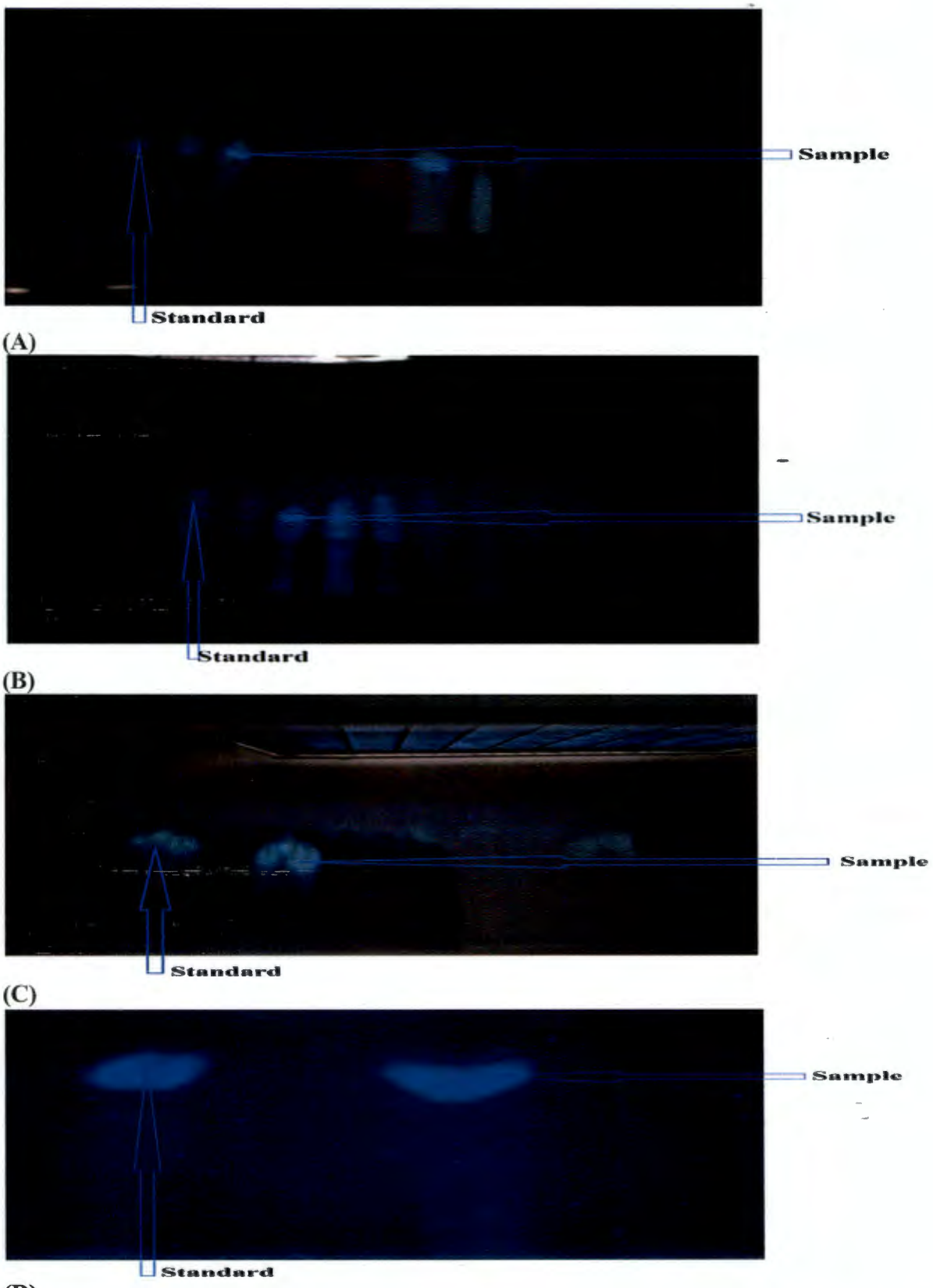


Figure 4.6: Detected samples for antibiotic residues in meat using TLC seen under U.V. light showing the presence of sulphonamides **(A)**, tetracyclines **(B)**, ciprofloxacin **(C)** and streptomycin **(D)**

Figure 4.6 shows results for the detection of ciprofloxacin, streptomycin, tetracycline and sulphonamides residues after a plate viewed under both long and short wave Ultra-Violet (UV wavelength) light at 254 nm and 365 nm.

4.1.3 Confirmation of antimicrobial residues using High Performance Liquid Chromatography (HPLC)

A screening test (ELISA) is really advantageous if it can be used rapidly on a large sample number, but is not reliable enough to be used as it can detect false positives but also because of cross reactivity. Therefore, there are othere of confirmatory tests such as TLC and HPLC to discriminate the falls from the true positive and for the obtention of true results. In this study, samples detected positive for antimicrobial residues by ELISA were further analysed using HPLC for confirmation and quantifications.

4.1.3.1 Confirmation of sulphonamides residues in meat

Table 4.7 shows the results of sulphonamides positive organs from different species, detailed report for a mean concentration of 62.14 µg/kg with the range of sulphonamides 32.5–87.9. The most prevalent organ is the liver with levels ranging between 32.5 and 87.9.

Table 4.6: Summary of the detection of sulphonamides in different meat samples using HPLC

		Sulphonamide						
Type of sample	**Organ**	**(n)**	**+ve (%)**	**Mean (µg/kg)**	**STD dev**	**Range (µg/kg)**	**Detection > MRL (%)**	**codex/SA MRL µg/kg**
Chicken	➢ Liver	7	7 (100)	32.5	±21.15	20.7–65.9	Nil	100
	➢ Muscle	3	2 (67)	45.81	±35.19	35.2–81.6	Nil	100
Beef	➢ Liver	5	5 (100)	33.2	±25.32	23.7–71.5	Nil	100
	➢ Kidney	5	3 (60)	48.2	±26.90	26.9–82.1	Nil	100
	➢ Muscle	1	1 (100)	62.14	Nil	Nil	Nil	100
Pork	➢ Liver	2	2 (100)	58.6	±35.23	35.2–73.9	Nil	100
	➢ Kidney	4	3 (75)	53.2	±27.69	27.9–77.9	Nil	100
	➢ Muscle	Nil	Nil	Nil	Nil	Nil	Nil	100

n=number of samples, **STD dev**=standard deviation, **+ve**=positive, **MRL**= maximum residue level, **SA**= South Africa

4.1.3.1.1 Calibration curve

A calibration curve was prepared from the peak areas obtained by injecting corresponding concentrations of sulphonamides standard solutions. Excellent recoveries ranging from 71% to 76% were determined.

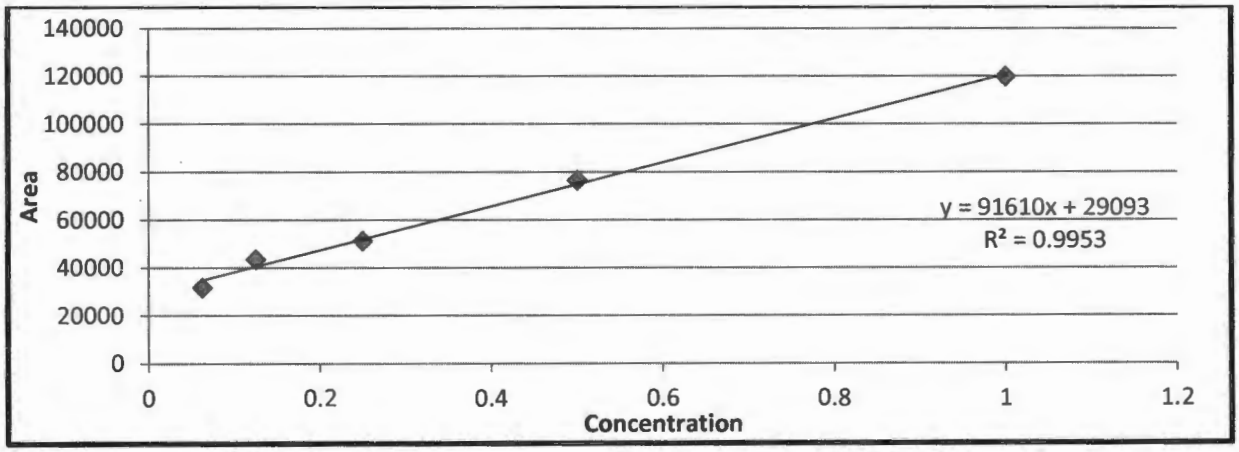
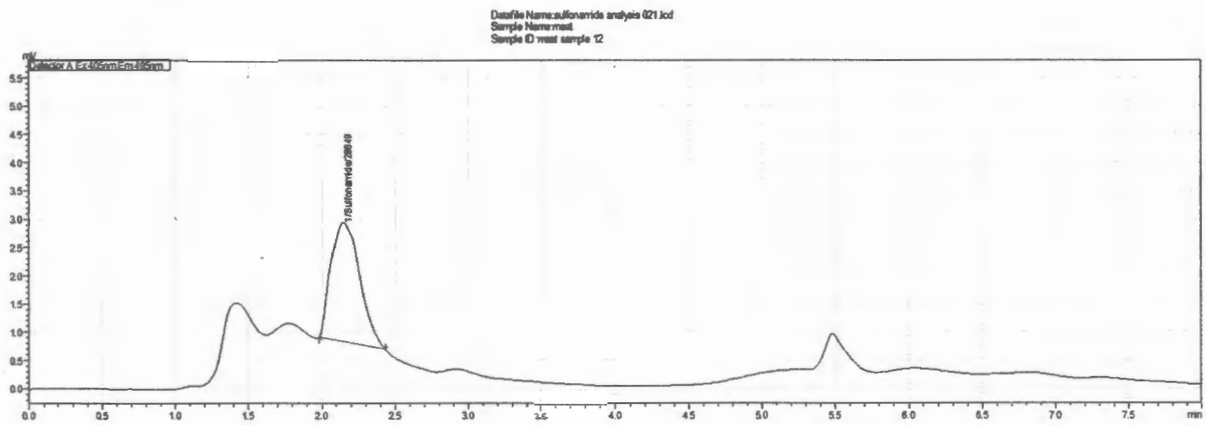
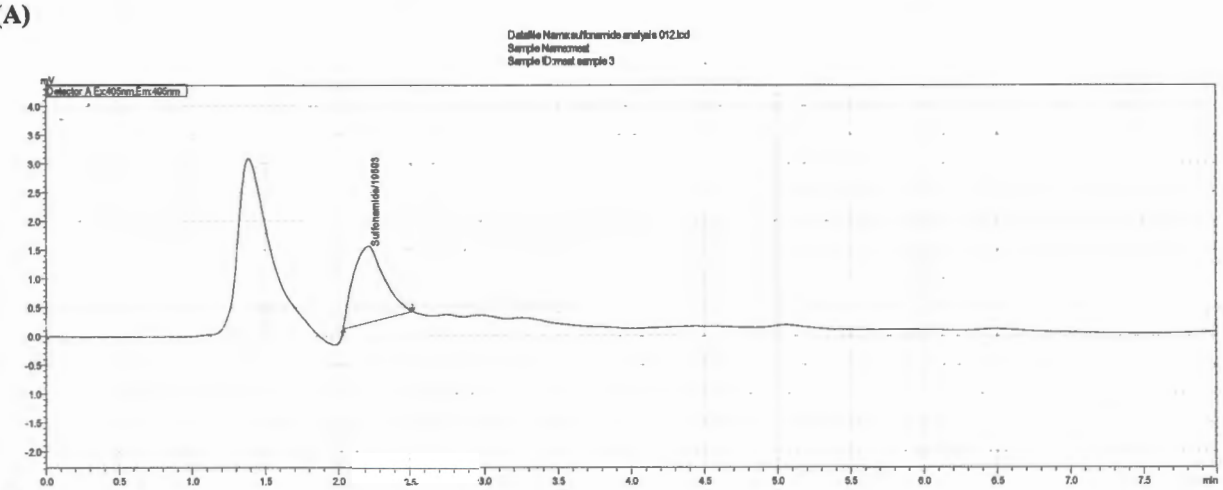


Figure 4.7: Calibration curve for sulphonamide standards at 1, 0.5, 0.25, 0.125 and 0.0625µg/kg run on HPLC under the RF detector

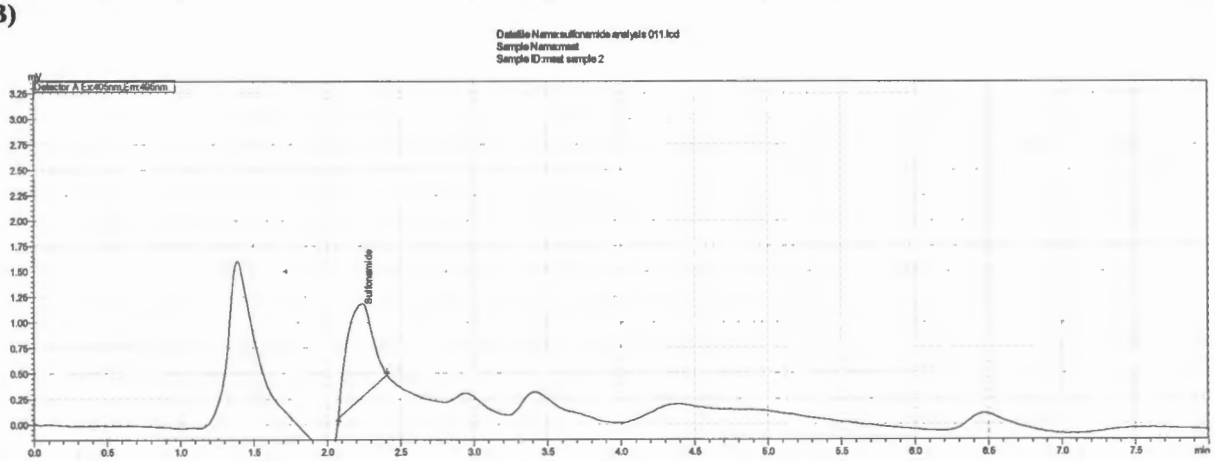
The chromatogram of the standards for sulphonamides was derived as shown in Figure 4.7.



(A)



(B)



(C)

Figure 4.8: (A) HPLC chromatogram of a standard at 0.025 µg/ml spiked with sulphonamide **(B)** Chromatogram of a spiked sample at 100 µg/kg spiked with sulphonamide, **(C)** Chromatogram of a meat sample spiked with sulphonamide with a flow rate of 1.0 mL/minute, Run on RF detector with 405 nm excitation and 495 nm emission wavelengths, ambient temperature (35°C) and the antibiotic was detected at approximately 2.0 to 2.8 minutes.

4.1.3.1.2 Determination of the Limit of Detection (LOD)

The present method provided the limit of detection (LOD) of sulphonamides residues in meat samples at 0.0156 µg/ml while the retention time was between 2.0 to 2.8 minutes.

4.1.3.1.3 Determination of the Limit of Quantification (LOQ)

The LOQ was 0.75 µg/ml for meat and calculated from the correlation coefficient, intercept and slope of the calibration curve.

4.1.3.2 Confirmation of ciprofloxacin residue in meat

Table 4.7 shows a present a summary of positive samples for ciprofloxacin in different samples organ from different species. The organ with the highest prevalence was the live with a mean of 13.85 µg/kg.

Table 4.7: Summary of detection of the quinolones (ciprofloxacin) in different meat samples using HPLC

		Ciprofloxacin						
Type of sample	**Organ**	**(n)**	**+ve**	**Mean (µg/kg)**	**STD dev**	**Range (µg/kg)**	**Detection > MRL (%)**	**EU-MRL µg/kg**
Chicken	➢ Liver	18	2 (11)	65.2	±13.85	34.7–95.6	Nil	200
	➢ Muscle	14	Nil	Nil	Nil	Nil	Nil	100
Beef	➢ Liver	10	1 (10)	44.39	Nil	Nil	Nil	200
	➢ Kidney	7	Nil	Nil	Nil	Nil	Nil	300
	➢ Muscle	14	Nil	Nil	Nil	Nil	Nil	100
Pork	➢ Liver	8	2 (67)	34.2	±13.82	32.8–35.6	Nil	200
	➢ Kidney	5	1 (9)	120.3	Nil	Nil	Nil	300
	➢ Muscle	8	Nil	Nil	Nil	Nil	Nil	100

n= number, **STD dev**= standard deviation, **+ve**= positive, **MRL**= maximum residue level, **EU**= European Union

4.1.3.2.1 Calibration curve

The calibration curve for ciprofloxacin showed good linearity with 0.9943 for the mean r^2 value (Figure 4.10). When the standard calibration curve was prepared for ciprofloxacin, a good dispersion of the standards on the curve was also obtained.

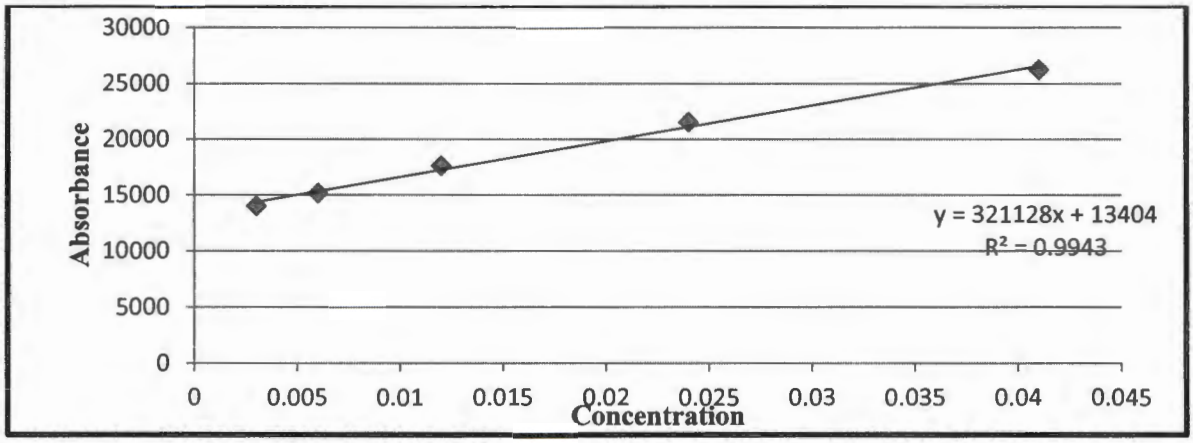
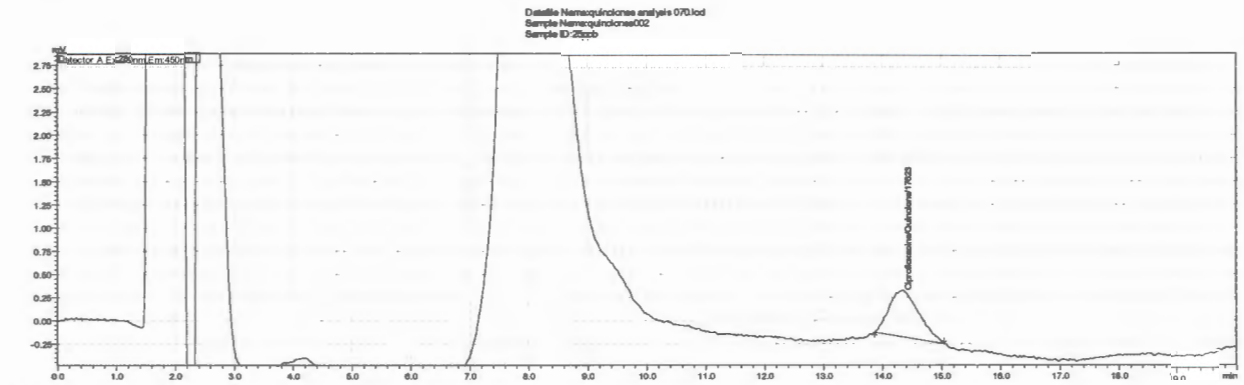
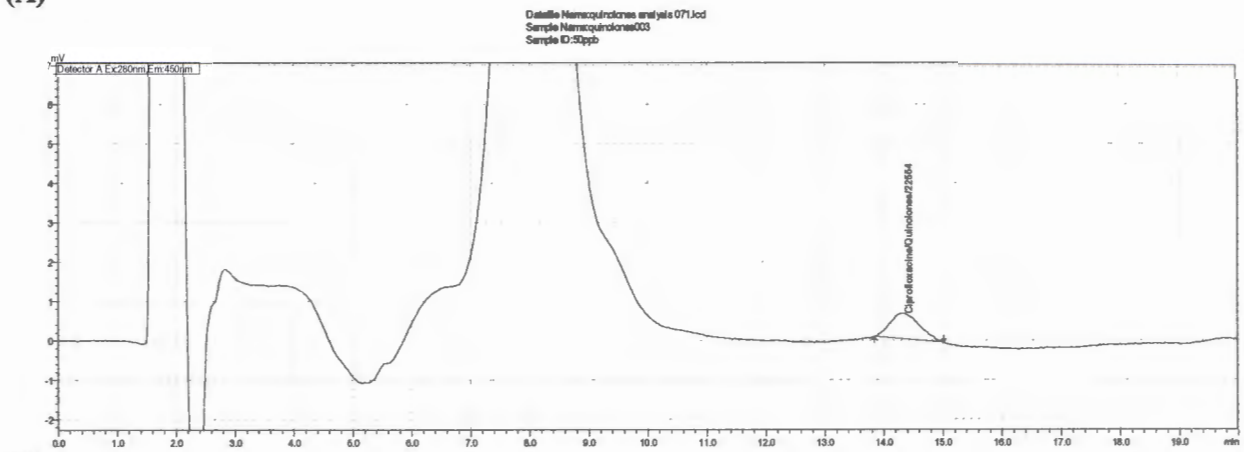


Figure 4.9: Calibration curve for ciprofloxacin standards at 0.003, 0.006, 0.012, 0.024 and 0.048 µg/kg run on HPLC under the RF detector

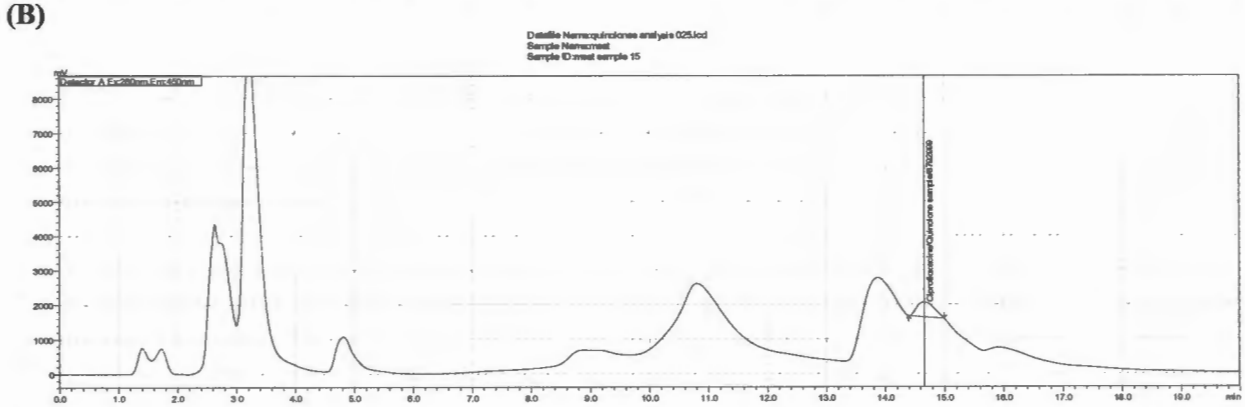
The results for ciprofloxacine are presented in Table 4.11 with excellent recoveries ranging from 75% to 84%, which is an indication that the sample preparation procedure was suitable for the analysis of quinolones (ciprofloxacin) in meat samples.



(A)



(B)



(C)

Figure 4.10: (A) HPLC chromatogram of a standard at 0.0244 µg/ml contaminated with ciprofloxacin, **(B)** Chromatogram of a spiked sample at 18µg/kg contaminated with ciprofloxacin, **(C)** Chromatogram of meat samples contaminated with quinolones (ciprofloxacin) with a flow rate of 1.0 mL/minute, Run on RF detector with 280 nm excitation and 450 nm emission wavelengths and ambient temperature (40°C) and the antibiotic detected at approximately 13.8 to 15 minutes

4.1.3.2.2 Determination the Limit of Detection (LOD)

The present method provided the limit of detection for quinolones (ciprofloxacin) residues in meat samples at 0.0015 µg/ml.

4.1.3.2.3 Determination the Limit of Quantification (LOQ)

The limit of quantification (LOQ) for ciprofloxacin was 0.0488 µg/ml for meat which was calculated from the correlation coefficient, intercept and slope of calibration curve.

4.1.3.3 Confirmation of tetracycline residue in meat

Table 4.8 shows a summary of the detection of tetracycline in various samples organs from different species (chicken, beef and pork) with their mean concentration (µg/kg). The organ with the highest prevalence level was chicken liver with a mean of 51.34 µg/kg.

Table 4.8: Summary of the detection of tetracycline in different meat samples using HPLC

			Tetracycline						
Type of sample	**Organ**		**(n)**	**+ve (%)**	**Mean (µg/kg)**	**STD dev**	**Range (µg/kg)**	**Detection > MRL (%)**	**Codex/SA MRL µg/kg**
Chicken	➢	Liver	9	8 (89)	98.34	±27.74	46.8–220.2	Nil	300
	➢	Muscle	5	3 (60)	52.31	±22.26	44.3–65.2	Nil	100
Beef	➢	Liver	5	4 (80)	71.65	±27.34	54.2–148.9	Nil	300
	➢	Kidney	5	2 (40)	181.3	±28.63	41.8–320.8	Nil	600
	➢	Muscle	Nil	Nil	Nil	Nil	Nil	Nil	100
Pork	➢	Liver	4	2 (50)	77.15	±44.31	68.6–80.3	Nil	300
	➢	Kidney	4	2 (50)	54.75	±16.55	44.3–65.2	Nil	600
	➢	Muscle	3	1 (33)	84.25	Nil	Nil	Nil	100

n= number of samples, **STD dev**= standard deviation, **+ve**= positive, **MRL**= maximum residue level, **SA**= South Africa

4.1.3.3.1 Calibration curve

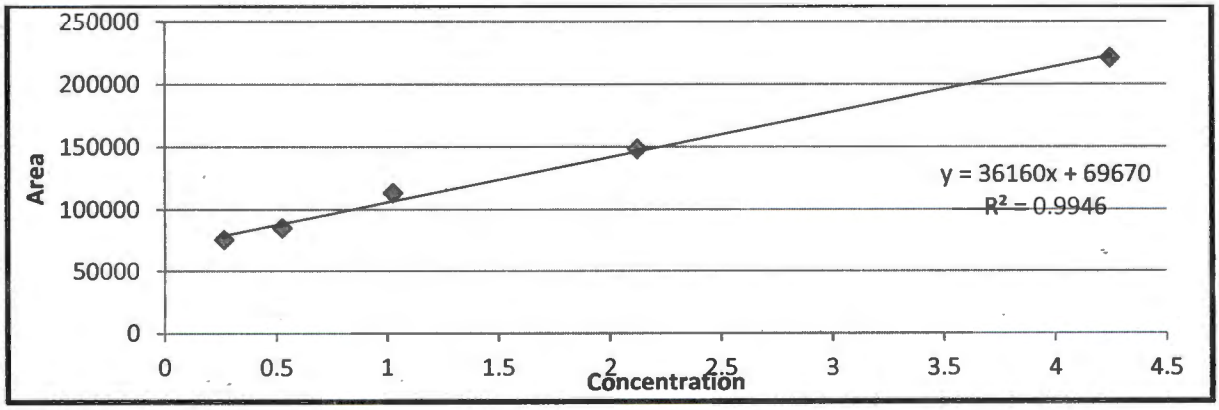
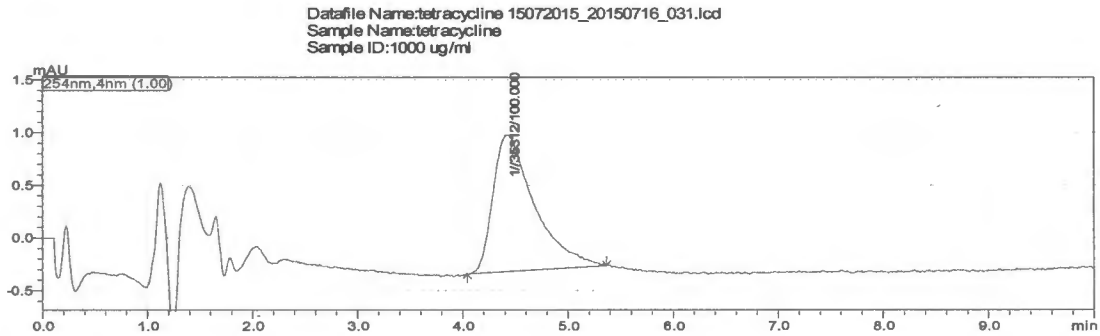
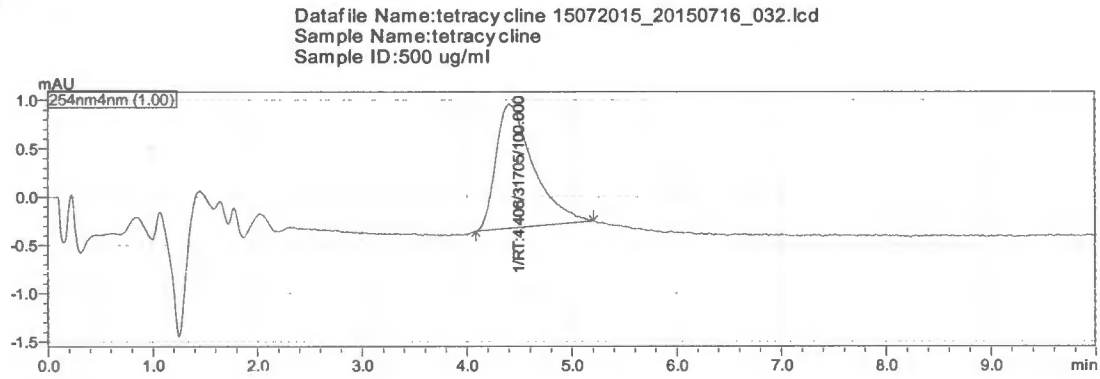


Figure 4.11: Calibration curve for tetracycline standards at 0.2654, 0.5308, 1.0616, 2.1232 and 4.2464 µg/ml run on HPLC under the RF detector

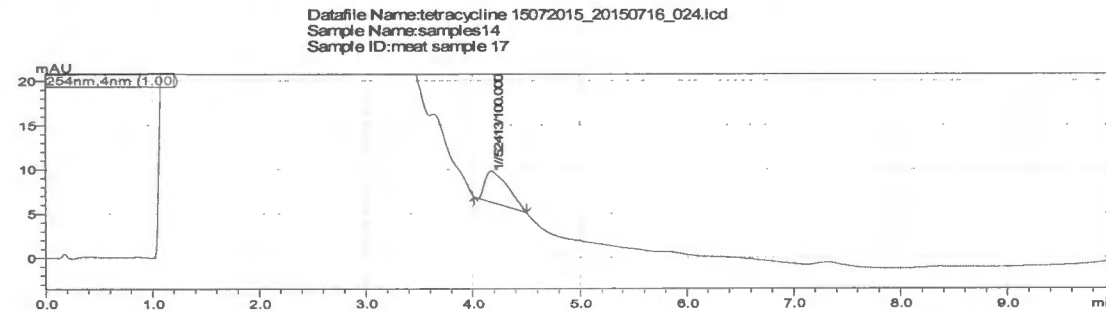
To determine the amount of tetracycline residues in meat samples, a standard calibration curve for tetracycline was obtained by running a standard solution on HPLC and then plotting peak areas against concentrations in µg/ml and later converted to µg/kg. A calibration curve, the best fit of the line was calculated by equation of line which was y= 59783 x + 8868.8. Linearity was evaluated through correlation coefficient at 0.9943 (Figure 4.11).



(A)



(B)



(C)

Figure 4.12: **(A)** HPLC chromatogram of a standard at 0.857 µg/ml contaminated with Tetracyclines **(B)** Chromatogram of a spiked sample at 100 µg/kg contaminated with Tetracyclines, **(C)** Chromatogram of meat samples contaminated with Tetracyclines with flow rate of 1.0 mL/minute, Run on RF detector with 405 nm excitation and 495 nm emission wavelength and ambient temperature (40°C) and the antibiotic detected at approximately 4 to 5 minutes.

4.1.3.3.2 Determination of the Limit of Detection (LOD)

The limit of detection for Tetracycline was 0.053µg/kg while the retention time was between 4 to 5 minutes for meat samples and calculated from the correlation coefficient, intercept and slope of the calibration curve.

4.1.3.3.3 Determination the Limit of Quantification (LOQ)

The limit of quantification (LOQ) for Tetracyclines was 4.075 µg/kg for meat and calculated from the correlation coefficient, intercept and slope of the calibration curve.

4.1.3.4 Confirmation of Streptomycin residues in meat

Table 4.9 presents a summary of the detection of streptomycin in different samples organs from different species (chicken, beef and pork) with their mean concentration (µg/kg). The organ with the highest prevalence level was chicken liver with a mean of 2.98 µg/kg.

Table 4.9: Summary of the detection of streptomycin in different meat samples using HPLC

		Streptomycin						
Type of sample	**Organ**	**(n)**	**+ve (%)**	**Mean (µg/kg)**	**STD dev**	**Range (µg/kg)**	**Detection > MRL (%)**	**Codex/SA MRL µg/kg**
Chicken	➢ Liver	12	6 (50)	187.2	±2.97	106.5–325.1	Nil	600
	➢ Muscle	9	3 (33)	120.8	±3.39	65.2–386.4	Nil	600
Beef	➢ Liver	8	5 (63)	201.2	±12.2	108.7–420.4	Nil	600
	➢ Kidney	8	3(38)	711.6	±3.39	625.9–952.2	Nil	1000
	➢ Muscle	5	Nil	Nil	Nil	Nil	Nil	600
Pork	➢ Liver	4	1(25)	30.21	Nil	Nil	Nil	600
	➢ Kidney	7	4(47)	421.5	±2.78	320.7–775.8	Nil	1000
	➢ Muscle	3	Nil	Nil	Nil	Nil	Nil	600

n= number of samples, **STD dev**= standard deviation, **+ve**= positive, **MRL**= maximum residue level, **SA**= South Africa

4.1.3.4.1 Calibration curve

The calibration curve for streptomycin showed good linearity with 0.9918 for the mean r^2 value (Figure 4.13). When the standard calibration curve was prepared for streptomycin, a good dispersion of the standards on the curve was also obtained.

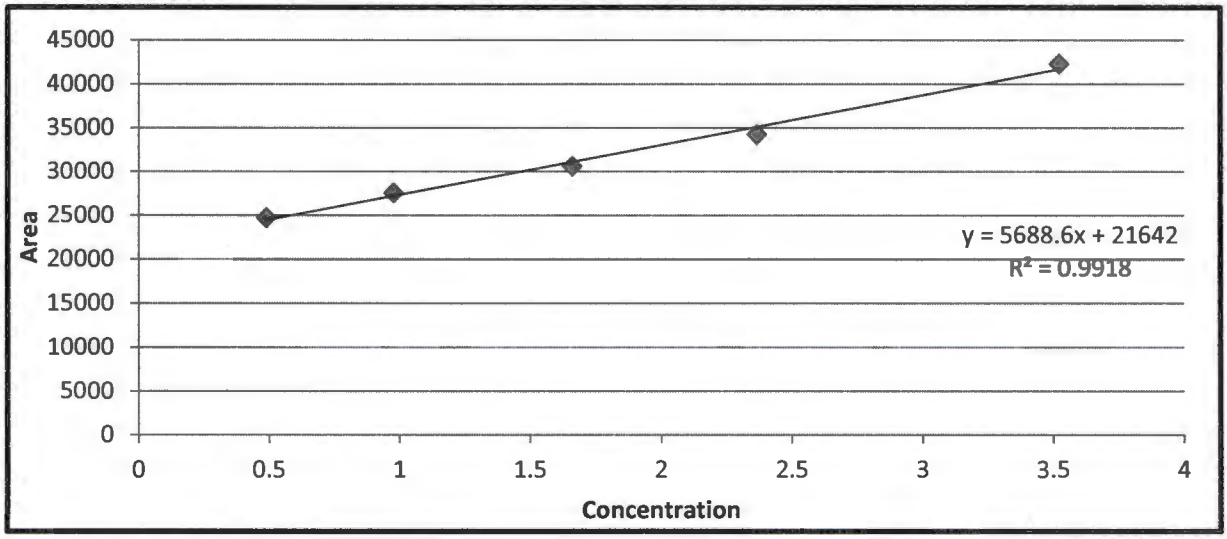


Figure 4.13: Calibration curve for streptomycin showing linearity over the concentration range of 0.488, 0.976, 1.953, 2.485 and 3.102 µg/kg.

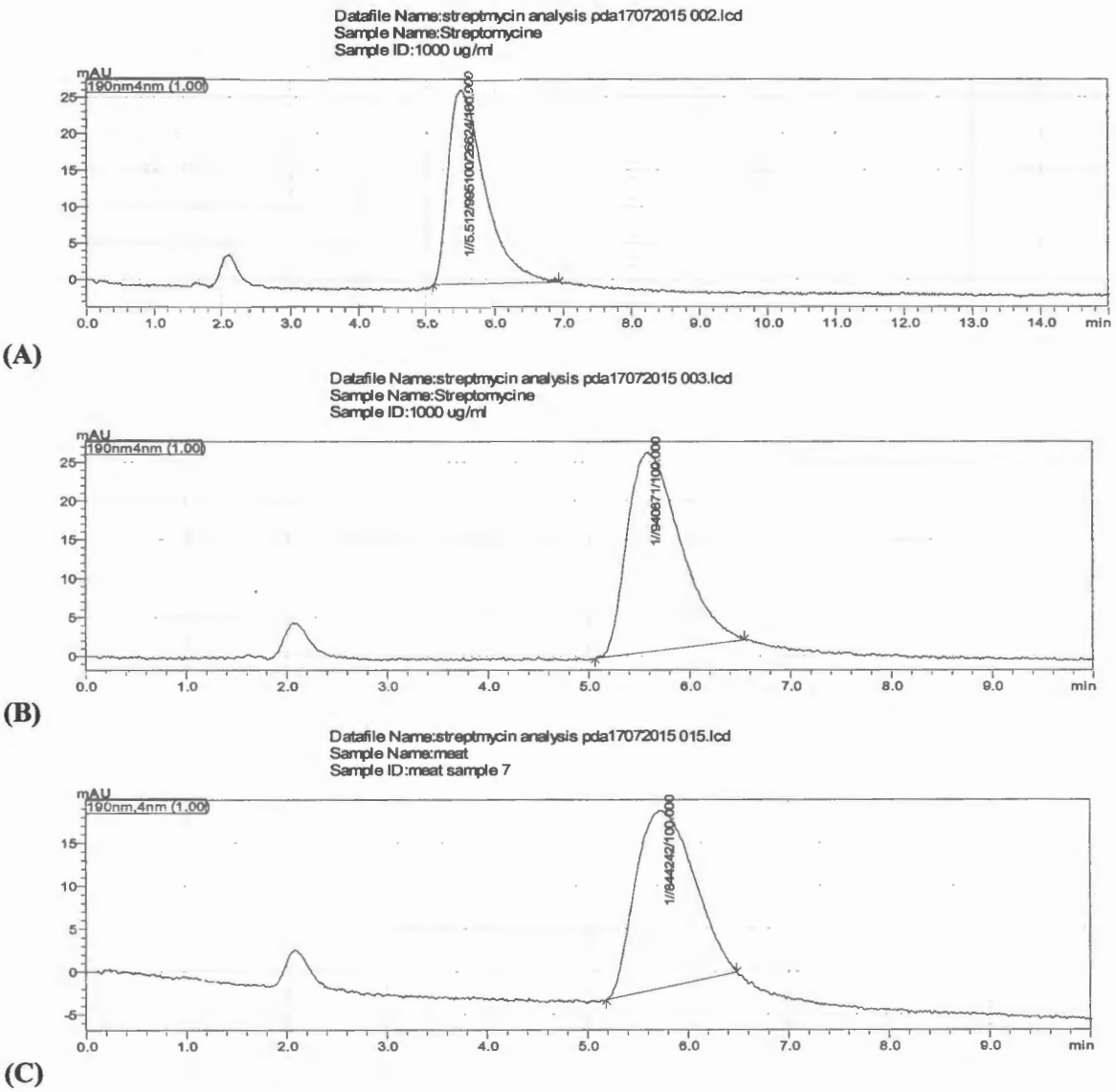


Figure 4.14: (A) HPLC chromatogram of a standard at 0.488 µg/ml contaminated with Streptomycin **(B)** Chromatogram of a spiked sample at 100 µg/kg contaminated with Streptomycin, **(C)** Chromatogram of meat samples contaminated with Streptomycin with flow rate of 1.0 mL/minute, Run on RF detector with 250 nm excitation and 450 nm emission wavelength and ambient temperature (40°C), the analysis was performed in 10 minutes and the antibiotic detected at approximately 5 to 7 minutes.

4.1.3.4.2 Determination of the Limit of Detection (LOD)

The limit of detection was 0.122 µg/kg for meat samples and calculated from the correlation coefficient, intercept and slope of the calibration curve.

4.1.3.4.3 Determination of the Limit of Quantification (LOQ)

The limit of quantification (LOQ) for streptomycin was 3.952 µg/kg for meat samples and calculated from the correlation coefficient, intercept and slope of the calibration curve.

4.1.4 Summary of all the results (multi-residues, statistics, Antimicrobial residues patterns of antimicrobial)

Table 4.10 shows the various samples, organs and methods used to detect antimicrobial residues in different meat samples.

Table 4.10: Summary of the detection of antibiotic residues in different samples using ELISA, TLC and HPLC

a) Ciprofloxacin

Type of sample	Organs	n	ELISA (%)	>MRL	TLC (%)	>MRL	HPLC (%)	>MRL
Chicken	➢ Muscle	25	14 (56)	11 (44)	3 (12)	Nil	Nil	Nil
	➢ Liver	25	18 (72)	12 (48)	7 (28)	Nil	2 (11)	Nil
Beef	➢ Muscle	15	14 (93)	5 (33)	Nil	Nil	Nil	Nil
	➢ Liver	17	10 (58)	9 (53)	4 (23.5)	Nil	1 (10)	Nil
	➢ Kidney	18	7 (38)	Nil	Nil	Nil	Nil	Nil
Pork	➢ Muscle	16	8 (50)	Nil	Nil	Nil	Nil	Nil
	➢ Kidney	11	5 (45.5)	Nil	1 (9)	Nil	1 (9)	Nil
	➢ Liver	23	8 (34.7)	3 (13)	3 (13)	Nil	3 (13)	Nil

b) Streptomycin

Type of sample	Organs	n	ELISA (%)	>MRL	TLC (%)	>MRL	HPLC (%)	>MRL
Chicken	➢ Muscle	25	9 (36)	Nil	5 (20)	Nil	3 (12)	Nil
	➢ Liver	25	12 (48)	9 (36)	8 (32)	Nil	6 (24)	Nil
Beef	➢ Muscle	15	5 (33.3)	5 (33)	Nil	Nil	Nil	Nil
	➢ Liver	17	8 (47)	7 (41)	6 (35.2)	Nil	5 (29.4)	Nil
	➢ Kidney	18	8 (44)	7 (39)	3 (16.6)	Nil	3 (16.6)	Nil
Pork	➢ Muscle	16	3 (19)	3 (19)	1 (6.25)	Nil	Nil	Nil
	➢ Kidney	11	7 (64)	1 (9)	4 (36.3)	Nil	4 (36.3)	Nil
	➢ Liver	23	4 (17)	Nil	1 (4.3)	Nil	1 (4.3)	Nil

c)			Sulphonamides					
Type of sample	Organs	n	ELISA (%)	>MRL	TLC (%)	>MRL	HPLC (%)	>MRL
Chicken	➢ Muscle	25	3 (12)	Nil	3 (12)	Nil	2 (8)	Nil
	➢ Liver	25	7 (28)	Nil	7 (28)	Nil	7 (28)	Nil
Beef	➢ Muscle	15	1 (6.6)	Nil	1 (6.6)	Nil	1 (6.6)	Nil
	➢ Liver	17	5 (2.9)	Nil	5 (29.4)	Nil	5 (29.4)	Nil
	➢ Kidney	18	5 (27)	Nil	4 (22.2)	Nil	4 (22.2)	Nil
Pork	➢ Muscle	16	Nil	Nil	Nil	Nil	Nil	Nil
	➢ Kidney	11	4 (36.4)	Nil	3 (27.7)	Nil	3 (27.7)	Nil
	➢ Liver	23	2 (8.7)	Nil	2 (8.6)	Nil	2 (8.6)	Nil

d)			Tetracycline					
Type of sample	Organs	n	ELISA (%)	>MRL	TLC (%)	>MRL	HPLC (%)	>MRL
Chicken	➢ Muscle	25	5 (20)	Nil	3 (12)	Nil	3 (12)	Nil
	➢ Liver	25	9 (36)	Nil	8 (32)	Nil	8 (32)	Nil
Beef	➢ Muscle	15	3 (20)	Nil	Nil	Nil	Nil	Nil
	➢ Liver	17	5 (29.4)	Nil	4 (23.5)	Nil	4 (23.5)	Nil
	➢ Kidney	18	5 (27.7)	Nil	2 (11.1)	Nil	2 (11.1)	Nil
Pork	➢ Muscle	16	3 (18.7)	Nil	1 (6.25)	Nil	1 (6.25)	Nil
	➢ Kidney	11	4 (36.3)	Nil	2 (18.1)	Nil	2 (18.1)	Nil
	➢ Liver	23	4 (17.3)	Nil	2 (8.69)	Nil	2 (8.69)	Nil

n=number of samples, **>MRL**= samples above MRL

Table 4.11, shows the samples that had more than one antimicrobial residue after being confirmed by HPLC, while being below the MRL.

Table 4.11: Samples which had more than one antimicrobial residue

S–ID	Organ/Area	Streptomycin	Tetracycline	Sulphonamides	Ciprofloxacin
M46	L^{S}	+	–	+	–
M96	L^{B1}	+	–	–	+
M13	L^{B2}	–	+	–	+
M56	K^{B1}	–	+	+	–

S–ID= sample Identity, **L**= liver, **M**= muscle, **K**= kidney, ^{B1}= butchery1, ^{B2} =butchery 2, ^{S}= supermarket

The results in Table 4.11, shows samples with more than one antimicrobial residue. Three out of the samples were from butcheries while one sample was from a supermarket.

Table 4.12: Fitness for four antimicrobial calibrations

Antimicrobial	R^2	Variation retention time
Streptomycin	0.9918	≤2
Sulphonamides	0.9953	≤2
Tetracycline	0.9946	≤2
Ciprofloxacin	0.9943	≤2

Correlation coefficient was between 0.9918 and 0.9953 as shown in Table 4.12. The closer the correlation coefficient to 1, the better the calibration curves is. The amount of detected compound is found closer to the real amount if correlation efficient is close to 1.

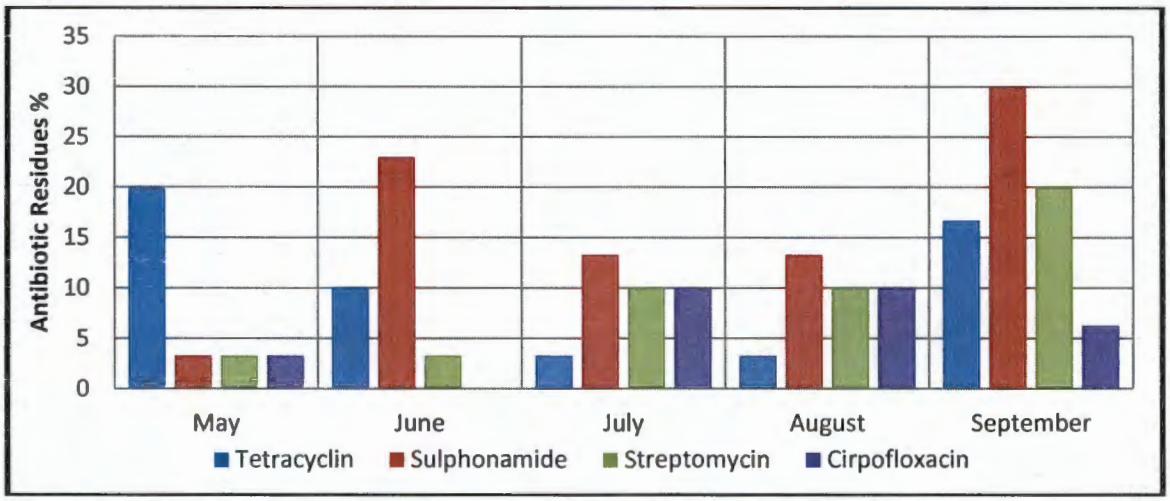


Figure 4.15: Antimicrobial residues patterns of antimicrobials detected from meat (pork, chicken and beef) from May–September

The antimicrobial residues patterns obtained for of tetracycline, sulphonamides, streptomycin and ciprofloxacin are shown in Figure 4.15.

Table 4.13: Correlation between ELISA and HPLC detectable concentrations of ciprofloxacin, streptomycin, sulphonamide and tetracycline residues in beef, chicken and pork

A)	**Beef**							
	ELISA A1	HPLC A1	ELISA A2	HPLC A2	ELISA A3	HPLC A3	ELISA A4	HPLC A4
ELISA A1		0.71*	-0.22^{NS}	-0.08^{NS}	0.10^{NS}	-0.16^{NS}	-0.08^{NS}	-0.09^{NS}
HPLC A1			-0.20^{NS}	-0.10^{NS}	0.03^{NS}	-0.11^{NS}	-0.06^{NS}	-0.05^{NS}
ELISA A2				0.40*	0.02^{NS}	-0.01^{NS}	0.04^{NS}	0.15^{NS}
HPLC A2					0.04^{NS}	-0.04^{NS}	-0.09^{NS}	-0.09^{NS}
ELISA A3						0.67*	0.07^{NS}	0.15^{NS}
HPLC A3							0.09^{NS}	0.17^{NS}
ELISA A4								0.93*
HPLC A4								

b) **Chicken**

	ELISA A1	HPLC A1	ELISA A2	HPLC A2	ELISA A3	HPLC A3	ELISA A4	HPLC A4
ELISA A1		0.03 ^{NS}	0.13 ^{NS}	0.37*	0.39*	0.25 ^{NS}	-0.01 ^{NS}	0.30*
HPLC A1			0.24 ^{NS}	-0.06 ^{NS}	0.26 ^{NS}	0.33*	0.05 ^{NS}	0.047*
ELISA A2				0.28*	0.021 ^{NS}	-0.19 ^{NS}	0.06 ^{NS}	0.19 ^{NS}
HPLC A2					-0.14 ^{NS}	-0.08 ^{NS}	0.03 ^{NS}	0.00 ^{NS}
ELISA A3						0.69*	0.22 ^{NS}	0.56*
HPLC A3							0.14 ^{NS}	0.27 ^{NS}
ELISA A4								0.61*
HPLC A4								

c) **Pork**

	ELISA A1	HPLC A1	ELISA A2	HPLC A2	ELISA A3	HPLC A3	ELISA A4	HPLC A4
ELISA A1		0.04 ^{NS}	-0.11 ^{NS}	-0.06 ^{NS}	-0.05 ^{NS}	-0.08 ^{NS}	0.01 ^{NS}	-0.05 ^{NS}
HPLC A1			-0.12 ^{NS}	-0.07 ^{NS}	-0.09 ^{NS}	-0.07 ^{NS}	-0.06 ^{NS}	-0.06 ^{NS}
ELISA A2				0.52 ^{NS}	0.10 ^{NS}	0.10 ^{NS}	-0.09 ^{NS}	-0.1 ^{NS}
HPLC A2					0.11 ^{NS}	0.34*	-0.04 ^{NS}	-0.06 ^{NS}
ELISA A3						0.70 ^{NS}	0.16 ^{NS}	0.11 ^{NS}
HPLC A3							-0.11 ^{NS}	-0.11 ^{NS}
ELISA A4								0.98*
HPLC A4								

NS = non significant, *= Correlation is significant at 0.05 level, **A1**= ciprofloxacin, **A2**= streptomycin, **A3**= sulphonamide, **A4**= tetracycline

Statisitical analysis revealed that there was a significant correlation ($p<0.05$) between results obtained on ELISA and HPLC for beef samples for all antimicrobial [ciprofloxacin (0.71), streptomycin (0.40), sulphonamide (0.67) and tetracycline (0.93)] (Table 4.13 a). While for chicken samples significant correlation ($p<0.05$) between ELISA and HPLC results was obtained for all antimicrobial but not for ciprofloxacin (Table 4.13 b). Moreover, only tetracyclin for those methods was positive correlated ($p<0.05$) in pork (Table 4.13 c).

Table 4.14: Correlation between ELISA and HPLC detectable concentrations of ciprofloxacin, streptomycin, sulphonamide and tetracycline residues in kidney, liver and muscle

a) Kidney

	ELISA A1	HPLC A1	ELISA A2	HPLC A2	ELISA A3	HPLC A3	ELISA A4	HPLC A4
ELISA A1		-0.06 ^{NS}	-0.25 ^{NS}	-0.14 ^{NS}	-0.21 ^{NS}	-0.17 ^{NS}	-0.11 ^{NS}	-0.11 ^{NS}
HPLC A1			-0.17 ^{NS}	-0.07 ^{NS}	-0.11 ^{NS}	-0.09 ^{NS}	-0.59 ^{NS}	-0.06 ^{NS}
ELISA A2				0.44*	0.05 ^{NS}	-0.04 ^{NS}	-0. 11 ^{NS}	-0.16 ^{NS}
HPLC A2					0.03 ^{NS}	0.22 ^{NS}	-0.14 ^{NS}	-0.12 ^{NS}
ELISA A3						0.49*	0.28 ^{NS}	0.23 ^{NS}
HPLC A3							0.06 ^{NS}	0.05 ^{NS}
ELISA A4								0.99*
HPLC A4								

b) Liver

	ELISA A1	**HPLC A1**	**ELISA A2**	**HPLC A2**	**ELISA A3**	**HPLC A3**	**ELISA A4**	**HPLC A4**
ELISA A1		0.36*	0.02 ^{NS}	0.19 ^{NS}	0.29*	0.05 ^{NS}	-0.03 ^{NS}	0.10 ^{NS}
HPLC A1			-0.17 ^{NS}	-0.01 ^{NS}	-0.02 ^{NS}	-0.08 ^{NS}	-0.11 ^{NS}	0.05 ^{NS}
ELISA A2				0.42*	-0.03 ^{NS}	-0.09 ^{NS}	0.06 ^{NS}	0.25 ^{NS}
HPLC A2					-0.05 ^{NS}	-0.01 ^{NS}	-0.00 ^{NS}	-0.11 ^{NS}
ELISA A3						0.73*	0.01 ^{NS}	0.32*
HPLC A3							0.03 ^{NS}	0.09 ^{NS}
ELISA A4								0.67*
HPLC A4								

c) Muscle

	ELISA A1	**HPLC A1**	**ELISA A2**	**HPLC A2**	**ELISA A3**	**HPLC A3**	**ELISA A4**	**HPLC A4**
ELISA A1		0 ^{NS}	-0.04 ^{NS}	0.12 ^{NS}	-0.05 ^{NS}	-0.10 ^{NS}	0.24 ^{NS}	0.21 ^{NS}
HPLC A1			0 ^{NS}	0 ^{NS}	0 ^{NS}	0 ^{NS}	0 ^{NS}	0 ^{NS}
ELISA A2				0.28*	0.00 ^{NS}	-0.13 ^{NS}	-0.02 ^{NS}	-0.09 ^{NS}
HPLC A2					-0.09 ^{NS}	-0.07 ^{NS}	0.04 ^{NS}	0.11
ELISA A3						0.53*	0.06 ^{NS}	0.19 ^{NS}
HPLC A3							-0.07 ^{NS}	-0.04 ^{NS}
ELISA A4								0.72*
HPLC A4								

NS = non significant, *= Correlation is significant at the 0.05 level, **A1**= ciprofloxacin, **A2**= streptomycin, **A3**= sulphonamide, **A4**= tetracycline

Data obtained also showed significant correlation ($p<0.05$) between ELISA and HPLC results for liver samples for all antimicrobials [ciprofloxacin (0.36), streptomycin (0.42), sulphonamide (0.73) and tetracycline (0.67)] (Table 4.14). Significant correlation ($p<0.05$) was also noted on for kidney samples for streptomycin (0.42), sulphonamide (0.73) and tetracycline (0.67), and not for ciprofloxacin. The analysis of muscle revealed significant

correlation ($p<0.05$) for streptomycin (0.28), sulphonamide (0.53) and tetracycline (0.72) and not for streptomycin.

Table 4.15: Correlation between ELISA, TLC and HPLC detectable concentrations of ciprofloxacin, streptomycin, sulphonamide and tetracycline residues in chicken, beef and pork

a)	Ciprofloxacin	
Species	**ELISA and TLC**	**TLC and HPLC**
Chicken	0.626*	0.685*
Beef	0.262 NS	0.434*
Pork	0.425*	0.808*

b)	Streptomycin	
Species	**ELISA and TLC**	**TLC and HPLC**
Chicken	0.494*	0.368*
Beef	0.506*	0.709*
Pork	0.592*	0.560*

c)	Sulphonamide	
Species	**ELISA and TLC**	**TLC and HPLC**
Chicken	0.792*	0.731*
Beef	0.839*	0.832*
Pork	0.788*	0.811 NS

d)	Tetracycline	
Species	**ELISA and TLC**	**TLC and HPLC**
Chicken	0.903*	1*
Beef	0.856 NS	0.944*
Pork	1*	1*

Correlation is significant at 0.05 level, **NS** = non significant, *= Significant

In analysing the results, samples were categorized into two as follows: category 1 representation of samples without concentration (0) while category 2 was represention of samples with concentration of residues (1). There was correlation ($p<0.05$) between TLC, ELISA and HPLC for all antimicrobials except for sulphonamides which shows no correlation ($p>0.05$) from pork samples for those two methods (Table 4.15). Also, streptomycin indicated significant correlation ($p<0.05$) between TLC, ELISA and HPLC for in data from beef and pork. While significant correlation was obtained for the three methods only with data obtained from chicken samples for ciprofloxacin (Table 4.15).

4.2 ISOLATION AND BIOCHEMICAL CHARACTERISATION OF STRAINS

4.2.1 Isolation and Biochemical Tests

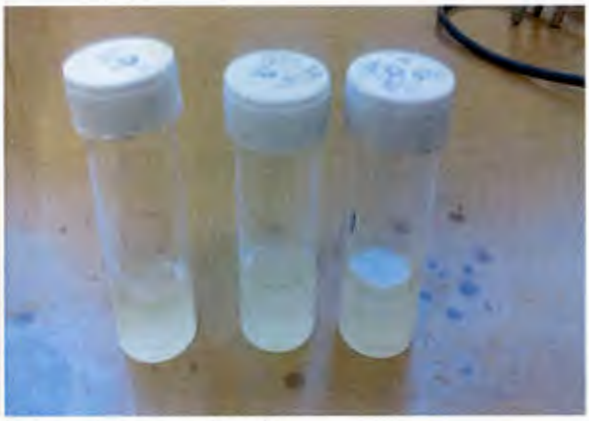
In this study, meat samples were cultured and thirty isolates obtained. The isolates were differentiated on the basis of cultural and cellular morphological studies, subsequent to a variety of primarily biochemical characteristics such as Voges-proskauer test, urease test, catalase, oxidase, indole and coagulase tests shown in Table 4.16.

Table 4.16: Preliminary results from primary Biochemical Tests

S–ID	GS	CAT	OX	IT	VPT	UR	COA
M49	–ve	+ve	–ve	+ve	–ve	–ve	–ve
M44Y	–ve	+ve	–ve	+ve	–ve	–ve	–ve
M5	–ve	+ve	–ve	+ve	–ve	–ve	–ve
M105	+ve	+ve	+ve	–ve	–ve	–ve	+ve
M77	+ve	+ve	+ve	+ve	–ve	–ve	+ve
M60	+ve	+ve	+ve	+ve	–ve	+ve	+ve
M73	+ve	+ve	+ve	+ve	–ve	–ve	+ve
M70	+ve	+ve	+ve	+ve	–ve	–ve	+ve
M3Y	+ve	+ve	+ve	–ve	–ve	+ve	+ve
M3W	+ve	+ve	–ve	+ve	–ve	–ve	–ve
M100	+ve	+ve	–ve	–ve	–ve	+ve	–ve
M48	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M4W	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M70	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M17	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M16W	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M44W	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M91	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M86	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M61Y	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M102	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M83	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M16Y	–ve	+ve	–ve	–ve	+ve	–ve	–ve
M46	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M96	–ve	+ve	–ve	+ve	+ve	+ve	–ve
M74	–ve	+ve	–ve	+ve	+ve	+ve	–ve
M13	–ve	+ve	–ve	+ve	+ve	+ve	–ve
M85	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M56	–ve	+ve	–ve	–ve	–ve	–ve	–ve
M12	–ve	+ve	–ve	–ve	–ve	–ve	–ve

S-ID = Sample Identity; **GS** =Gram staining; **VPT**= Voges Proskauer Test; **UR**=Urease Test; **CAT**= catalase, **OX**=oxidase; **IT**=Indole Test; **COA**=Coagulase. +, Activity; –, No activity

(A) Indole test (+ve)

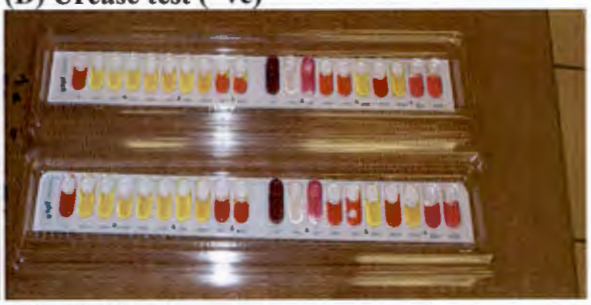


(B) Indole test (−ve)

(C) Urease test (+ve)

(D) Urease test (−ve)

(E) Catalase test (+ve)



(F) API Staph

(G) API 20E

Figure 4.16: Some pictures of biochemical results performed in this study

Figure 4.16 above shows results of some biochemical tests performed in order to identify some of the organisms in this study. **A** shows the Indole positive, due to the red colour ring at the upper layer of liquid while **B** shows that there was no colour change, which

indicates indole negative. **C** shows the production of urease which indicates pink-red colour throughout the broth. When organisms utilise urea, ammonia is formed during incubation which makes the reaction of these media alkaline, producing a pink-red colour while **D** is urease negative since the broth remains yellowish-orange after incubation. Moreover, the bubbling seen in **E** indicates catalase positive, which is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell. Because of this, any cell that uses O_2 or can live in the presence of O_2 must have a way to get rid of the peroxide. One of these ways is to make catalase. **F** shows the confirmatory test (API Staph) which was used as one of the confirmatory biochemical tests. API web TM identification software was used to confirm *Staphylococcus spp*. Lastly **G**, is the confirmatory test API 20E.

4.2.2 Molecular identification

4.2.2.1 Genomic DNA from the isolates

After running the gel, an automatic UV transilluminator (UV tec, Sigma, Germany) was used to view the bands of the genomic DNA and photographed using a Bioprofile gel documentation system. The aim was to check the presence of DNA, and confirm successful extraction (Figure 4.17).

Figure 4.17: Image of an Agarose (2%w/v) gel depicting genomic DNA extracted from positive isolates

4.2.2.2 Detection of specific 16S rDNA gene by PCR

Thirty one (31) isolates were selected and subjected to PCR analysis. Figure 4.18 shows a 1% (w/v) agarose gel representing 16S rDNA gene fragments. The desired 1.5 kb base pairs fragments were obtained after running the gel on electrophoresis for 65 minutes.

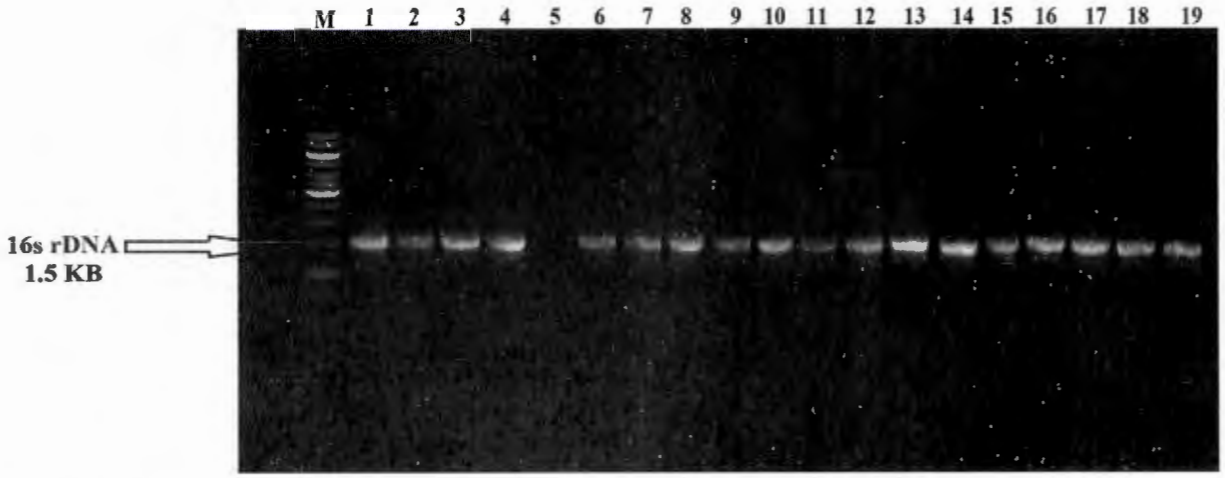


Figure 4.18: Electrophoresis in a 1% agarose gel of PCR amplified 16S rDNA of *Macrococcus caseolyticus* (1–6), *Enterococcus spp.* (7–9), *Enterococcus faecalis* (10–13), *Enterococcus mundtii* (14), *Escherichia spp.* (15), *Bacillus spp.* (16), *Bacillus cereus* (17), *Clostridium subterminale* (18) and *Citrobacter freundii* (19) *species* strains. Molecular weight marker (1kb DNA ladder Lane M)

The entire bacteria that had been classified by biochemical tests as shown in Table 4.13 and morphological assay were analysed using PCR profile to verify their strain identification as shown in Figure 4.18. Table 4.14 compares results of 30 isolates as determined by MALDI-TOF and 16S sequencing, detected to be positive for antibiotic residues after High Performance Liquid Chromatography (HPLC) confirmation.

Thirty isolates (30) using PCR were obtained in this study and identified as follows: *Macrococcus caseolyticus* 18 (60%), *Enterococcus faecalis* 4 (13%), *Enterococcus mundtii* 1 (3.3%), *Bacillus spp.* 1 (3.3%), *Bacillus cereus* 1 (3.3%), *Clostridium subterminale* 1 (3.3%), *Enterococcus spp.* 3 (10%), *Citrobacter freundii* 1 (3.3%) and *Escherichia spp.* 1 (3.3%) as shown in Table 4.17. For API there were 29 isolates positive at the API 20E and API staph 98.9% likelihood level, 30 were also positive by PCR (16S rRNA sequencing). For results

obtained from MALDI-TOF, there were no picks for some samples; M5, M102, M16Y and M85. However, seven common results were obtained for both tests, samples number M13, M96, M46, M83, M16W, M44W and M49. The findings are reliable because MALDI-TOF identification was not reliable, amplification and sequencing of the 16S rDNA gene was performed. All isolates showed 96%–100% identity according to 16S rDNA BLAST sequence homology results.

Table 4.17: Results for 30 isolates; API, MALDI-TOF and 16S sequencing (PCR)

S–ID	MALDI-TOF identity	API identification (% assurance)	16S rDNA gene sequence based Identity	Acc. No. in GenBank (PCR)
M49	*M. caseolyticus*	*M. caseolyticus*	+	FJ263452
M44Y	*S. condimenti*	*Enterococcus spp.*	+	KJ726743
M5	NP	*M. caseolyticus*	+	NR074941
M105	*Escherichia coli*	*Bacillus spp.*	+	KP100327
M77	*Hafnia alvei*	*Bacillus cereus*	+	KJ882426
M60	NP	*Clostridium spp.*	*C. subterminale*	NR113027
M73	*Hafnia alvei*	*M. caseolyticus*	+	JCSC5402
M70	*Hafnia alvei*	*M. caseolyticus*	+	FJ263452
M3Y	*Hafnia alvei*	*M. caseolyticus*	+	KJ783380
M3W	*Hafnia alvei*	*Enterococcus faecalis*	+	KF598930
M100	*Proteus vulgaris*	*Enterococcus spp.*	+	JF799879
M48	*Hafnia alvei*	*Enterococcus spp.*	+	DQ118026
M4W	*C. braakii*	*M. caseolyticus*	+	NR074941
M70	*M. caseolyticus*	*M. caseolyticus*	+	NR074941
M17	*Hafnia alvei*	*M. caseolyticus*	+	NR074941
M16W	*M. caseolyticus*	*M. caseolyticus*	+	KP058399
M44W	*M. caseolyticus*	*Enterococcus faecalis*	+	AB898316
M91	*Hafnia alvei*	*Enterococcus spp.*	+	JF799879
M86	*Klebsiella oxytoca*	*M. caseolyticus*	+	NR074941
M61Y	*Hafnia alvei*	*Enterococcus faecalis*	+	KP090135
M102	NP	*M. caseolyticus*	+	FJ263452
M83	*M. caseolyticus*	*M. caseolyticus*	+	NR074941
M16Y	NP	*M. caseolyticus*	+	NR074941
M46	*M. caseolyticus*	*M. s caseolyticus*	+	NR074941
M96	*M. caseolyticus*	*M. caseolyticus*	+	FJ263452
M74	*C. braakii*	*Escherichia spp.*	+	KF917161
M13	*M. caseolyticus*	*M. caseolyticus*	+	FJ263452
M85	NP	*M. caseolyticus*	+	NR074941
M56	*M. caseolyticus*	*Citrobacter freundii*	+	KM509080
M12	*Escherichia coli*	*Enterococcus mundtii*	+	AB898311

S-ID = Sample Identity, **NP**= No pick, **+ve**= positive samples

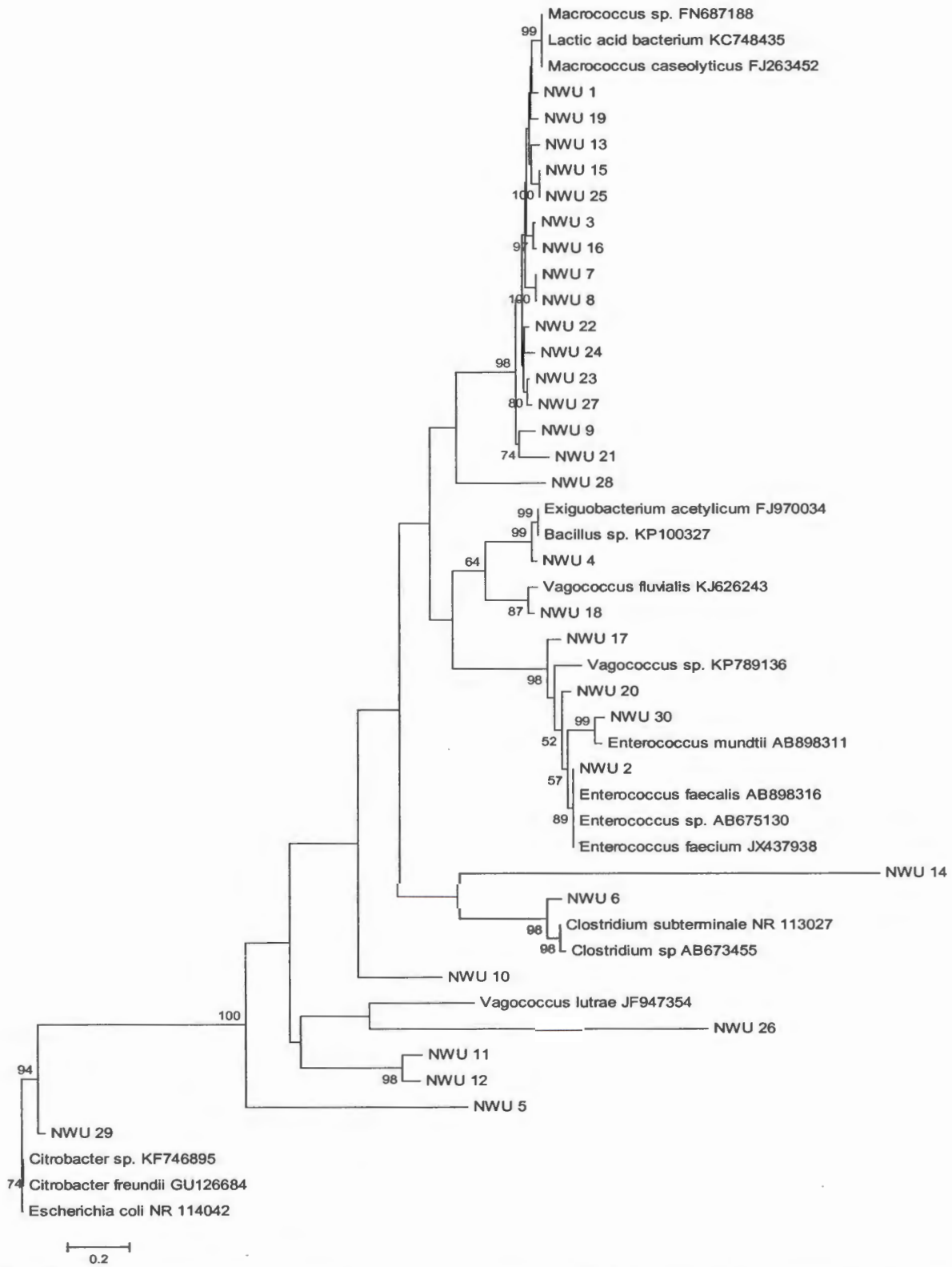


Figure 4.19: Phylogenetic tree based on comparison of 16S rDNA constructed using 30 isolates from chicken, beef and pork. The tree was clustered with the neighbour joining method using MEGA 5.2 package.

Table 4.18: Frequency of potential bacterial pathogens in samples

Microorganisms	n of Isolates (%)
Macrococcus caseolyticus	18 (60)
Enterococcus faecalis	4 (13)
Enterococcus mundtii	1 (3.3)
Bacillus spp.	1 (3.3)
Bacillus cereus	1 (3.3)
Clostridium subterminale	1 (3.3)
Enterococcus spp.	3 (10)
Escherichia spp.	1 (3.3)
Citrobacter freundii	1 (3.3)

n= number of isolates

Table 4.19: Number of isolates and their percentages isolated from different organs: Liver, muscle and kidney

Organisms	Liver	Muscle	Kidney
M. caseolyticus	10 (33%)	5 (16.6%)	3 (10%)
E. faecalis	1 (3%)	3 (10%)	–
E. mundtii	1 (3%)	–	–
C. subterminale	–	–	1 (3%)
B. cereus	–	1 (3%)	–
Bacillus spp.	–	1 (3%)	–
Enterococcus spp.	1 (3%)	–	2 (7%)
Escherichia spp.	1 (3%)	–	–
C. freundii	1 (3%)	–	–

4.2.3 Antimicrobial susceptibility test

The antimicrobial profile of isolated bacteria to various antibiotics was determined using the disk diffusion method following recommendations of the clinical laboratory institute standards (CLIS, 2012; 2013). Antibiotic susceptibility profiles of *Macrococcus caseolyticus*, *Enterococcus faecalis*, *Enterococcus mundtii*, *Bacillus spp.*, *Bacillus cereus*, *Clostridium subterminale*, *Enterococcus spp.* and *Escherichia spp.* isolates were subjected to

antibiotic susceptibility tests. The resistance patterns obtained for the isolates tested are shown in Table 4.20.

Table 4.20: Information on antibiotics used in this study to investigate antibiotic resistance obtained from clinical laboratory institute standards

Antibiotic	Abbreviation	Antibiotic concentrations disc(μg)	Inhibition zone (mm)		
			Resistant	Intermediate	Susceptible
Streptomycin	S	(300 μg)	≤11	12–14	≥15
Tetracycline	TE	(30 μg)	≤11	12–14	≥15
Sulphonamides	S3	(300 μg)	≤12	13–16	≥17
Ciprofloxacin	CIP	(5 μg)	≤15	16–20	≥20

Antibiotic susceptibility profiles of different species isolated were tested against four different antimicrobial agents and outcomes reported as percentages. The antibiotic resistance profile obtained for the isolates tested are shown in Table 4.21.

Table 4.21: Antibiotic-resistance profiles of isolates from meat obtained from butcheries and supermarkets

Antimicrobial	Drug con (μg)	Resistance	Intermediate	Susceptibility
Streptomycin	(300 μg)	–	1	29
Tetracycline	(30 μg)	25	3	2
Sulphonamides	(300 μg)	4	10	16
Ciprofloxacin	(5 μg)	1	1	28

Con=Concentration

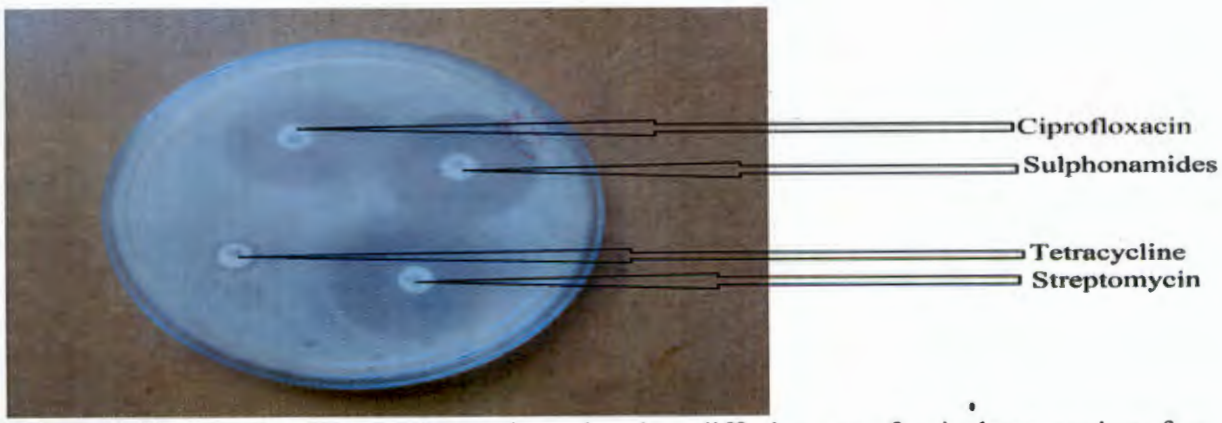


Figure 4.20: Mueller Hinton Agar plate showing diffusion test for isolates against four antibiotic discs

According to Bilatu (2012), despite the fact that some isolates which showed zones of clearance a little higher than resistance and less than susceptible were grouped as intermediate, statistically these isolates were considered to be resistant. A multidrug resistant strain of isolates from butcheries and supermarket meat around Mafikeng has been reported, from thirty (30) isolates and the resistance patterns obtained are shown in Table 4.22. Most of these strains were resistant to tetracycline (86.2%). Figure 4.20 shows the results of inhibition zones for antimicrobial drugs. The data obtained in this study revealed that multiple antibiotic resistant strains were obtained, especially for tetracycline. One can therefore suggest that these isolates may act as reservoirs or enviromenta contaminants for the transmission of antibiotic resistant genes within bacterial species and human residents.

The results in Table 4.22 show the antimicrobial profile of eight isolates which showed a high resistance rate for *Macrococcus caseolyticus* 66.6% (20/30), followed by *Enterococcus spp.* 10% (3/30), *Enterococcus faecalis* 10% (3/30), *Enterococcus mundtii* (3.33%), *Escherichia spp.* 3.33% (1/30), *Bacillus spp.* 3.33% (1/30), *Bacillus cereus* 3.33% (1/30) and *Citrobacter freundii* (0%).

Table 4.22: Resistance, susceptible and intermediate resistance patterns of microorganisms in the meat samples to sulphonamides, tetracyclines, ciprofloxacin and streptomycin.

S–ID	Microorganisms	Organ /Area	Streptomycin S_{300}	Tetracycline TE_{30}	Sulphonamide $S3_{300}$	Ciprofloxacin CIP_5
M49	*M. caseolyticus*	L^{B1}	18mm	8mm	19mm	31mm
M44Y	*Enterococcus spp.*	M^{B1}	23mm	6mm	19mm	28mm
M5	*M. caseolyticus*	L^{B1}	17mm	10mm	15mm	23mm
M105	*Bacillus spp.*	K^{S}	26mm	26mm	26mm	29mm
M77	*Bacillus cereus*	L^{B1}	14mm	13mm	31mm	24mm
M60	*Clostridium spp.*	M^{B2}	19mm	26mm	17mm	32mm
M73	*M. caseolyticus*	M^{B2}	13mm	6mm	19mm	21mm
M70	*M. caseolyticus*	M^{S}	26mm	10mm	15mm	28mm
M3Y	*M. caseolyticus*	L^{B2}	31mm	7mm	26mm	28mm
M3W	*E. faecalis*	L^{B2}	17mm	31mm	11mm	21mm
M100	*Enterococcus spp.*	L^{S}	13mm	23mm	22mm	25mm
M48	*Enterococcus spp.*	L^{S}	15mm	17mm	25mm	29mm
M4	*M. caseolyticus*	M^{B1}	26mm	10mm	8mm	29mm
M70	*M. caseolyticus*	M^{B2}	23mm	8mm	10mm	21mm
M17	*M. caseolyticus*	M^{B2}	26mm	6mm	6mm	28mm
M16	*M. caseolyticus*	K^{B2}	18mm	5mm	18mm	28mm
M44	*E. faecalis*	K^{S}	23mm	23mm	19mm	21mm
M91	*Enterococcus spp.*	L^{B1}	26mm	9mm	15mm	9mm
M86	*M. caseolyticus*	M^{B2}	31mm	11mm	26mm	33mm
M61Y	*E. faecalis*	M^{B2}	23mm	4mm	31mm	25mm
M102	*M. caseolyticus*	K^{B2}	17mm	9mm	17mm	32mm
M83	*M. caseolyticus*	L^{B2}	19mm	6mm	14mm	32mm
M16Y	*M. caseolyticus*	M^{S}	15mm	10mm	15mm	24mm
M46	*M. caseolyticus*	L^{S}	26mm	7mm	26mm	26mm
M96	*M. caseolyticus*	L^{B1}	31mm	8mm	23mm	16mm
M74	*Escherichia spp.*	L^{B2}	23mm	6mm	26mm	26mm
M13	*M. caseolyticus*	M^{B2}	17mm	6mm	18mm	26mm
M85	*M. caseolyticus*	L^{S}	26mm	9mm	23mm	26mm
M56	*C. freundii*	K^{B1}	17mm	21mm	19mm	32mm
M12	*E. mundtii*	L^{B1}	19mm	6mm	19mm	19mm

S–ID= sample Identity, red= Resistance, green= Intermediate and black= Susceptible, **L**= liver, **M**= muscle, **K**= kidney, ^{B1}= butchery1, ^{B2} =butchery 2, ^{S}= supermarket

In addition, the antimicrobial profile of the isolates provided the following antibiotics resistance level 83.3% for tetracycline. Out of this number 56.5% was for a non pathogenic *micrococcus caseolyticus*, which is an indication that the overall level of resistance was low from the pathogenic (26.6%), 13.3% for sulphonamides and 3.3% for ciprofloxacin. All isolates were sensitive to streptomycin (Table 4.22).

Table 4.23: Multi-resistance relating to specific residue concentrations

Isolates		Tetracycline		Sulphonamide		Ciprofloxacin	
	S-ID	R-con (µg/kg)	R	R-con (µg/kg)	R	R-con (µg/kg)	R
Enterococcus faecalis	M3W	89.2	R	81.6	R	–	–
Enterococcus spp.	M91	123.5	R	–	–	95.6	R
M. caseolyticus	M4	96.8	R	77.9	R	–	–
M. caseolyticus	M70	186.9	R	69.2	R	–	–
M. caseolyticus	M17	110.3	R	82.1	R	–	–

S-ID= Sample identity, **R-con**= Residue concentration, **R**= resistance

Table 4.23 shows the strains which were multi-resistance and their relationship between the specific residue concentrations. Sample identity M3W (liver), M91 (liver) and M17 (muscle) were collected from butchery 1 while sample identity M70 (muscle) and M4 (muscle) were collected from butchery 2.

Table 4.24: Correlation between the antimicrobial residues and antimicrobial resistance from ciprofloxacin, streptomycin, sulphonamide and tetracycline

a)	Ciprofloxacin
Species	**Residue and resistance**
Chicken	0.37 NS
Beef	0.23 NS
Pork	3 NS

B)	Sulphonamide
Species	**Residue and resistance**
Chicken	0.21 NS
Beef	0.23 NS
Pork	0.20 NS

b)	Tetracycline
Species	**Residue and resistance**
Chicken	1.00*
Beef	1.00*
Pork	1.00 NS

Correlation was significant at 0.05 level, NS = non significant, *= Significant

In analysing the results, samples were categorised into two as follows: category 1 representation of samples without concentration (0) and category 2 (concentration of residues). There weas no significant correlation ($p>0.05$) between residues and resistance on

ciprofloxacin from all species (chicken, beef and pork). For sulphonamides there was no significant correlation ($p>0.05$) from all the species. For tetracycline, there was correlation ($p<0.05$) between residues and resistance from two species; chicken and beef. However, there was no correlation ($p>0.05$) for pork (Table 4.24).

CHAPTER FIVE

5.0 DISCUSSION

In this study, antibiotic residues were analysed using three methods (ELISA, TLC and HPLC). In addition, microbial contaminations were assessed using molecular methods (PCR) and antibiotic resistance conducted.

5.1 ANALYSIS OF ANTIMICROBIAL RESIDUES

Analysis of drug resistance of meat using ELISA test was done for qualitative screening of quinolones, streptomycin, tetracycline and sulphonamides. The positive samples obtained from ELISA were analysed through TLC and HPLC for quantification of sulphonamides, ciprofloxacin, streptomycin and tetracycline residues. The data obtained in this study revealed that tetracycline, streptomycin, sulphonamides and quinolones (ciprofloxacin) residues were detectable in kidneys, liver and muscle of pork, chicken and beef sold around Mafikeng with 47.3% of positive samples and ranged between 22.5–982.2 µg/kg as shown in Table 4.10. However all the samples from HPLC were below the MRLs set by the European Union, South Africa regulations governing the maximum limits for veterinary medicine and stock remedy residues and the Codex Alimentarius Commission, making them sale for human consumption.

In this study, different organs (liver, kidney and muscle) were tested for antimicrobial residues. With regard to pork tissues, the result showed that 8 (6%), 1 (%) and 10 (20%) samples of liver, muscles and kidneys contained tetracycline, streptomycin, sulphonamides and ciprofloxacin residues, respectively. Moreover, with regard to chicken tissues, the result showed that 22 (44%) and 10 (20%) samples of liver and muscles respectively, contained tetracycline, streptomycin, sulphonamides and ciprofloxacin residues. Although, in beef, tissues, 15 (30%), 5 (10%) and 1 (2%) samples of liver, kidney and muscles respectively contained tetracycline, streptomycin, sulphonamides and ciprofloxacin residues. Dispite this,

similar higher findings were obtained by Sultan (1995). In his study he detected tetracycline residues in muscles and kidneys (58%) and the samples were below MRLs. Although the results obtained in this study are different from those reported by Elnasri *et al.* (2014), where positive samples were higher in muscles followed by liver with 29% and 28.3% respectively, with a lower percentage in kidneys (21.4%) for tetracycline residues. Moreover, Hala (2006) conducted study in Khartoum and found a higher percentage (24.6%) of residues in kidneys. Furthermore, in Tabriz, Iran Mesgari Abbasi *et al.* (2012) found that 25.8% of muscle samples, 31.8% of liver samples and 22.7% of kidney samples contained amounts of tetracycline residues beyond the maximum residue limits (MRLs).

For ciprofloxacin residues, six samples (12%) of livers and one sample (0.2%) of kidney were positive for ciprofloxacin residues. In the case of liver, sulphonamides residues were detected in fourteen samples only (28%), seven samples (14%) of kidneys and three samples (6%) of muscles were positive for sulphonamides residues. Data of streptomycin residues of meat sold around Mafikeng showed that twelve samples (24%) of liver, three samples (6%) of muscles and seven samples (14%) of kidneys were positive for streptomycin residues. Moreover, the results in Table 4.10 reveal that only fourteen samples (28%) of liver, four samples (8%) of kidney and four samples (8%) of muscles were positive for tetracycline. Despite the detection of antimicrobial residues from all the organs, the liver stood out as the organ with the highest detection level (87%). Results obtained in this study also concur with the findings of Naeem *et al.* (2006) who found ciprofloxacin in liver samples using HPLC. Muriuki *et al.* (2001) conducted in Nairobi, Kenya, found tetracycline residues from beef at 60 (24%) in liver; 35 (14%) in kidney, and 19 (7.6%) in muscle samples, HPLC was used to detect residues. Olatoye & Ehinmowo (2010) conducted a study in Nigeria and also found that residue level in liver was the highest followed by kidney and lastly muscle with 80%, 55.0% and 28.3% respectively. The results obtained in this study differ from those reported

by Elnasri *et al.* (2014), who found that positive samples tetracycline residues were higher in muscles followed by liver with 29% and 28.3% respectively and lower percentage in kidneys (21.4%).

The findings of this study are in agreement with the recent report from a survey carried out in South Africa by Eagar, Swan & Van Vuuren (2012), who also fund that tetracyclines constituted the second largest group of antimicrobials and penicillin the majority of the parenteral dosage forms sold and constiduted the most important residue problem. Moreover, Hala (2006) conducted study in Khartoum and found a higher percentage 24.6% in kidneys. Furthermore, in Tabriz, Iran Mesgari Abbasi *et al.* (2012) found that 25.8% of muscle samples, 31.8% of liver samples and 22.7% of kidney samples contained amounts of tetracycline residues beyond the maximum residue limits (MRLs). Despite this, there were some organ samples (muscle, liver and kidney) which had more than one antibiotic residue (Table 4.11). Several factors that may have contributed to this such as the presence of antibiotic additives in food fed to animals in order to promote growth (Sarmah *et al.*, 2006). Moreover, animals are usually given antibiotics to cure/prevent diseases and the main aim is to improve their health status (Chang *et al.*, 2000; Mehtabuddin *et al.*, 2012; Er *et al.*, 2013).

For the detection of tetracycline, the High Performance Liquid Chromatography with spectroscopic fluorometric detection (HPLC–RF) was used. HPLC is one of the most widely used techniques for determining tetracycline residues in beef, chicken and pork (De Wasch *et al.*, 1998; Shalaby *et al.*, 2011). In order to detect antimicrobial residues, the RF wavelength was carried out as in the range of 280–450 nm, as those in previous research (Blanchflower *et al.*, 1997; Shalaby *et al.*, 2011; Bedada & Zewde, 2012). The extraction and sample clean-up process was time consuming, laborious, making use of a large amount of samples and dangerous organic solvents, in particular for biological matrices such as pork which is rich in lipid. Previous studies have shown a variation in recovery values of

approximately 65–99% (Navratilova *et al.*, 2009; Bedada & Zewde, 2012). In this study, there was a recovery of 64–68% as shown in Table 3.3 and the antibiotic was detected at approximately 4 to 5 minutes as shown in Figure 4.12. Linearity was evaluated through the correlation coefficient (0.9899) as shown in Figure 4.11. These values were in correlation with the range of published data on tetracyclines (Navratilova *et al.*, 2009; Shalaby *et al.*, 2011). The different results obtained in this study, as well as others revealed that the difference could be partially due to differences in detection methods, sampling size and techniques and lastly, the geographical location of the environment. Moreover, the other reasons might be explained by the survey conducted by Eagar *et al.* (2012) in South Africa which showed that 68.5% of the antimicrobials were administered as in-feed medications which is the highest percentage of administration of antimicrobial compared to administration such as 1.5% of topical and aural dosage, 12% of antimicrobials for water medication.

Tetracyclines are mainly used as antimicrobials, mostly in livestock and poultry production, due to their affordability, safety and for their broad spectrum activity against acute disease caused by Gram positive and Gram negative bacteria such as mycoplasma, chlymedia and rickettsia (Neilsen & Gurd-Hansen, 1996; Rajaian *et al.*, 2008; Dang *et al.*, 2013). Furthermore, they are used as additives in animal food stuffs (Mesgari *et al.*, 2009). Moreover, they are easily administered in mass, either as feed or water (Hakimzadegan *et al.*, 2014). For Tetracyclines to be lower than MRL, it might be because of their metabolism since it is known that tetracyclines bind to plasma proteins at varying degrees in different species of animals (Neilsen & Gurd-Hansen, 1996). In addition, tetracycline has a short half-life time which range from 7–10 hours (Davis *et al.*, 2006). This is because the major elimination pathway of tetracycline is through renal excretion, with about 60% of tetracycline administrated being excreted in the urine in an unchanged form (Oh & Han, 2006).

In this study, a sulphonamide (Table 4.10) was found to be prevalent in 18% of results obtained using ELISA. 15.3% prevalence of sulphonamides was obtained using HPLC. Table 4.6 shows results of sulphonamides positive per organ from different species. It also represents detailed report with a mean concentration of 48.2 µg/kg and a range of 19.8–87.9. In addition, 16.7% prevalence of sulphonamides was obtained using TLC. The organ with high prevalence of sulphonamides was found in the liver (23.7–73.9 µg/kg) (Table 4.6). To detect the presence of sulphonamides in this study, high performance liquid chromatography with spectroscopic photodiode array detector (HPLC–PAD) were used. The reason for high detection of sulphonamides might be because most studies used UV detector for the presence of sulphonamides (Horii *et al.*, 1990; Pensabene *et al.*, 1998; Shaikh *et al.*, 1999; Mehtabuddin *et al.*, 2012; Ebrahimpour *et al.*, 2015). In this study, the diode detector wavelength was carried out in the range of 405–495 nm, as in previous studies (Baere *et al.*, 2000; Pang *et al.*, 2003; Garcia *et al.*, 2004; Salisbury *et al.*, 2004). However, the diode detector wavelength in this study was higher than that of previous studies (Pensabene *et al.*, 1998; Shaikh *et al.*, 1999, Mehtabuddin *et al.*, 2012; Ebrahimpour *et al.*, 2015). There was a high yield recovery of 71–76% as shown in Table 3.3. The antibiotic was detected approximately 2 to 2.8 minutes as shown in Figure 4.10. The difference of the results might be due to the use of different methods. These researchrs used a UV detectors while in this study a fluorescence detector was used. Moreover, the different mobile phases and wavelength might be one the reasons for different results for recoveries.

A review conducted by Mungroo & Neethirajan (2014) revealed that among other antibiotics, 20% of sulphonamides is classified as an antibiotic mostly present in food (Figure 2.1). Moreover, Eagar *et al*, (2012) indicate the percentages of volume (kg) for sales of classes of sulphonamides for the period 2002–2004 in South Africa. It stood at 12.40%, which is lower than other antibiotics such as tetracyclines (16.70%) and macrolides (42.40%). the presence

of sulphonamides in analysed samples might be explained by the fact that they are widely used in the livestock industry as growth promoters (Furusawa & Hanabusa, 2002; Mubito *et al.*, 2014; Mungroo & Neethirajan, 2014). They are mixed with feed several times a day to prevent bacterial contamination and are also injected to animals at high levels to increase/promote animal growth (Long *et al.*, 1990; Gentili *et al.*, 2004). The presence of sulphonamides, either higher concentration may impose health threats due to toxicity and skin allergies (Wang *et al.*, 2006; Shareef *et al.*, 2009). These results show that sulphonamidess administered to animals may not have been fully excreted and metabolised before being slaughtered.

In addition, the high detection of sulphonamides might be its long half-life which is 10.1 hours in cattle (Shoaf *et al.*, 1987). This is because the withdrawal time of a drug is recognised based on the drug and blood is a central pool of drug distribution to body compartments and removal from tissues through biological fluids (Lee *et al.*, 2000). Several reports deal with other sulphonamidess, showing that N_4-acetyle metabolite formation is one of the most common ways to eliminate these drugs from the organ. The major routes for sulphonamides metabolism are conjugations with acetyl, hydroxyl or glucuronic acid groups, in order to obtain more polar compounds which are eliminated in urine (Mouricio & Lins, 2012). The presence of this antibiotic may lead to hypersensitivity reactions to human beings (Paige *et al.*, 1997).

Analysis of ciprofloxacin residues in edible tissues is very important for human health. Using antibiotic such ciprofloxacin for prophylaxis and the treatment of chicken and beef is authorised (Buket *at al.*, 2013). In this study quinolones (Table 4.1) was found to be prevalent for 65.3% from results obtained from ELISA. Thin Layer Chromatography results obtained in this study revealed 12 % prevalence of ciprofloxacin as shown in Table 4.5. Results obtained using HPLC (Table 4.7) show a 3.3% positive prevalence of organs from

different species, a detailed report of mean concentration of 46.29 µg/kg ranging between 32.8–95.6.

In this study, high yield recovery of 75–84% as shown in Table 3.3, and the antibiotic was detected approximately 13.8 to 15 minutes (Figure 4.10). For the detection of this antibiotic, the RF wavelength was carried out as in the range of 280–450 nm similar to that of previous studies (Kassab *et al.*, 2005; Kirbis *et al.*, 2005; Wu *et al.*, 2008; Hu *et al.*, 2012). The difference seen between the three methods might be explained by the lower detection limit of each methods with TLC limit being high whicle the HPLC one is low (Kirbis *et al.*, 2005). The use of the HPLC to detect ciprofloxacin remains one of the most widely used techniques for determining residues in beef, chicken and pork (Gigosos *et al.*, 2000).

Enrofloxacin is known to be partially metabolized to ciprofloxacin in cattle, and ciprofloxacin achieves 25 to 35% of the concentration of the parent drug in blood (Kaartinen *et al.,* 1995). Enrofloxacin is metabolised in the liver to its main metabolite ciprofloxacin (Kaartinen *et al.,* 1995). Both Enrofloxacin and ciprofloxacin are effective against microorganisms which are resistant to other antimicrobial agents, such as aminoglycosides, tetracyclines, macrolides and β-lactams (Idowu *et al.*, 1995). Quinolones are licensed for the treatment of *Salmonella* infections in some animal species (WHO, 1998). The use of quinolones in food producing animals is because it has been approved for therapeutic use in food producing animals (FDA, 2001). Collignon (2003) maintains that the therapeutic use of antibiotics in some countries such as Denmark remains much lower per kilogram of meat produced than in nearly all other countries in the European Union. Furthermore, in some region such as Latin America, Asia and South Africa quinolones are licensed for use in food animals (WHO, 1998).

For the detection of streptomycin, High Performance Liquid Chromatography with Photodiode Array Detector (HPLC–PAD) was used in this study. HPLC is one of the most widely used techniques for determining streptomycin residues in beef, chicken and pork (Codex, 2010). To detect this antibiotic, the PAD wavelength was carried out as in the range of 280–450 nm, similar wavelength in previous studies (Ferguson *et al.*, 2002; Chang-Won Pyun *et al.*, 2008). In this study, there was a high yield recovery of 74–81% as shown in Table 3.3 and the antibiotic was detected at approximately 5 to 7 minutes (Figure 4.14). Linearity was evaluated through the correlation coefficient (0.9918) as shown in Figure 4.13. Enzyme Linked Immunosorbent Assay (ELISA) was used for the detection of streptomycin residues according to the manufacturer's instructions. However the procedure used was similar to the ones used by other researchers (Amjad & Naeem, 2005; Sattar *et al.*, 2014).
In general previous studies have shown a variation in recovery values of approximately 65–99% (Gigosos *et al.*, 2000; Kassab *et al.*, 2005; Kirbis *et al.*, 2005; Wu *et al.*, 2008; Hu *et al.*, 2012) while in this study, the recovery ranged between 64–84% (Table 3.3) for all four antimicrobial residues.

The antimicrobial residues patterns obtained for tetracycline, sulphonamides, streptomycin and ciprofloxacin are shown in Figure 4.15. In this study, 30 samples including chicken, pork and beef were collected monthly and detected for residues. Sample collected in May were showed higher percentage of tetracycline 6 (20%) while in June sulphonamides 7 (23%) were the highest detectable antibiotic followed by tetracycline 3 (10%) and streptomycin 1 (3.3%) however, ciprofloxacin was not detected. In August sulphonamide 4(13.3%) had more samples detected for residues. Lastly, September was dominated by sulphonamide 9 (30%) followed by streptomycin 6 (20%) then tetracycline 5 (16.7%). In generally tetracycline and sulphonamide were the most detected and had high percentage of residues detection. These results in this study concur with the study/survey conducted by

Eagar *et al.* (2012) whereby (16.17%) tetracyclines and (12.40%) sulphonamides were sold in South Africa between 2002–2004.

This study revealed that with ELISA, quinolones (Table 4.1), streptomycin (Table 4.2) and sulphonamide (Table 4.3) detected in beef, chicken and pork samples tested were above the MRL international standards, the reason for sample above MRL might be that the test was determined by analysing the cross-reactivities corresponding substance in the buffer system. Moreover, the test screened the class of compound of antimicrobial such as: sulphonamide, tetracycline and quinolones. However, results using HPLC revealed a low incidence of sulphonamides, ciprofloxacin tetracycline and, streptomycin residues below MRLs in the organs analysed as shown in Table 4.6, 4.7, 4.8 and 4.9. This is because the standards used for HPLC were for individual compound. The low concentrations of antimicrobial residues in meat samples analysed by TLC and HPLC, indicated the possible cross-reaction between chemicals themselves and their metabolites which can be detected in ELISA and not by TLC and HLPC. The differences of concentrations levels of ciprofloxacin and sulfonamide noted in this study between the ELISA and HPLC methods might be be explained by the cross reactivity or inability by ELISA to discriminate between ciprofloxacin and enrofloxacin, between all antibiotics of the sulfonamide group or between tetracycline and oxytetracycline (Mensah *et al*, 2014).

Statisitcal analysis showed the presence of a significant correlation ($p \leq 0.05$) (Table 4.13) for selected antibiotics such as [ciprofloxacin (0.36), streptomycin (0.42), sulphonamide (0.73) and tetracycline (0.67) for the liver, streptomycin (0.42), sulphonamide (0.73) and tetracycline (0.67), for kidney while streptomycin (0.28), sulphonamide (0.53) and tetracycline (0.72) were found for muscle]. This is an indication of a good degree correlation between ELISA and HPLC results. There was significant correlation ($p \leq 0.05$) also between ELISA and HPLC for all antimicrobial as shown on Table 4.13. However no significant

correlation ($p>0.05$) was observed for beef (ciprofloxacin), beef (tetracycline) and pork (sulphonamide) for results obtained on ELISA and HPLC. Moreover, there was a good correlation between HPLC and TLC (Table 4.14), also ciprofloxacin and streptomycin indicated a positive correlation ($p\leq0.05$) between TLC and HPLC for all the species (chicken, beef and pork). Moreover, there was a correlation ELISA and TLC from all the species apart from beef on ciprofloxacin and tetracycline, there were no correlation ($p>0.05$). HPLC is very producible, reliable and sensitive for drug analysis considering the fact that it can quantify the presence of very low amount of drug in samples (Huang *et al.*, 2012; Mei *et al.*, 2012) compare to those two tests. Moreover, the other major advantage over ELISA and TLC is that the inferior detection limit makes it a highly accurate device. The chromatograms for HPLC shown in Figures 4.8, 4.10, 4.12 and 4.14 for a range of matrix extracts, obviously confirmed that the method is good for meat. In the present study the potential of ELISA for the monitoring of selected antimicrobial residues meat samples was demonstrated. Immunoassay method provided excellent sensitivity and selectivity for the determination of ciprofloxacin, streptomycin, sulphonamide and tetracycline in meat samples. Also a good correlation of results with the TLC and HPLC method has been proven in our experiments. ELISA can be used as a screening method for determining the ciprofloxacin, streptomycin, sulphonamide and tetracycline residues in meat. The ELISA results should be confirmed by chromatographic methods, such as TLC and HPLC, to eliminate the false positive and false negative results.

The results from this study revealed that the tissues had the maximum amount of antimicrobial residues in ELISA compare to TLC and HPLC analysis (Shahbazi *et al.*, 2015). Additionally, the levels of antimicrobial which detected by TLC and HPLC method was extremely lower than that of ELISA. The ELISA test can detect one substance or a group of related chemicals. This confirms the fact that a screening test is really advantageous if it can

be used rapidly on a large sample number. It turned out that TLC had been a necessary test as more accurate, and most samples detected positive from TLC can also be detected positively by HPLC. The results of this study revealed that all samples (100%) which were positive on TLC were also detected in most samples using HPLC. These findings are in line with previous study conducted by Bedada & Zewde (2012) where both TLC and HPLC were used to detect tetracycline in Ethiopia. It is important to mention that differences noted between TLC (low number of positive) and HPLC (high number of positive) results for all antibitics might be explained by the detection limit differences low for HPLC opposite for TLC method (Mwanza, 2012). These findings are also in line with the results obtained by Badada *et al.* (2013). Samples with low concentrations will always not be positive on TLC. Hence the use of TLC as a screening method while the HPLC is used as confirmatory one.

5.2 MICROBIOLOGICAL ANALYSIS OF MEAT SAMPLES

The occurrence of bacteria in meat has been extensively reported from different parts of the world (Kinsella *et al.*, 2008) and it is a rising public health concern in developing as well as developed countries (Saulat, 2012). Mead *et al.* (1999) maintain that 13.8 million cases are estimated to cause food-borne illnesses due to known agents; approximately 30% are due to bacteria. This study reveales the relatively high frequency of occurrence of pathogenic organisms (40%) in meat sold from butcheries and supermarket around Mafikeng. Among the pathogenic organisms; *Enterococcus mundtii*, *Bacillus spp.*, *Bacillus cereus*, *Enterococcus spp.* and *Escherichia spp.* were the most prevalent (Table 4.18). Results obtained in this study showed that *M. caseolyticus* was the most contaminant in liver in (33%); muscle (16.6%) and kidneys (10%) *and Enterococcus spp.* were isolated in liver (3%)and muscle 2 (7%). The low incidence of contaminants in analysed samples might be explained by the fact that these are mostly originating from poor handling in butcheries.

In addition, *Micrococcus* species are known to cause spoilage in food, moreover, these microbes are known as indicators of faecal contamination which suggest poor handling and hygiene status of workers Oudiz (2004) which could have occurred in the abattoirs or butcheries. Bacterias such as Macrococcus caseolyticus originate from animal sources cows and a food-processing factory (Kloos *et al.*, 1998; Baba *et al.*, 2009) and it can be isolated from animal skin and meat. This has been confirmed by Tsubakishita *et al.* (2010) who found that this bacterium was isolated from a skin swab of chicken from Japan. This bacterium does not cause human or animal diseases (Kloos *et al.*, 1998; Baba *et al.*, 2009) hence it presence in analysed samples is not a threat to consumers. In addition, other findings showed that the presence of *Micrococcus* spp. in analysed samples can be associated with central venous catheter infection in patients with pulmonary hypertension (Marr *et al.*, 2015).

In this study just like in previous studies, the 16S rRNA gene sequencing was shown to be the most suitable test for the identification of less common isolates (Rhoads et al., 2012). HIV/AIDS is a major concern as these pathogen organisms induce immunosuppression in human beings (Ateba & Setona, 2011). Therefore, the outcome of microorganisms on human health has been reported (Saadia & Hassanein, 2010). The current study was conducted to provide information on the presence of microorganisms in meat sold in butcheries and supermarkets around Mafikeng, their importance to human beings. It also evaluated the prevalence of multidrug resistant bacteria isolates from meat samples detected positive for antibiotic residues. Results obtained revealed contamination in meat which may have an incidence on food-borne illnesses.

In this study, biochemical tests, MALDI-TOF and PCR identifications were performed in order to identify organisms. Preliminary results from primary biochemical tests were confirmed by the Analytical Profile Index API 20E strips and API Staph-Ident test for the identification of isolates. API 20E was used to classify species of *Enterobacteriaceae* and

others Gram negative rods (Forbes *et al.*, 2002) while API Staph-Ident was used to identify Gram positive species (Downes & Ito, 2001). In order to resolve the discrepancy between results of the biochemical test for the isolates, MALDI-TOF identification and the PCR confirmation were performed, the 16S rRNA gene of 30 selected isolates was sequenced (Table 4.17). Tweenty nine (29) isolates were positive at the API 20E and API Staph-Ident 98.9% likelihood level, 30 were also positive using PCR (16S rRNA sequencing). Validation of both PCR and API (20E and staph) as accurate diagnostic tests suggests that either of them can be used with similar results (Table 4.17). Consistence with the findings from previous studies (Nucera *et al.*, 2006; Bagge *et al.*, 2010) indicated that both PCR and API (at the 99.9% likelihood level) were demonstrated to be accurate diagnostic tests for bacteria identification.

The study, conducted by Angelakis *et al.* (2014) compared MALDI-TOF mass spectrometry with PCR. In this study, the researchers considered the molecular biological procedure as an axiomatic procedure to resolve discordant findings. Angelakis *et al.* (2014) also maintain that for isolates not clustered with other bacteria when using MALDI-TOF, PCR amplification and sequencing of the 16S rRNA gene was independently performed to identify the isolates. In addition, De Bruyne *et al.* (2011) indicate that, MALDI-TOF is a valuable screening and rapid identification method. MALDI-TOF has been used in the analysis of different biomolecular for decades, application to microbiological diagnosis seems to be promising (Murray, 2012; Mimica *et al.*, 2013). In this study, since MALDI-TOF identification was not reliable, amplification and sequencing of the 16S rDNA gene was performed in order to obtain accurate results. The advantage of the 16S rDNA gene sequence allows for a superior identification of poorly described and rarely isolated strains (Clarridge, 2004). This molecular technique is routinely used in the food industry (Handschur *et al.*, 2005; Ngoma *et al.*, 2013), microbial ecological studies (Muyzer & Smalla, 1998), clinical

studies (Handschur *et al.*, 2005), for the identification of novel pathogens and uncultured microbes (Ngoma *et al.*, 2013).

Polymerase Chain Reaction (PCR) was used for the detection of pathogens and non pathogen organisms in different kinds of meat. PCR was used as one of the most powerful technologies in molecular biology in this study as a confirmatory test. A 16S DNA primer specific sequence within a DNA template was amplified and all amplicons viewed in agarose gel at a molecular weight of 1kb to verify their strain identification. Sequence information from the 16S rDNA gene of 30 isolates representative was analysed through BLAST (www.ncbi.nlm.nih.gov/BLAST) from the National Centre for Biotechnology Information (NCBI). The closest match from the Gen-Bank database for each of the 30 isolates is reported in Table 4.17. The phylogeny of all isolates based on 16S rRNA gene sequences has been described in detail in previous studies (Baba *et al.*, 2009, Tamura *et al.*, 2011; Ngoma *et al.*, 2013). The phylogenic tree was constructed using 30 isolates along with their closest sequences in the NCBI Gen-Bank and clustered with the neighbour joining method using MEGA 5.2 package (Figure. 4.19). Morphological and biochemical identifications showed a higher rate of strains of *Macrococcus caseolyticus* (60%). Table 4.20 shows the frequency of occurrence of *M. caseolyticus* carriers highest in live (33%) followed by muscle (16.6%) and lastly kidney with (10%).

The various pathogenic organisms acknowledged in this study are among those that cause food-borne illnesses (Mor-Mur & Yuste, 2010; Nyenje *et al.*, 2012; Saulat, 2012; Osibote *et al.*, 2014). The findings of this study concur with results of most researchers (Zakpaa *et al.*, 2009; Ajiboye *et al.*, 2011; Iroha *et al.*, 2011; Bradeeba & Sivakumaar, 2013). Moreover, results obtained in this study are also in line with findings of the study conducted by Ateba & Setona (2011) in which they demonstrated that pathogenic bacteria species isolated from the study were higher in samples obtained from butcheries than supermarket in

mafikeng. This basically reflects the level of hygiene practiced in the two types of settings as acknowledged during the collection of samples.

Despite isolating different organisms, pathogenic organisms such as *Escherichia coli* were isolated. As shown in Table 4.19, the frequency of occurrence of *E. coli* was detected in kidney samples (3%). Some studies have indicated the prevalence of *E. coli* in meat (Haimanot *et al.*, 2010) and this bacterium remain a public concern. The findings of this study concur with previous studies conducted by Effler *et al.* (2001). The study was conducted after a great outbreak of bloody diarrhoea caused by *E. coli* 0157 occurred in Swaziland. This was the first recorded outbreak of *E. coli* 0157 in Africa, in fact, in a developing country. This could compare to the findings reported of *E. coli* from a previous study conducted in Ethiopia in which prevalence was higher 24.0% (Bradeeba & Sivakumaar, 2013). Moreover, the similar results obtained in a study conducted by Ateba, & Setona (2011) in Mafikeng, North West Province, the researchers found *E. coli* (10.0%) in mince meat. Millard & Rockliff (2000) reported the prevalence of 22.2% for *E. coli* in Australia, Iroha *et al.* (2011) reported *E. coli* (8%) in beef in Abakaliki, Ebonyi State Nigeria. Moreover, in a study conducted by Zarei *et al.* (2013) in Ahvaz, South West of Iran, 28% *E. coli* was detected from beef. This is contrary to what was previously reported by Hassan Ali *et al.* (2010) who found 35% of *E. coli* as the first predominant bacterial isolates. In addition Odu & Ameweiye (2013) found 13.3% *E. coli* as the second major bacterial isolates in street-vended-ready-to-eat meat.

E. coli is part of the normal flora in the colon of humans and other animals. However, it can be pathogenic both within and outside the gastrointestinal tract (Feng *et al.*, 2007). Adzitey *et al.* (2010) also maintain that *E. coli* can cause illnesses ranging from gastrointestinal tract related complications such as urinary tract infections, meningitis,

dysentery, diarrhoea to urinary tract infection. The isolation of *E. coli* in this study should be taken as a considerable threat (Mukhopadhyay *et al.*, 1998; Haimanot *et al.*, 2010).

The other pathogenic isolate in this study is *Enteroccoci spp.* which is the third most generally isolated healthcare pathogen (Tyne *et al.*, 2013). As shown in Table 4.18, the frequency of occurrence of *Enterococcus spp.* carriers was highest in kidneys (7%) followed by liver (3%) while *Enterococcus faecalis* carriers were highest in muscles (10%) followed by liver (3%). *Enterococcus mundtii* carriers were isolated in liver (3%). These species are capable of causing a range of infections including sepsis, endocarditis, and urinary tract infections (Maki *et al.*, 1988; Jett *et al.*, 1994). So far aproximatly ninety percent (90%) of *Enterococcal* infections are caused by two species: *E. faecalis* and *E. faecium* (Tyne *et al.*, 2013). Enterococci have been recognised as an important cause of nosocomial infections, the most common cause of this is *E. faecium* and *E. faecalis* (Furtula *et al.*, 2013). Isolation of these organisms should be taken as a considerable threat to the human population.

In this sstudy *Bacillus spp.* were found to be in a very low frequence. Out of 30 isolates, it was detected in one sample (muscles with 3%) as shown in Table 4.19. *Bacillus cereus* is an opportunistic pathogen, one of the causes of food poisoning as shown by the emetic syndrome (Tahmasebi *et al.*, 2014) and very important in public health. Some of the enterotoxins produced by *B. cereus* are known to cause vomiting, nausea, diarrhoea and abdominal cramp (Nortjé *et al.*, 1999; Kapute *et al.*, 2012). It is an opportunistic organism and considered as one of the causes of food poisoning (Tahmasebi *et al.*, 2014). Lund *et al.* (2000) maintain that foods that are rich in protein have been associated with food-borne outbreaks of diarrhoea caused by *B. cereus*.

However, Table 4.19 shows the frequency of occurrence of *Clostridium subterminale*. It was detected in one sample (liver 3%). *C. subterminale* is one of the species of *Clostridium* that is most often isolated from soil (Tappe *et al.*, 2009). Due to its low pathogenicity,

nevertheless, it is very rarely associated with human infections (Miyazaki *et al.*, 2003). In general, in comparing results of this study with other studies, the dissimilarities could be partially due to differences in detection methods, sampling techniques, size and source as well as type of samples. During sample collection and observation of operations of the butcheries selected for the study had similar operating conditions and hygiene practices. Floors were not clean, knives and other cutting tools were handled carelessly, weighing scales were unclean and the two butcheries lacked hand washing facilities. Butchers handled meat and collected money at the same time. Unlike the hygienic condition in the supermarket, where the floor was clean, the cashier did not handle money and protective cloves were in place.

The chain of meat productions from the abattoir to the consumers is regulated South African legislation. Animals are then sent to abattoirs for slaughtering where processes are regulated by the South African Meat Safety Act (Act 40 of 2000) as well as other local and international regulations. The carcases from abattoirs are then transported to butcheries in cold trucks, where they are offloaded. For the safety and hygenien of meat, the South African Regulation 918 of 30 July 1999 framed under the Health Act, 1997 (Act no. 63 of 1977), National Health Act 61 of 2003 and the Meat Safety Act 2000 (Act no. 40 of 2000) clearly reflect and stipulate that all food specified under the regulation and act must be kept at a low temperature (4°C) during storage, transportation and while on display. Regarding microbial standards, regulations governing microbiological standards for foodstuffs and related matters are governed under the foodstuffs, cosmetics and disinfectants Act, 1972 (Act No. 54 of 1972).

5.3 ANTIMICROBIAL SUSCEPTIBILITY PROFILE

Antimicrobial susceptibility patterns of isolated strains were also conducted on isolated pathogenic bacteria. Resistance of bacterial isolates to a series of offered antibiotics

of these isolates was usually observed. The problem may be the use of sub-therapeutic doses of antibiotics in animal feeds to improve animal productivity, which could also be used to select resistant strains (Khan *et al.*, 2009) and also the natural resistance of species to certain antibiotics (Wong, 1998). Microbial populations develop resistance to antimicrobials through several mechanisms. The rate at which an individual gene mutates to express an antimicrobial resistance phenotype is a complex phenomenon in which cell physiology, environment, bacterial genetics, and population dynamics all play roles (Martinez & Baquero, 2000). Furthermore, for full resistance to occur, mutations must develop within multiple genes because of genetic redundancy in the antimicrobial targets. A primary example is the fluoroquinolone target gene gyrA, gyrB, parA, and parC that are all targets for the fluoroquinolone antimicrobial (Martinez & Baquero, 2000). The frequency with which bacteria acquire DNA from the environment depends on several factors including cell wall structure and bacterial species with transfer frequencies being as low as 10−7 (Elkins *et al.*, 1991). Bacteria can also exchange and acquire genetic material through conjugation of self replicating extra chromosomal DNA or plasmids. This requires physical contact between cells, which allows the plasmids to be exchanged between donor and recipient cells (Roe & Pillai, 2003).

The antibiotics used in this study were selected because they have been able to show great resistance to majority of isolates (Ateba *et al.*, 2008; Moneoang *et al.*, 2009; Ateba *et al.*, 2010; Ndeddy & Babalola, 2011). Furthermore, they are commonly used as veterinary drugs, and widely used to treat and prevent infections such as gastrointestinal and respiratory tract infection. Furthermore, they are used to promote the growth of livestock (Botsoglou & Fletouris, 2001; Mahmoud *et al.*, 2014). Since antibiotics are used for the prevention or treatment of the diseases in humans, the constant use of these antibiotics may lead to the acquisition of microbial resistance and thus be a public health thread. There is therefore a

need to determine the level of antibiotic resistance among bacterial isolates (Ateba *et al.*, 2008).

A total of 30 isolates were analysed to determine antimicrobial resistance profiles using breakpoints from the Clinical Laboratory Standards Institute (CLSI). The results obtained in this study revealed that 83.3% of isolates were more resistant to be of tested drug. However 56.5% of this resistance was for the non-pathogenic *Macrococcus caseolyticus*, which is an indication of the low level of resistance on pathogenic organism. Nonetheless, this varies with the findings of a study conducted by Enayat *et al.* (2012) in Iran. It was reported in the study that 26% of isolates were resistant to tetracycline. Results obtained in this study are in line with a similar study conducted by Smith *et al.* (2007) on antimicrobial resistance of *E. coli* which showed resistance to tetracycline. However, *E. coli* did not show any resistance to ciprofloxacin (Hassanein Easa, 2010), an indication that, ciprofloxacin is one of drugs of choice for *E. coli* infection. Moreover, tetracycline was found to be the most resistant for various pathogens such as *Bacillus spp.*, *E. coli* and *Enterococcus spp.* In summary a large number of isolates were resistant to tetracycline (Table 4.21). Resistance to tetracycline is highly associated with the acquisition and expression of efflux pumps that reduce toxic levels of the drug in bacterial cells (Bilatu, 2012). In most isolates, tetracycline resistance efflux pumps are encoded by the *tet* genes (Bush *et al.*, 2003).

Results obtained in this study, correlate with findings of a study conducted by Darwish *et al.* (2013) as shown in Figure 2.1; that tetracyclines constitutes 41.17% of antibiotic distributed in African countries. Tetracyclines are used for the prevention of disease as well as the promotion of growth in different animal production processes (Dang *et al.*, 2013). It is not surprising to find tetracycline to be the most resistant in this study. A survey conducted by Van Vuuren *et al.* (2007), revealed high levels of resistance for *Enterococcus* and *E. coli*. *E. coli* showed a 67% resistance to one or more commonly used antimicrobials, particularly

fluoroquinolones, tetracyclines and sulphonamides. Moreover, the survey showed that the resistance level of *Enterococcus* isolates were particularly high for tetracyclines and sulphonamides. The prevalence of *Enterococcus spp.* and *E. coli* isolates resistant to tetracycline in this study can moderately cheaper on the market which is of concern because of the limited access and high cost of antibiotics such as quinolones (Bedada & Zewde, 2012). Mesgari *et al.* (2009) reported that, due to enterohepatic circulation, a small amount of administrated dosage of tetracycline may persist in the body for an extended time after administration. Therefore, microorganisms can produce a gene which will lead to resistance to certain antibiotics such as tetracycline. Only 10% of the isolates were resistance to sulphonamide. The slow metabolism noted on sulphonamides is linked to the production of new genes for microorganisms to become resistant to this antibiotic (Sulejmani *et al.*, 2012).

Quinolones are mostly used to prevent and treat diseases due to their broad spectrum against both Gram positive and Gram negative microorganisms (Dang *et al.*, 2013). Results obtained in this study reveal a low prevalent of resistance to quinolones (3.4%). This is in line with a study conducted by Darwish *et al.* (2013) on antibiotic residues in African countries as shown in Figure 2.1. This study is different from the one conducted by Hassan Ali *et al.* (2010) where there was no quinolones (ciprofloxacin) resistant pathogen isolated. A study conducted by Gales *et al.* (2002) found that *E. coli* isolates exhibited high ciprofloxacin resistance rates (nearly 20%), which is contrary to the results obtained in this study.

Antimicrobial resistant bacteria may be present in animals and in food that are free of antimicrobial residues (Report of the Scientific Committee of the Food Safety Authority of Ireland, 2015). Despite the resistance of other antibiotics from different isolates, all isolates in this study were susceptible to streptomycin. This is a relief to human beings as streptomycin is well-known as anti-tuberculosis activity (Ishii *et al.*, 2008; Unusan, 2009). However, results obtained in this study differ from other studies conducted by Bilatu (2012) in Addis

Ababa, Ethiopia where 58% of streptomycin were found in raw and ready-to-eat meat. Thong & Modarressi (2011) also found 58% of isolates resistant to streptomycin. The greater the number of resistant bacteria in different meat organs, higher is the possibility for the transfer of the encoding resistant genes to pathogenic bacteria and their distribution in the environment and foods of animal origin. The acquisition of bacteria resistant to aminoglycoside antibiotics is associated with modification of enzymes such as aminoglycoside adenyltransferases which are responsible for resistant streptomycin antibiotics (Poole, 2005). The differences in results might be the differences in the methods of analysis employed the number of sampling, the differences in sampling climate/locations and the quality of hygienic conditions in the processing plant.

As shows in Table 4.23, the some strains were multi-resistance and their relationship between the specific residue concentrations. Sample identity M3W (liver), M91 (liver) and M17 (muscle) were collected from butchery 1 while sample identity M70 (muscle) and M4 (muscle) were collected from butchery 2. All these samples were from butcheries, this is an indication that contamination was highly in butcheries regarding to isolates. Different prevalence rates of multidrug resistant bacteria have been found in raw meat from different animal species, and bacterial contamination rates also differ between supermarkets and wet markets (Mathew et al., 2007). Big supermarkets and retailers have professional food technologists (Business Monitor International, South Africa Food and Drink Report Q2 2012). This multi-resistance shows a link to multi samples residues; this might bring more concern to public health. From the resistance perspective, the resistance of this isolates might be in two factors, contamination from the meat (Bhandare *et al.*, 2007) or contamination from the environment/handling (Duffy *et al.*, 1999; Hassan Ali, *et al.*, 2010).

In this study, samples were detected for antibiotic residues and for antibiotic resistance. For stastical analysis regarding correlation between residues and resistance,

among four antimicrobials, only tetracycline showed a strong correlation ($p<0.05$) between residues and resistance from two species; chicken (1.00) and beef (1.00) as shown in Table 4.24. Tetracycline is The second largest group of antimicrobials sold in South Africa (16.7% of the total volume sold), as they are broad-spectrum antimicrobials, active against some species such as *Mycoplasma* spp. and *Chlamydophila spp.* and are also effective against erhlichias, rickettsias and anaplasmas (Eagar, 2008). Moreover, Jonker & Picard (2010) found that, tetracyclines are broadly used in the poultry industry in South Africa, as they are broad-spectrum in activity, cheap and can easily be administered in food and water.

This study also revealed that few samples (4/150) (Table 4.11) had more than one antibiotic residues. This results correlate whith the one of Ibrhim *et al.* (2009) who in theior study in Nigeria beefs also obtained similar results. These results also correlate with the data obtained in this study on antimicrobial resistance where some bacteria revealed multi antibiotic resistance (Table 4.23). These results are also confirmed by the study of Ibrahim e*t al.* (2009).

CHAPTER SIX

6.0 CONCLUSION

The main objective of this study was to determine antimicrobial residues in meat from butcheries and supermarkets around Mafikeng with regard to the occurrence of antimicrobial residues and the health risks associated with exposure to such residues. To reach the above-mentioned objective, meat samples (pigs, chicken and beef) were collected from butcheries and super market in Mafikeng and analysed using ELISA and chromatographic methods such as TLC and HPLC. All samples confirmed using HPLC for ciprofloxacin, streptomycin, tetracycline and sulphonamides detected in beef, chicken and pork meat were lower than the international MRL standards. There were no samples above MRL as prescribed by the European Union, and South Africa in terms of the maximum limits for veterinary medicine and stock remedy residues and the Codex Alimentarius Commission. However, studies have shown that chronic exposures to low doses of antibiotics might induce antimicrobial resistance. According to these limitations, low levels (lower than the standards of MRL) of antimicrobial residues in pork, chicken and beef meat is not a surprising but a good result. These results are valid for the samples obtained during in the study. The results may change periodically. Therefore, the steps in food processing should be kept under continuous monitoring for preventing over expressed levels of these antimicrobial residues.

Significant statitstical correlations ($P \geq 0.05$) between different methods (TLC, ELISA HPLC) used as well as among sample types (muscles, liver and kidney), and species (beef, chicken and pork) were observed. This showed the regularity, consistancy repeatability and quality of techniques used in this study. In addition the relaibility of results obtained in this study is shown by the recovery rate as well as by the calibration curves of each antibiotic and for each method used. The novelity of this work is that three different techniques (TLC, ELISA and HPLC) were used to analyse samples and results obtained are generally in correlation. Even

though the results obtained in this study are below the MRL, there is a need to respect the withdrawal periods of antimicrobial in order to obtain low/absence of antimicrobial residues and also the reinforce controls through regular sampling and analysis control order to reduce consumers exposure health risk.

Furthermore, confirmed positive meat samples for residues were cultured for bacterial isolation and tested for antimicrobial resistance. The identification of isolates was performed using the conventional biochemical test, as well as molecular methods based on 16S rRNA species specific gene amplification by PCR. This study revealed that PCR is a very useful tool for the detection of pathogens and non-pathogenic bacteria. The method represents a rapid and cost effective way for the detection of these pathogens and non-pathogenic bacteria in different kinds of meat. The relatively high frequency of occurrence of pathogenic organisms; *Enterococcus mundtii*, *Bacillus spp.*, *Bacillus cereus*, *Enterococcus spp.* and *Escherichia spp.* and pathogenic organisms observed in this study are among the causes of food-borne illnesses and danger to public health. However, their prevalence was very low; *Enterococcus faecalis* 4 (13%), *Enterococcus mundtii* 1 (3.3%), *Bacillus spp.* 1 (3.3%), *Bacillus cereus* 1 (3.3%), *Clostridium subterminale* 1 (3.3%), *Enterococcus spp.* 3 (10%), *Citrobacter freundii* 1 (3.3%) and *Escherichia spp.* 1 (3.3%).

The other objective of this study was to evaluate microbial susceptibility to different antibiotics commonly used in veterinary medicine. The results obtained from the exercise revealed that of all the isolates, eight of them showed resistance to antibiotics rates for *Macrococcus caseolyticus*, *Enterococcus spp.*, *Enterococcus faecalis*, *Enterococcus mundtii*, *Escherichia spp.*, *Bacillus spp.* and *Bacillus cereus*. Most of these isolates were resistant to tetracycline at 83.3%. However, 56.5% of this resistance was for a non-pathogenic *Macrococcus caseolyticus*, which is an indication low level of resistance on pathogenic organism then followed by sulphonamides (13.3%) and lastly ciprofloxacin (3.33%).

Continuous exposure at low doses might induce mutability in both animals and consumers. There was only one antimicrobial (tetracycline) which showed a strong correlation ($p<0.05$) between residues and resistance from two species; chicken (1.00) and beef (1.00). The presence of some of these bacteria in meat poses a health risk to consumers especially in immune compromised, elderly and young children if not properly prepared (Sofos, 2008). Laboratory reports of resistance frequently result from treatment failures rather than treatment successes, as animals that have been treated previously will be more probable to yield resistant bacteria than those that have not (Blunt *et al.*, 2013). It was observed that more bacteria were isolated from the butcheries than supermarket. Some isolates showed resistance to multiple antibiotics. The results for this antimicrobial resistant might be the natural progression in the resistance development from general use over a number of years and also the integrone gene sequencing (Stokes & hall, 1989). Moreover, the antimicrobial resistant observed with some of the microorganism might also be explained by long exposure to low doses or over used of these antibiotics by animal health petitioners and veterinaries in order to treat or prevent diseases in animals. This leads to resistance induction by mutagenesis. A comprehensive residue testing programmed should performed on all animal products for export as well as for domestic consumption.

Limitations of the study

- Assessment of kidney from chicken samples was not done in this study because not accessible from butcheries.
- Not measuring the other quinolones such as enrofloxacin may also be accounted as the other limitations of this study
- The sampling should have been done at the source of the abattoir and not a commercial environment
- Detection of a highly used group of antibiotics in South Africa such as macrolides
- Bacteria that exhibit antimicrobial resistance will continuously evolve

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