Addis Ababa University

College of Natural Sciences

Center for Food Science and Nutrition

A thesis submitted to Addis Ababa University in partial fulfillment of the requirement for MSc degree in Food Science and Nutrition

Title: Determination of the level of oxytetracycline residues in chicken meat from one commercial poultry farm in Bishoftu town

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Declaration

I hereby declare that this research report being presented for MSc. degree in Food Science and Nutrition has not been previously submitted for other degree at this or any other university nor is it being currently submitted for any other degree.

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Approval

This thesis of Shimelis Mamo is approved in partial fulfillment of requirement for the degree of Master of Science (MSc.) in Food Science and Nutrition, Submitted to Addis Ababa University.

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Abstract

Antibiotics are used in poultry farms for treatment of diseases, prevention of diseases and to increase the growth-rate and productivity of the chicken. Oxytetracycline (OTC) is one of the tetracycline (TCs) broad-spectrum antibiotics widely used in poultry farms. However, improper use of OTC with excessive dose, improper withdrawal time, age and health chickens and pharmacokinetics of antibiotics can lead to residue formation in chicken meat that can be harmful to consumers in the form of developing drug resistant pathogens, allergy, carcinogenic, mutagenic teratogenic and bones and teeth discolouration.

In Ethiopia there are no formal control mechanisms and rules to protect consumers from these antibiotics residues in the chicken meat products. The aim of the study is to determine the level of the OTC residue in chicken meat in the slaughterhouses in Bishoftu town and evaluating the knowledge, attitude and practice of the workers of the poultry farm.

About 2g of sample was taken from each meat (leg muscle, breast muscle and gizzard) and cut into smaller pieces by knife on clean plastic board separately. Each cut meat sample was homogenized by *IKA T-25 ULTRA TURRAX® Digital Homogenizer* with disperser set at 15rpm for 5 minutes. Then to extract 10ml sample solution, 2g of homogenized meat sample was placed in 15 ml plastic centrifuge test tube and to this sample 0.1g citric acid, 1ml of nitric acid (30%), 4ml of HPLC grade methanol and 5ml of deionized water were added respectively.

The suspension with solid particles was vortexed and then kept in an ultrasonic bath for 15 minutes. The sample was centrifuged for 10 minutes at 5300 rpm and 10ml supernatant was decanted into a clean 15ml test tube and then it was filtered on 0.45µm nylon micro filter. Clean solution was transferred into 1.5ml HPLC autosampler amber vials. Finally 20µl of the solution was injected into HPLC for analysis.

The analyzed meat samples of 30 chickens showed that all were at non-detectable level for the OTC residue. There is also knowledge gap among the workers of the farm. The finding of the study shows that the chicken meat samples collected for the analysis from the poultry farm do not pose risk to consumers. However, unless corrective measures on KAP are put in place, there is a potential risk that OTC residue may reach consumers.

Key words: Oxytetracycline; chicken meat; residue; HPLC; analysis

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Abbreviations and Acronyms

ADI………… Acceptable Daily Intake

CAC…………Codex Alimentarius Commission

CFR................Code of Federal Regulations

CFIA………...Canadian Food Inspection Agency

CFU/g……… Colony form unit per gram

CRLs ……….Community Reference Laboratories

TC…………..Chlortetracycline

DAD ………..Diode array detector

DC…………..Doxycycline

DNA………...Deoxyribonucleic acid

EC…………..European Community

ELISA……… Enzyme linked immunsorbent assay

ELU………...Extra-label Drug Use

EU…………..European Union

FAO………...Food and Agriculture Organization

USFDA……..United State Food and Drug Administration

FDA-CVM….Food and Drug administration Center for Veterinary Medicine

GC……….....Gas chromatography

GIT………….Gastro intestinal tract

HPLC ……….High pressure liquid chromatography

IgE…………..Immunoglobulin E

KAP…………Knowledge, Attitude, Practice

LCM SMS…..Liquid chromatography- mass spectrometry

LOD…………Limit of detection

LOQ…………Limit of quantification

MOH………..Ministry of Health

MRL………...Maximum residue limit

nm…………...Nano meter

OTC….……...Oxytetracycline

ppm………… Parts per million

RNA………...Ribonucleic acid

RSD…………Relative standard deviation

SPE …………Solid phase extraction

SD ……… Standard Deviation

TC…………...Tetracycline

TLC…….…...Thin liquid chromatography

UV ………….Ultra violet

v/v…………..Volume-volume ratio

WHO...............World Health Organization

μg/g………… Microgram per gram

μl…………......Microliter

Chapter One

1. Introduction

1.1 Background

The world poultry population is estimated to be about 16.2 billion, with 71.6% in developing countries, producing 67,718,544 metric tons of chicken meat and 57,861,747 metric tons of hen eggs (Raney *et al.*, 2009). In Africa, village poultry contributes over 70% of poultry products and 20% of animal protein intake. In East Africa, over 80% of human population in rural areas and over 75% of these households keeps indigenous chicken and Ethiopia is no exception to this situation (Kitalyi *et al.*, 1998; Bushra, 2012).

Chicken is the most common type of poultry in the world. The term broiler is applied to chickens that are especially bred for meat; they grow rapidly. Chickens farmed for eggs are called egg-laying hens or layers. The worldwide commercial poultry industry is the largest supplier of animal protein in the form of meat and eggs. Its significance is even greater in the developing countries, usually providing both protein and income for small families (Raney *et al.*, 2009; Kitalyi *et al.*, 1998).

Over the past few decades poultry industry has gone through tremendous growth, however, with the increase in production, the use of certain drugs and feed additives has become crucial in order to prevent diseases, treatment and growth promotion (Chapman and Johnson, 2002;Mumtaz *et al.*, 2000).

Different studies stated that by-products of antibiotic production, which contain a high level of vitamin B12, when fed to poultry animals resulted in higher growth. Eventually, it was discovered that the trace amounts of antibiotics remaining in these byproducts accounted for this growth. Since then antibiotics have been used on poultry in large quantities to enhance production (Chapman and Johnson, 2002; Mumtaz *et al.*, 2000; WHO, 2000).

Antibiotics are substances that can destroy or inhibit the growth of microorganisms. They are widely used in the prevention and treatment of infectious diseases. They are therapeutically used to protect the health and welfare of humans and animals. Some antibiotics are produced by microorganisms but most of them are now manufactured synthetically. The term antibiotic originally referred to any agent with biological activity against living organisms; however, ‘‘antibiotic” now refers to substances with antibacterial, anti-fungal, or anti-parasitical activity (Prescott *et al.*, 2002; Kemper, 2008).

However, one of the drawbacks of using antimicrobial drugs is that they get accumulated in tissues and organs of treated animals as residues and eventually become part of the food pyramid and thus transferring to humans through food and the environment. Excessive use of antibiotics thus resulted in the emergence of bacterial resistance (Jallailudeen *et al*., 2015; Mitchell, 2000; Donoghue, 2003; Nisha, 2008)

To prevent any residues of antibiotics in food and food products of animal origin, withdrawal time for different antibiotics is determined by different regulatory agencies. Withdrawal time is labeled on package of each type of antibiotics that are used for animals. The dose of antibiotics given to animals at a time is also prescribed and therefore excessive usage has been recognized as illegal and prohibited by the food regulatory agencies and health authorities. Because improper withdrawal time and overdose can cause antibiotics residues in food of animal origin which poses adverse human health effect and develop antibiotic resistant pathogens (EC, 2001; Apley, 2003).

1.2. Statement of the problem

A study of the quality of veterinary antibiotics in most African countries revealed that almost 61% of veterinary antibiotics do not comply with international standards. The quality affects pharmacokinetic and this is one of the reasons for antibiotics residue in animal origin food (Grasswitz *et al*., 2004).

In Ethiopia, the control of antibiotics by government authorities and information on the actual use of veterinary antibiotics is very limited. Tetracyclines are one of the commonest antibiotics used in poultry farms in the country (Habte *et al.,* 2017). But chicken meat consumed in the country is not checked for the presence of residues in most poultry farms. Similarly, no legal control mechanisms exist to protect the consumers against the consumption of chicken meat containing tetracyclines residues in the country (Beyene *et al*., 2015).

To our knowledge no study has been done on edible tissues of chicken to determine the antibiotics residue levels including tetracyclines in Ethiopia. Therefore, the present study is designed to check the level of oxytetracycline (OTC) residue in chicken meat from slaughterhouse in Bishoftu.

1.3. Significance of the study

This study will produce information on the status of the OTC residue in chicken meat consumed in Ethiopia. The information is useful in formulating strategic regulation to address the level of antibiotics residue in chicken meat. The finding from this study will contribute to knowledge on antibiotics residue in food of animal origin. The knowledge will be useful to the Ministry of Health (MOH), poultry industries and other organizations that want to design regulatory program in poultry industries in Ethiopia.

1.4. Objectives

1.4.1. General objective

The main objective of this study is to determine the safety of oxytetracycline residue level in chicken meat from slaughterhouse in Bishoftu and state the awareness of farm workers about antibiotics residues.

1.4.2. Specific objectives

- To determine Knowledge, Attitude and Practice (KAP) of the people engaged in the poultry farm on antibiotics residue in chicken meat.
- To detect and quantify the OTC residues in chicken muscles meat and gizzard of slaughterhouse in Bishoftu.
- To determine safety by comparing the result obtained against the Maximum Residue Limit (MRL) for OTC recommended by different countries and organizations

Chapter Two

2. Review of literature

2.1. Major classes of antibiotics used in poultry farms

There are many different classes of antibiotics which differ either in their chemical structures or the mechanism they act on microorganism. Antibiotics which have the same molecule may have different function depending on the pH conditions they act in. Antibiotics categorized as Quinolones, Aminoglycosides, β-Lactams, Tetracyclines, Sulfonamides, Bambermycin, Glycopeptides, Ionophores, Linocosamides, Macrolides, Polypeptides and Streptogramins (Jacob, 2015). A few of the major categories of antibiotics are described as follows.

Quinolones *e.g.* Fluorquinolones have fluorine attached to the central rings system. Fluoroquinolones are types of synthetic antimicrobial drugs which are active against both gram-positive and gram-negative bacteria. They are easily absorbed into cells and are used to treat intracellular pathogens. The representative's examples of fluoroquinolones which are used to treat intracellular pathogens are *Enrofloxacin, Ciprofloxacin* and *Norfloxacin*. Fluoroquinolones are classified as 'critically important in human medicine' by the WHO, due to their importance for treating infectious diseases caused by *Campylobacter, Salmonella* and *E. coli* (WHO, 2009; Jacob, 2015).

Aminoglycosides are antibiotics which consist of an aminocyclitol ring connected to many amino sugar linked through a glycoside link. Aminoglycosides are derived from bacteria of the genus *Streptomyces* and *Micromonospora*. Aminoglycosides are associated with a post antibiotic effect in a number of bacteria, principally gram-negative (*e.g.*, *E. coli*, *Klebsiellap neumoniae*) (Guardabassi *et al.*, 2008; Benveniste and Davis, 1973; Mingeot-Leclercq *et al.*, 1999).

Beta-Lactams are antibiotics that contain a β-lactam ring nucleus with a hetero atomic ring structure. They consist of three carbon atoms and one nitrogen atom (e.g. Penicillin, Ampicillin, Cloxacillin, Amoxacillin). Beta-Lactamantibiotics are active against gram-negative and gram-positive bacteria effectively (Jacob, 2015; Guardabassi *et al.*, 2008)

Tetracyclines are a group of broad spectrum antibiotics with four hydrocarbon rings. They are an important group of antibiotic agents used in humans and veterinary medicine. All tetracyclines are active against gram-positives and gram-negatives bacteria. They interfere with the bacterial protein synthesis in rapidly growing and reproducing bacterial cells and inhibit their metabolism.

Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC) are the most commonly used tetracyclines compounds. Oxytetracyclineis four hydrocarbon ring is shown in Fig.2.1 (Chopra and Roberts, 2001).

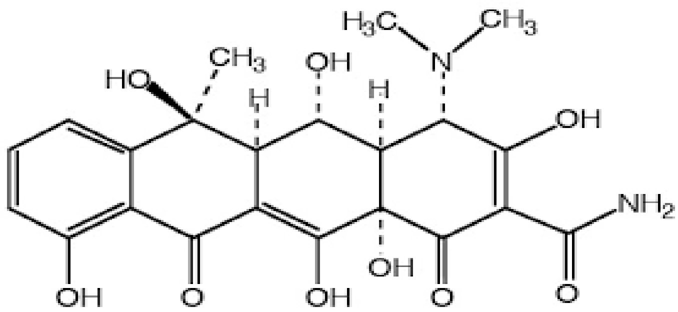

Fig.2.1 Structural formula of oxytetracycline

Sulfonamides are antibiotics that contain sulfur amides on its rings. They have bacteriostatic activity against a broad spectrum of pathogens. They interfere with RNA and DNA, which are necessary for cell growth and replication. Sulfonamides, such as trimethoprim, are effective against *Staphylococcus* species, *Streptococcus* species, *Pasteurella*, *Salmonella*, and *E.coli* (Jacob, 2015).

2.2. Uses of antibiotics in poultry farming

Antibiotics are used in poultry farming as:

Therapeutic Agents (Treatment of disease). Infected poultry receive a course of antibiotics, which usually involves high doses for a relatively short period of time (Al-Mastafa and Al-Ghamdi, 2002; Riviere and Papich, 2009).

Prophylactic Agents (For prevention of disease). This involves sub-therapeutic doses of antibiotics to poultry with feed or drinking water, when signs and symptoms of infection are absent but suspected. Antibiotics are given periodically for several days during the life cycle of the chicken (Riviere and Papich, 2009).

Growth promoters (to increase growth-rate and productivity). The use of growth promoters is characterized by administration of very low-dose of antibiotics on a regular basis, mostly over a lifetime of the food-producing animal and given through feed and water. This is distinguished from therapeutic and prophylactic antibiotic use, which is delivered at relatively higher doses and generally administered with feed and water. Growth promoter antibiotics are known to suppress the gut bacteria leaving more nutrients for chicken to be absorbed for greater weight gain (Dibner and Richards, 2005).

2.3. Withdrawal time for antibiotics

Withdrawal time is the time required for the residue of toxicological concern to deplete or reduce to safe concentration in meats, eggs, organs, or other edible products of food animals or the time between the last dose given to the animal and the time when the concentration of residues in animal edible products is lower than or equal to the maximum residue limit (MRL) (FDA-CVM, 2006). It is also referred to as the interval from the time an animal is removed from medication until the permitted time of slaughter (Löhren *et al*., 2009).

This interval is required to minimize levels of drug residues in edible tissues for human consumption rather than working for the health of the animal. Withdrawal time intervals vary with each drug preparation and among the different animal species. Depending on the drug product, dosage form, and route of administration, the withdrawal time may vary from a few hours to several days or weeks (Apley, 2003).

Withdrawal of antibiotics is necessary to avoid drug residue above tolerance level in chicken meat marketed for human consumption to prevent humans from unnecessary exposure to antimicrobials residue (Hsu, 2008). Whenever antibiotics are produced and given to food producing animals, the veterinarian has to give attention to the necessity of preventing animals from market or slaughterhouse during the treatment period. From a public health welfare standpoint, veterinarians have the responsibility of controlling the proper use of drug they prescribe or use in food animals (Löhren *et al*., 2009).

2.4. Some commonly used antimicrobials in poultry farms in Ethiopia

According to assessment on chemicals and drugs residue in 42 poultry farms in Bishoftu and Modjo towns, all of them used oxytetracycline for treatment of diseases and as growth promoter and 71.4% of them used amoxicillin as prophylaxis and/or control of bacterial infections, amprollium (100%) and sulfa drugs (26.3%) are also used for the treatment of coccidiosis and piperazine (31.0%), albendazole (9.1%) and tetramisole (7.1%) to control endoparasites (Beyene *et al*., 2015)

Therefore, the antimicrobials in use in poultry farms around Bishoftu and Modjo are tetracyclines, beta-lactams, quinolones and sulfonamides with oxytetracycline being used in all farms. The tetracyclines are the most widely used antimicrobials in poultry industry (Chopra and Roberts, 2001). This is largely due to their affordability, a wide margin of safety and broad-spectrum (Mycoplasma, gram-positive and gram-negative bacteria) and intracellular activity. It is also easily administered to a mass in either feed or water (Chopra and Roberts, 2001).

Tetracyclines also acts against some pathogenic agents unaffected by other antibiotics *e.g.* rickettsia in animals and lymphgranuloma venerurn group in humans. Tetracyclines are reversibly bound to plasma membrane proteins and are widely distributed and diffuse throughout the animals' body but are found in highest concentrations in kidney, liver, spleen and lungs (Michalova *et al.*, 2004).

2.5. Regulations for Tetracyclines residue in chicken meat

Tetracyclines (TCs) are commonly used for the treatment and control of bacterial infections in poultry farms. As a feed additive in sub-therapeutic doses, it contributes to the maintenance of optimal health and thus promotes growth in food animals (Dibner and Richards, 2005). But the use of this compound will result in residues in chicken derived food products. These residues pose a health threat to consumers. For this reason, regulatory agencies have established maximum legal tolerance levels for this drug in animal derived food products (Chopra and Roberts, 2001).Different countries have established their own maximum legal tolerance levels for tetracyclines (oxytetracycline, chlortetracycline and doxycycline) in both domestic and export chicken meat as shown on Table 2.1. But most countries have adopted the acceptable Maximum Residue Limit (MRL) for tetracyclines recommended by the joint FAO/WHO Expert Committee on food additive (WHO, 2009) such as Canada (Health Canada, 2013).

	EU (ppm)	WHO and Health Canada(ppm)	USFDA (ppm)
Oxytetracycline	0.1 in muscle 0.3 in liver	0.2 in muscle 0.6 in liver 1.2 in kidney	2 in muscle 6 in liver 12 in kidney
Chlortetracycline	0.1 in muscle 0.3 in liver 0.6 in kidney	0.2 in muscle 0.6 in liver 1.2 in kidney	2 in muscle 6 in liver 12 in kidney
Doxycycline	0.1 in muscle 0.3 in liver 0.6 in kidney		

Table 2.1.Maximum residue limits of various tetracyclines for poultry meat set by various regulatory agencies (CAC, 2014; FDA, 2012; EU, 2009).

2.6. Screening methods of tetracyclines residue in meat

A screening method is defined as the first procedure that is applied to sample analyses. The purpose is to assure the presence or absence of veterinary drug (OTC) residue. This procedure should be as simple as possible (Aerts *et al*., 1995; CRLs, 2014). The following 3 methods of screening are mostly used:

Biological methods: detect cellular responses to antibiotic residue (e.g. inhibition of bacterial growth). These methods are not selective and can cover several chemical classes of active antibiotics. They do not allow the identification of individual antibiotics such as oxytetracycline, tetracycline, chlortetracycline and doxycycline.

Biochemical methods: detect molecular interactions (e.g. antigens, proteins) between antibiotics and antibodies or receptor proteins (e.g. ELISA), chemical labeling of either the antibiotics or antibody/receptor allows the interaction to be monitored and measured. These methods are either selective for a family of antibiotics having related molecular structures or are sometimes antibiotics specific.

Physicochemical methods: used to distinguish the chemical structure and molecular characteristics of antibiotics by separation of molecules (e.g. TLC, GC, HPLC) and the detection of signals related to molecular characteristics (e.g. UV, DAD, *etc.*). They are able to distinguish between similar molecular structures and allow the simultaneous analysis of several antibiotics. This method was used to detect OTC in this study by using HPLC with UV-detector.

2.7. Factors responsible for oxytetracycline residue in chicken meat

There have been public health concerns on the antibiotic residues in poultry edible tissues and products like meat and egg and the attendant adverse effects of these residues on the public, who directly consume these products (Weaver, 1992; Kaneene and Miller, 1997). A number of factors may be responsible for the persistence of OTC residues in chicken meat.

2.7.1. Extra-label drug use (ELU)

Extra-label Drug Use (ELU) refers to the use of drug other than the approved label directions. ELU occur when a drug only approved for human use is used in animals, when a drug approved for one species of animal is used in another, when a drug is used to treat a condition for which it was not approved, or the use of drugs at levels in excess of recommended dosages (Schwark, 2014; Martin-Jimene *et al*., 1997).

2.7.2. Improper withdrawal time

The withdrawal time (also known as the depletion or clearance period) is the time required for the residue of toxicological concern to reach a safe concentration as defined by WHO (2009).Different antibiotics have their own withdrawal time, so if breeder do not see their withdraw time while they are used, it can be lead to develop antibiotics residues in edible tissue of animals (Schwark, 2014).

2.7.3. Pharmacokinetics

Pharmacokinetics is the movement of drug into, through and out of the body: the time course of its absorption, bioavailability, distribution, metabolism, and excretion (Boothe and Reevers, 2012).

Absorption is influenced by many factors such as lipophilic property of drugs, route of administration and the health state of the animal. The bioavailability of the drugs is affected by route of administration, the drug properties and the feed ingredient which the animals feed on.

Distribution is the process whereby a drug is transported to the tissues and organs. There are four major factors that affect rate of distribution. These are the properties of the drug, the concentration gradient established between the blood and tissue, the ratio of blood flow to tissue mass, and the affinity of the drug for tissue constituents and serum protein binding (Riviere, 2017).

Age: Pharmacokinetic can also be affected by age of animals (Riviere, 2017). The ability and rate of chicken to remove antibiotic from their bodies, to a lesser extent, depend on their age. In younger aged animals, the removal rate of antibiotics is slower because of the immaturity of the drug clearance system (Schwark, 2014). So age may be a factor for the residue of antibiotics in chicken muscles.

Feeding: in some cases, the rate of clearance of antibiotics from bodies of animals depends on the food they eat because the diet can affect the bioavailability of drugs (Bushra *et al*., 2011). Different studies conducted to determine the effects of diet content on the bioavailability of orally administered antimicrobial drugs to animals showed that different ingredients of feeds have different effect on the bioavailability of the drug. Tetracyclines have a high affinity for metallic ions such as calcium, iron, magnesium and zinc (Ziołkowski *et al*., 2016) which will impair absorption if present in feed or in the digestive system. Thus, the drugs stay in the gut and are released with the feces. The actual gut contents (type of feed in the gut regardless of mineral content) can also affect drug uptake and pharmacodynamics (Weaver, 1992; Kaneene and Miller, 1997).

Disease status: diseases can affect the pharmacokinetics of drugs administered, which can increase the potential for persistence of residues (Boothe and Reevers, 2012). This could occur when the disease affects the metabolic system of an animal or when the presence of disease causes the drug to accumulate in affected tissues. The main diseases that cause the development of antibiotics residue in animal are the liver and kidneys diseases which affect the metabolic and excretion function of the animals (Schwark, 2014).

Biotransformation is the principal mechanism of elimination by the transformation of drugs into metabolites through chemical reaction. Liver plays an extremely important role in the metabolism of drugs that are foreign to the body, some of which are toxic. The liver converts drugs into hydrophilic compounds by using processes including oxidation, reduction, hydrolysis, hydration and isomerization reactions and finally conjugate the drug to substrate such as glucuronic acid, sulfate, acetate and methyl group. The liver can also change the drug into inactive form and easy to excrete but some drugs may be converted to an active form (Boothe and Reevers, 2012).

Excretionis the process by which the parent drug or its metabolites are removed from the body fluids. The kidney is the most important site of drug excretion. Renal insufficiency usually significantly affects drug excretion. Although most compounds are excreted primarily by the kidneys, some drugs are partially or completely excreted through the bile.

It has been reported that there is an extensive species variation among animals in their general ability to excrete drugs in the bile; example, chicken are characterized as good biliary excretes (Serrano *et al*., 1999).

2.8. Toxicological and public health hazards of consuming poultry tissues with oxytetracycline residue

It is estimated that approximately 3kg chicken meat is consumed on average by each individual in Addis Ababa in a year (Tadelle *et al.,* 2003). Thus such types of food of animal origin must be safe and wholesome for human consumption. OTC residues in foods of animal origin (eggs, meat and milk) that are used for human consumption are one of the sources of concern among public and medical health professionals (Jallailudeen *et al*., 2015; Nisha, 2008).

Human beings are the ultimate consumer of this antibiotic residue in these products which cause significant health problems that might be toxicological, microbiological or immunological (Smith *et al.,* 2007).

Health hazards in humans that result from the consumption of chicken tissues with OTC residues include the transfer of antibiotic resistant strain bacteria known to be food borne pathogen (e.g. *Salmonella* spp., *Escherichia coli* and *Campylobacter* spp.), immunological effects, imbalance of intestinal micro-flora and carcinogenicity (Smith *et al*., 2007; Jallailudeen *et al*., 2015).

2.8.1. Development of drug resistance

Antibiotics resistant pathogens are one of world's most pressing health problems, because the number of bacteria resistant to antibiotics has increased in the last decade and many bacterial infections are becoming resistant to the most commonly prescribed treatment. The WHO has identified antibiotic resistance as one of the three greatest threats to human health (WHO, 2009).

The public health implication of the use of antimicrobials in animals could be direct or indirect. It is direct when humans are exposed to low doses of antimicrobials and consequent development of resistant strains of microorganisms (Cerniglia and Kotarski, 1999). The consumption of meat containing residues of antibiotics over a long period of time may lead to emergence of resistant gut flora and pathogens in human beings. Indirect exposure could be from consumption of foods contaminated with resistant microorganism originating from the use of antimicrobials in animals. Studies have shown that the feeding of antimicrobials to chicken resulted in an increase in a population of resistant *E. coli* (Boisseau, 1993; Smith *et al*., 2007).

2.8.2. Drug allergy

Drug allergy is defined as an immune mediated response to a drug agent in a sensitized patient, and it is restricted to a reaction mediated by IgE. Hypersensitivity or allergy reactions have been reported for many antibiotics including tetracyclines. However, they are not as common as those due to penicillin. These reactions can be fatal if they are very severe. Allergic reactions to drugs may include anaphylaxis, serum sickness, cutaneous reaction and many others. About 10% of the human population is considered hypersensitivity to some types of antibiotics, but in animals, the extent of hypersensitive to the drug is not well known (Donoghue, 2003; Dayan, 1993).

2.8.3. Carcinogenic effect

The term carcinogen refers to any substance, radionuclide, or radiation that promotes carcinogenesis, the formation of cancer. Antibiotics residues in food of animal origin can cause cancer if they are consumed regularly. The antimicrobial residues cause cancers by their interaction or covalently binding to various intracellular components such as proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), glycogen, phospholipids and glutathione (Kushi *et al*., 2012).

2.8.4. Mutagenic effect

The term mutagen is used to describe chemical or physical agents that can cause a mutation in a DNA molecule or damage the genetic component of a cell or organisms. The antibiotic residues in food of animal origin may pose a chronological effect to the human population by causing gene mutation or chromosome breakage (Celik and Eke, 2011) which affects human inheritance.

2.8.5. Teratogenic effect

The term teratogen applies to drug or chemical agent that produces a toxic effect on the embryo or fetus during a critical phase of gestation. Consequently, a congenital malformation that affects the structural and functional integrity of the fetus is produced. Exposure to OTC during the second month of pregnancy presents a teratogenic risk to the fetus (Celik and Eke, 2011).

2.8.6. Blood cell damage

Chronic exposure to OTC also causes blood changes such as leucocytosis, atypical lymphocytes, toxic granulation of granulocytes and thrombocytopenia purpura. Liver injury and delayed blood coagulation may also occur (Chi *et al*., 2014; Celik and Eke, 2011).

2.8.7. Bones and teeth discolouration

OTC can damage calcium rich organs like teeth and bones. Children under 7 years of age may develop a brown discolouration of the teeth. Infants born from the mothers treated with OTC during pregnancy may develop discolouration of the teeth. The deposition of the drug in the teeth and bones is probably due to its chelating property and the formation of a tetracycline-calcium orthophosphate complex (Requa-clark and Holroyd, 1995; Milch *et al.*, 1957).

2.9. Effects of tetracyclines on human GIT microbiota

Bifidobacteria are among the dominant populations of the human GIT microbiota, where they are thought to play a vital role in maintaining the microbial balance necessary for intestinal health. Bifidobacterial strains are therefore frequently used as probiotics in the prophylaxis and therapy of GIT disorders (Bunešová *et al*., 2014).

The microbiotas in the human GIT are large in number, containing more than 400 bacterial species. The concentration of anaerobic microbiota is 10^{11}-10^{12}CFU/g of feces, and the concentration of aerobic microbiota much lower, less than 0.1% of the normal microbiota (Sullivan *et al*., 2001)

The normal microflora acts as a barrier against colonization of potentially pathogenic microorganisms and against overgrowth of already present opportunistic microorganisms. Administration of antimicrobial agents causes disturbances in the ecological balance between the host and the normal microflora (Cerniglia and Kotarski, 1999). At relatively low dose; tetracycline may have some impact on the fecal anaerobic microbiota of humans. According to Jung *et al*., (2017), a close relationship between tetracyclines residues in food and the resistance of bacteria isolated from fecal samples was found; suggesting that the presence of low levels of tetracyclines might exert a positive pressure towards the selection and expression of resistance in bacteria colonizing animal gut. Therefore, the consumption of trace levels of tetracyclines residues in foods from animal origin may have consequences on the indigenous human intestinal microflora (Jung *et al*., 2017).

2.10. Incidence of antibiotic residues in food in African countries

In many African countries, antibiotics are used indiscriminately for the treatment of bacterial diseases or as feed additives for domestic animals and birds (Darwish *et al*., 2013). Antibiotics residues in animal-derived foods are frequently recorded in several African countries and most exceed the WHO limits in many cases. Tetracyclines, such as OTC, are highly predominant antibiotics accounting for about 41% (Fig. 2.1) of all antibiotic contaminants, followed by ß-lactams at 18% (Darwish *et al*., 2013).

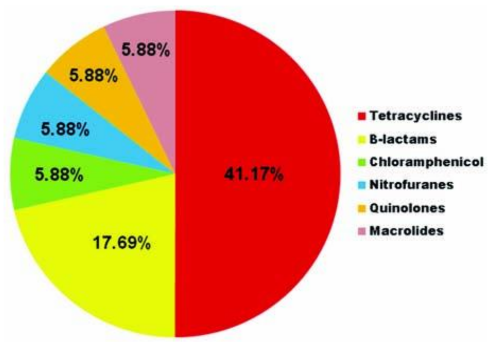

Fig.2.2. Distribution of antibiotic residues in African countries.

The incidence of tetracyclines residues (e.g., oxytetracycline in fresh chicken meat and liver samples) collected and recorded over the course of one year from retail shops in Cairo revealed that 66 samples (44%) contained tetracyclines residues including 21 breast (42%), 19 thigh (38%), and 26 liver (52%) samples. An 8% of breast, 7% of thigh, and 13% of liver had tetracyclines residues above the WHO maximum residue limits (Darwish *et al.,* 2013).

In Ethiopia, a cross-sectional study was conducted from October 2006 to May 2007 to estimate the proportion of tetracycline levels in beef; the study focused on Addis Ababa, Debre Zeit, and Nazareth slaughterhouses. Out of the total 384 samples analyzed for tetracycline residues, 71.3% had detectable oxytetracycline levels. Among the meat samples collected from Addis Ababa, Debre Zeit, and Nazareth slaughterhouses, 93.8%, 37.5%, and 82.1% tested positive for oxytetracycline (Bedada *et al*., 2012).

2.11. Safety Evaluation for Antibiotics Residue

2.11.1. Acceptable daily intake (ADI)

ADI is the amount of a substance that can be ingested daily for a long period of time without noticeable health risk. Calculation of ADI is based on the toxicological safety evaluations that consider acute and long-term exposure to the drugs (EC, 2001). FDA calculate the safe concentration for each edible tissue using ADI, the weight in kg of an average adult (60 kg), and the amount of the product eaten per day in grams as follows;

Safe concentration= [ADI (µg/kg/day) x 60 kg] / [Grams consumed/ day] (FDA, 2006).

2.11.2. Maximum residue limit (MRL)

MRL is the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical, which is recommended to be legally acceptable in a food. The concentration is expressed in milligrams per kilogram of the commodity (or milligrams per liter in the case of a liquid commodity (EC, 2001; EU, 2009). Some countries have their own MRL and some follow WHO/FDA. Ethiopia does not have its own MRL and there is also no information on that the country adapted MRL from others.

2.11.3. Withdrawal time

The withdrawal time is determined when the tolerance limit on the residue concentration is at or below the acceptable dose in edible tissue. During withdrawal time studies, the target organ is determined and animals are sampled at various times after drug administration is stopped.

Statistical procedures are used to determine when almost every animal given the drug would be below the drug tolerance concentration in the target organ (FDA-CVM, 2006; Apley, 2003).

2.12. Control and preventive measures

In the EU, self-monitoring and the control of residues are based on standardized analytical methods. The regulatory framework in force in the EU is based on Directive 96/23/EC, which structures the network of laboratories approved for official residue control, laying down requirements in terms of quality and performance of analytical methods (Decision 2002/657) (EU, 2002). In general, the residue control strategy is based on preventing the violation of residues in meat recommended for human consumption by proper drug use guide developed for use by food animal producers. These guides include: flock health management, use of approved drugs, establishment of valid veterinarian-client-patient relationship, proper drug administration and identification of treated animals, proper maintenance of treatment records and identification of treated animals, having proper drug residue testing capabilities really available on and off the farm and creating awareness of proper drug use, principally educational(EU, 2002).Persons involved in raising, handling, transporting, holding and marketing food producing animals are encouraged to establish systems to ensure that veterinary drugs are used properly and to prevent potentially hazardous drug residues in edible animal products(FDA, 2009). These control systems involve the following practice

- Identifying and tracking animals to which drugs were administered, in order to preclude the sale of edible animal tissues containing legally unacceptable residues.
- Maintaining a system of medication and/or treatment records that at a minimum, identifies the animals treated, the date of treatment, and the drug administered, who administered the drug, the amount administered and the withdrawal time prior to slaughter can be used, if appropriate.
- Properly storing, labeling and accounting of all the drug products and medicated feeds.
- Obtaining and using veterinary prescription drugs only through a licensed veterinarian based on a valid veterinarian/client/patient relationship, and
- Educating all employee and family members involved in treating, handling and selling the animals on proper administration techniques, observance of withdrawal times and methods to avoid marketing adulterated animal tissues and products destined for human consumption.

Chapter Three

3. Methods and Materials

3.1. Study Area

This study was conducted in Bishoftu town of East Shoa zone of Oromia regional state from November 2016 to June 2017. Bishoftu is located at 47 km southeast of Addis Ababa and at latitude and longitude of 8°45′N 38°59′E and elevation of 1885 meters above sea level. There are 7 poultry farms and 5 slaughterhouses in this town. In this study a poultry farm with its own slaughterhouse was included. The poultry farm and slaughterhouse have 20 employees consisting 12 professionals and 8 non-professional workers. About 5000 chickens are raised on the farm and more than 600 of chickens are slaughtered per week on Fridays.

3.2. Study design

A cross-sectional study was undertaken in a poultry slaughterhouse located in Bishoftu town. The research was conducted to determine the level of oxytetracycline residue in 3 different meat samples from 30 freshly slaughtered broiler chicken that are collected randomly.

Study Knowledge, Attitude and Practice (KAP): A total of 20 respondents (8 from farm management and 12 from feeders and other workers) participated in the survey. Purposive sampling technique and semi-structured questionnaires were used to gather information. The data obtained was evaluated for awareness and knowledge on oxytetracycline residue in chicken meat.

3.3. Sample size determination for OTC residue determination

Sample size was calculated using single group sample size calculation formula ($n=\frac{\log \beta}{\log \mathrm{p}}$) where1−β is the chosen power that is 95% chance of detecting chicken with OTC residue (β = 0.05).p represents the proportion of the chicken in the group that have no detectable OTC residue and *n* represents the estimated size of sample (Dell *et al*., 2002; Naing *et al*., 2006).

According to different studies in Africa on the prevalence of antibiotics residues in chicken meat and egg, tetracyclines accounted for 6.8% to 8% of which the dominant type is oxytetracycline (Darwish *et al.,* 2013; Mensah *et al*., 2014). Therefore, at 10% expected prevalence of OTC residue is estimated (p =90%), and a sample size of 30 chickens were accordingly determined to be sufficient for the investigation. n= $\frac{\log 0.05}{\log 0.9}$n=28.4

3.4. Sampling method

A total of 30 chicken meat samples (leg muscle, breast muscle and cuts of gizzard from each chicken) were taken randomly from a slaughterhouse immediately after slaughtering. Samples were packed in polyethylene bags, sealed, labeled and kept in dry ice box immediately after collection and transported to the laboratory under frozen condition. The samples were then stored in -20^{o}c refrigerator in the laboratory until analysis.

3.5. Method for oxytetracycline residue analysis

3.5.1. Materials and Chemicals

Chemicals and reagents

All the solvents used (acetonitrile and methanol) were HPLC grade. Citric acid, H_2SO_4, and nitric acid were purchased locally. Other chemicals such as standard oxytetracycline hydrochloride (OTC-HCL) were purchased from Sigma-Aldrich. Distilled water used was of HPLC grade (deionized) obtained from the laboratory stock of Center for Food Science and Nutrition.

Materials and apparatus

Knife, electronic balance, tissue homogenizer,15ml test tubes, racks, centrifuge, ultrasonicator, vortex mixer, water bath, 0.45µm nylon filters, volumetric devices with different volume, 10ml syringes, micropipettes, micropipette tips, stirrer, parafilm, disposable gloves, sample labeling materials, vials with screw cap.

Shimadzu HPLC with SIL-20A/20AC auto-sampler, injector, oven, hypersil BDS-C18 (3 µm, 100×4 mm) column, link, online degasser, UV-detector and computer with lab solutions chromatography software.

Mobile phase

The mobile phase consisted of deionized water (pH = 2.1 with H_2SO_4): acetonitrile, 85:15 (v/v) isocratic methods because of the single analyte (only oxytetracycline). The mobile phase was prepared, filtered and degassed by ultrasonicator before use.

3.5.2. Standard solutions

Standard OTC solution of 100µg/ml (100ppm) was prepared by dissolving 10.8mg of oxytetracycline hydrochloride in 100ml HPLC grade methanol and kept in the freezer (-4^{0}C). Working solutions of (50ppm→ 10ppm→1ppm) were prepared by serial dilution from the stock solutions using ultrapure water (pH = 2.1 with H_2SO_4) in 10ml volumetric flasks. Then all other working standards (0.05, 0.08 0.1, 0.2, 0.4, 0.5 and 0.6ppm) were prepared from 1ppm by further dilution for method validation. The prepared standards were transferred into amber vials and stored at -4^{o}C to protect the deterioration of OTC in the solutions.

3.5.3. Method validations

Validation is testing a method to demonstrate that it is suitable for its intended purpose and the results obtained are reliable, accurate and reproducible. It is used to provide confidence that the method will perform properly under intended conditions. Method validation involves identification, accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ) determination.

Identification

Identification of oxytetracycline was done based on its retention time by preparing the working standard solution of different concentration and injecting under the same chromatographic condition to get the acceptable percent of relative standard deviation(%RSD) (FDA, 2002; CRLs, 2014).

Precision

Precision is the measure of agreement among test results when the method is applied repeatedly to the sample of the same concentration. The method was evaluated through repeatability of method by injecting 1ppm of the standard working solution six times under the same condition in the same day to get the acceptable %RSD (FDA, 2002).

Recovery and Accuracy

Accuracy is the measure of the closeness of test results obtained by the method to the true value. It involves spiking the sample with known amount of standard analyte, then extract it and calculating for the recovery (FDA, 2002; CRLs, 2014). It was done by spiking 1ppm and 0.5ppm oxytetracycline standards with the meat sample and extracting and calculating the percent recovery compared to the injected working standard of pure 1ppm and 0.5ppm oxytetracycline.

Linearity

Linearity is the measure of the ability of the method to elicit test results that are directly proportional to concentration within a given range. It is expressed as the variance of the slope of the regression line. The linearity of the method was determined by injecting six different concentrations of oxytetracycline standards in the concentration range of (0.05-1) ppm. Standard curve was prepared using peak area (Y) vs. concentration of standard solutions (X). The data was determined using linear regression from Microsoft Excel that uses the equation $y = mx+b$: Where m is the slope of the line and b is the y-intercept (CRLs, 2014).

LOD and LOQ

Limit of detection (LOD) is the lowest concentration of OTC in a sample that can be detected under stated operational conditions and limit of quantification (LOQ) is the lowest concentration of OTC in a sample that can be quantified with acceptable precision and accuracy under stated operational conditions. Both of them were determined by injecting standard working of0.04, 0.05, 0.06 and 0.08ppm solution prepared by further dilution of 1ppm in triplicate and standard deviation (SD) of the response and slope (S) was calculated. Then LOD was determined as 3(SD/S) and LOQ was determined as 10(SD/S) (FDA, 2002; CRLs, 2014).

3.5.4. Sample preparation and extraction

Application note from Agilent technologies (Gratzfeld-Hüsgen and Schuster, 2001) was used for the sample extraction. About 4g of sample was taken from each meat (leg muscle, breast muscle and gizzard) and cut into smaller pieces by knife on clean plastic board separately. Each cut meat sample was then transferred to clean 10ml volume beaker and homogenized by *IKA T-25 ULTRA TURRAX®Digital Homogenizer* with disperser set at 15rpm for 5 minutes. Then to extract 10ml sample solution, 2g of homogenized meat sample was placed in 15 ml plastic centrifuge test tube and to this sample 0.1g citric acid, 1ml of nitric acid (30%), 4ml of HPLC grade methanol and 5ml of deionized water were added respectively.

The suspension with solid particles was vortexed and then kept in an ultrasonic bath for 15 minutes. The sample was centrifuged for 10 minutes at 5300 rpm and 10ml supernatant was decanted into a clean15ml test tube. The purification of sample was aqueous based extract. It was filtered on 0.45µm nylon micro filter. Clean solution was transferred into 1.5ml HPLC autosampler amber vials. Finally 20µl of the solution was injected into HPLC for analysis.

3.5.5. Analysis

Each breast muscle, leg muscle and gizzard of all chicken sample were individually tested for oxytetracycline residue. Each chicken sample was analyzed in duplicate for oxytetracycline using HPLC with UV detector at 360nm wavelength. Gratzfeld-Hüsgen and Schuster, (2001) methods was used for the analysis.

3.5.6. Chromatographic conditions

Isocratic separation was achieved using Shimdzu HPLC SIL-20A/20AC with UV detector using the analytical column hypersil BDS-C18 (3 µm, 100×4 mm) column. The mobile phase (**A** = consisting of distilled water (pH = 2.1 with H_2SO_4): **B** = Acetonitrile, 85:15 (v/v)) was pumped at a flow rate of 1ml/min. Each sample was detected at 360 nm using UV detector.

The injected volume was 20µl and the chromatographic column compartment temperature was set at 24 ºC.

3.6. Data Analysis

The eluted OTC solution was determined at parts per million (µg/g) levels in chicken meat sample by high performance liquid chromatography with UV-detection and calculated according to the following equation. Amount of OTC in µg/g = m_a (V_f/V_i)* (1/m_t)

Where: m_a= OTC level in µg corresponding to the area of peak of the sample elute

V_f= Final test solution elute volume in (µl)

V_i= Volume of elute injected into HPLC in (µl)

M_t= weight of meat sample used in final extract in (g)

The obtained data was inputted in Excel and descriptive analysis was done.

Chapter Four

4. Results and Discussion

4.1. Knowledge, Attitude and Practice Survey

Knowledge, Attitude and Practice (KAP) of the poultry farm workers and management regarding antibiotics residue in chicken meat was evaluated separately, with the results shown in Table 4.1.

Table 4.1. Workers and management knowledge survey response.

No.	Questions	Yes		No	
		Mgmts.	Worker	Mgmts.	Worker
1	Do you know about antibiotics residues in meat?	8	-	-	12
2	Do you know that withdrawal time is needed for antibiotics used for chicken?	8	-	-	12
3	Do you know the factors that increase antibiotics residue in chicken meat?	4	-	4	12
4	Do you know the risk of antibiotics residue on human?	4	-	4	12

NB: There were 8 management and 12 farm workers surveyed

According to the result shown in Table 4.1, all the survey participants do not know about the residues of OTC and others veterinary drugs in meat and even they do not know the withdrawal time of each antibiotic used for chickens the poultry farm. They also have no knowledge about factors that increase residues of antibiotics including OTC in meat and the problem it causes on people when they consume the products with OTC residue. But the managers and professional workers of the farm have knowledge on the question provided even though they do not participate directly in feeding and administering antibiotics to chickens. The farm has no onsite laboratory to check their chicken meat for antibiotics, as per my observation.

Regarding ingredient of the feeds, antibiotics used in the farm, mechanism of providing (route of drug administration) and withdrawal time of each antibiotics used, the results of survey are shown in Table 4.2.

Table 4.2. Practice survey of workers' and managers' response about the feed and antibiotics of the chicken.

No.	Questions	Responses
1	What are the ingredients of the chicken feeds?	Corn, ground bone, sands, soya bean and oil seed
2	What are the types of antibiotics used in the farm?	Tetracyclines (tetracycline and oxytetracycline), Amoxacillin and Amprolium
3	What are the mechanisms of supplement of antibiotics to chicken used?	Feed and water
4	What is the withdrawal time of each antibiotic used in the farm?	No withdrawal time for tetracycline. 3 days for Amoxacillin and Amprolium.

The results in Table 4.2 show that the chicken feed are prepared mainly from ground corn, soya bean and oil seeds as sources of carbohydrates, proteins and fats, respectively. As the sources of minerals, bone meal is used and sand is also used as additive in the feed to facilitate digestion. The main antibiotics used in the farm are tetracyclines such as tetracycline and oxytetracycline and other antibiotics such as amoxicillin and amprolium. These antibiotics are supplied for chicken by adding into their feed and water. In the farm, according to the information gathered, the withdrawal time used for amprolium and amoxacillin is 3 days but in the farm, no withdrawal time for oxytetracycline.

According to the data presented in the Tables 4.1 and 4.2, the KAP survey of antibiotics residues in chicken meat shows that there is knowledge gap among the farm workers as well no commitment on managers to create awareness on other workers; there is also poor practice on the withdrawal time for oxytetracycline.

4.2. Oxytetracycline residue in chicken meat

4.2.1. Method validation

The analytical performance of the HPLC and the validity of the method used to analyze OTC residue in chicken meat were confirmed by various parameters: identification, accuracy, linearity, LOD, LOQ and recovery of the method used.

Identification

The retention time of 1ppmOTC standard was identified on HPLC chromatogram (Figure 4.1a and Figure 4.1b). Mean of the retention time of OTC and percent of relative standard deviation (%RSD) are shown in Table 4.3.

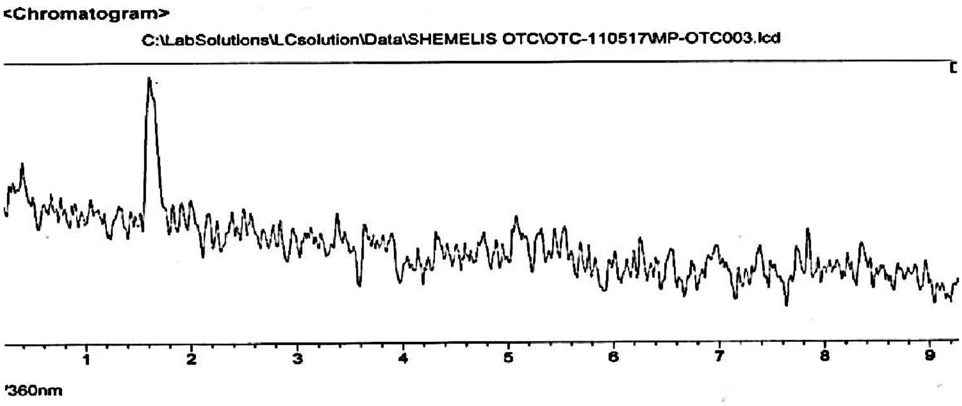

Fig.4.1a: Mobile phase chromatogram

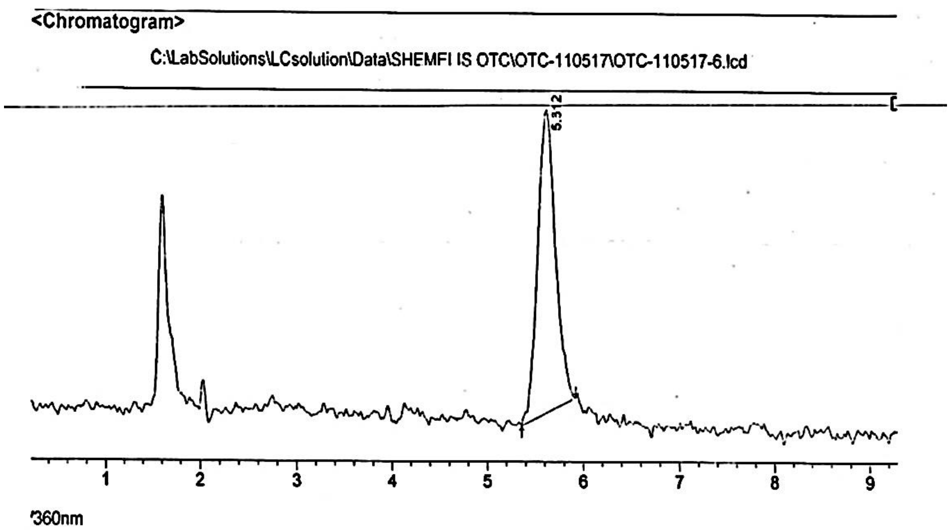

Fig.4.1b: Standard OTC 1ppm chromatogram (representative)

Table4.3. Oxytetracycline retention time identification and its %RSD

OTC 1ppm injection	Retention time
1	5.623
2	5.613
3	5.615
4	5.620
5	5.612
6	5.614
Average	**5.616**
Standard Deviation (SD)	**0.0044**
Relative standard deviation (%RSD)	**0.078**

Table 4.3 shows that the mean of the retention time of OTC was 5.616. The precision of the retention time was measured by using %RSD. The percent relative standard deviation is 0.078 and according to Food and Drug Administration and Community Reference Laboratories (FDA, 2002; CRLs, 2014), %RSD below 2% is within the acceptable threshold.

Precision

Precision of absorbance (quantification) ability of HPLC and its retention time was evaluated by injecting 1ppm standard working solution of OTC six times under the same condition in the same day and the following results, shown in Table 4.4, were obtained.

Table4.4. Precision of the method using 1ppm OTC

Injection	Peak area	Retention time
1	26793	5.712
2	26514	5.713
3	26600	5.710
4	26609	5.715
5	26780	5.718
6	26550	5.708
Mean	**26641**	**5.713**
Standard Deviation	**107.66**	**0.00325**
Relative SD	**0.404%**	**0.057%**

Table4.4 shows that relative standard deviation for peak area is about 0.4% and for the retention time, it is less than 0.06%. According to FDA and CRLs, the acceptable level of %RSD for precision is ≤ 5% for significant number of injections, *i.e* more than 5 injections (FDA, 2002; CRL, 2014). So the method is precise to be used for analysis

Recovery and accuracy

The performance of the extraction and filtration method used during sample preparation was checked by spiking 1ppm and 0.5ppm OTC standard in 2g of breast muscle. Then 20µl of the extracted solution from each spiked sample was injected into HPLC and the results shown in Table 4.5 were obtained. The recovery peak chromatogram is shown on Figures 4.2a and 4.2b.

Table4.5.Oxytetracycline recovery test

Spiked concentration	N	Measured concentration in (ppm)	% Recovery
0.5ppm	1	0.44	88.0
	2	0.46	92.0
	3	0.43	86.0
Average1			**88.67**
SD			**3.05**
%RSD			**3.44%**
1.0ppm	1	0.92	92.0
	2	0.92	92.0
	3	0.91	91.0
Average2			**91.67**
SD			**0.52**
%RSD			**0.57%**

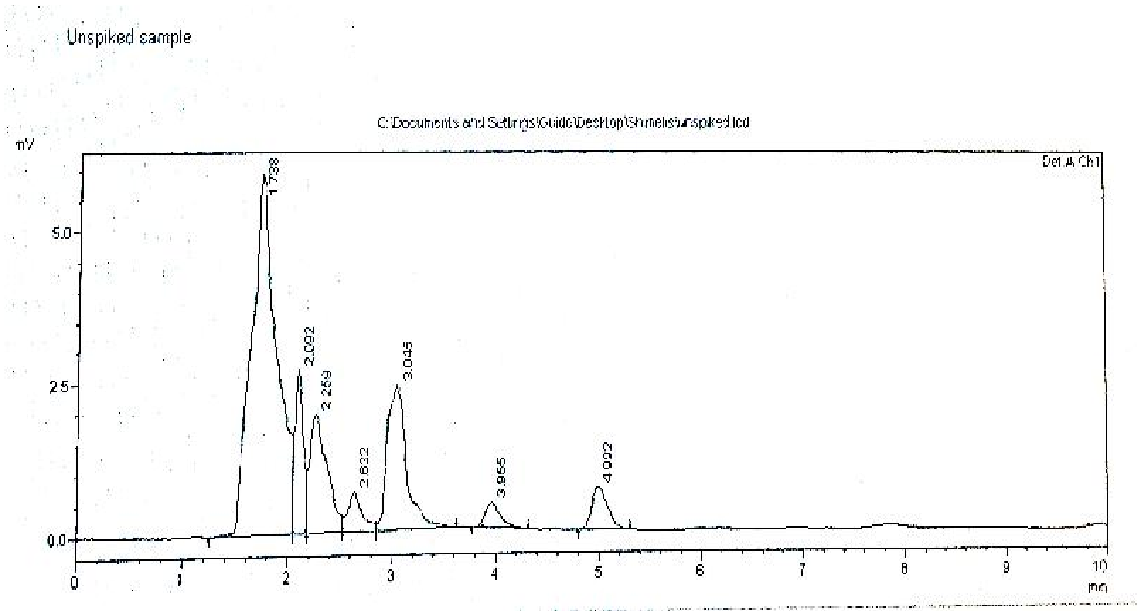

Fig.4.2a.Unspiked breast meat sample chromatogram

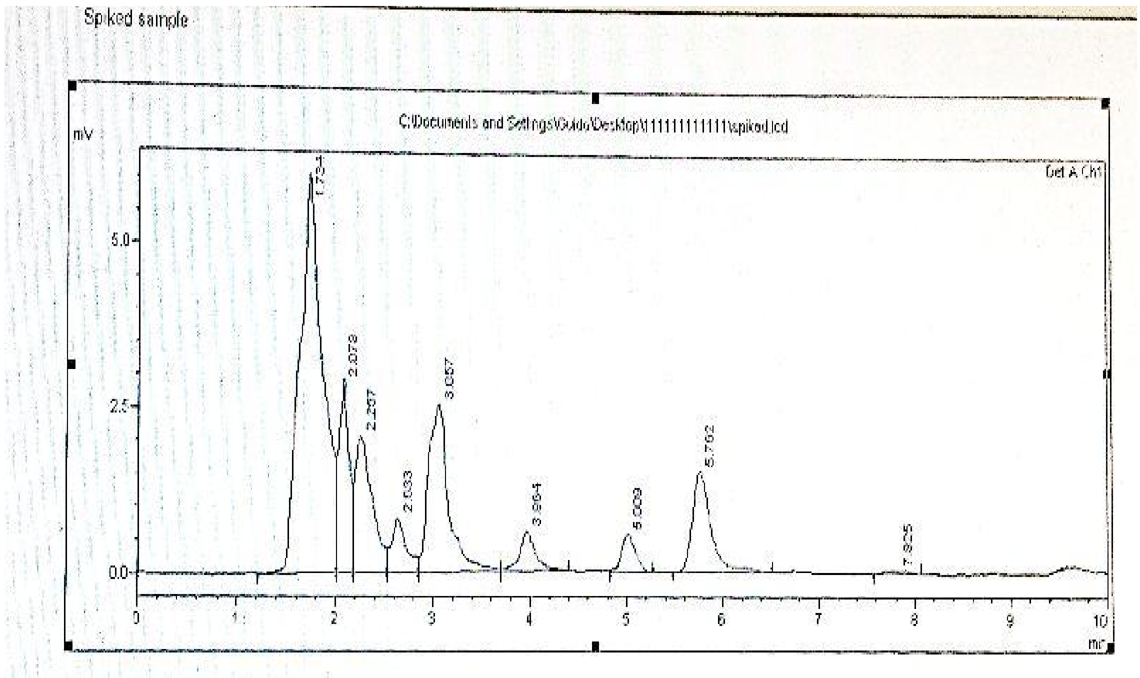

Fig.4.2b. Chromatogram of breast meat sample spiked with 1ppm OTC

According to Table 4.5, recovery ranges from 86%-92% and %RSD ranges from 0.57%-3.44%, which indicates that the method is accurate within the acceptable recovery range set by FDA and CRL (FDA, 2002; CRL, 2014) and the %RSD is less than 5%.

Linearity

The linearity of the method was evaluated by injecting 0.05, 0.1, 0.2, 0.4, 0.6 and 1.0ppmoxytetracycline standard solutions to determine the presence of linear relationship between the concentration of OTC and the peak area. The peak areas for each injected concentration of standard solution were obtained as shown on Table 4.6. A standard curve was generated using peak area (y) vs. concentration of standard OTC in ppm (x) as shown in Figure 4 3. The data was determined using linear regression from Microsoft Excel that uses the equation y = mx+b.

Table 4.6: OTC standard concentration and their relative absorbance

Concentration (ppm)	Replication	Average peak area at 360nm
1.00	2	26514
0.60	2	16371
0.40	2	10281
0.20	2	5276
0.10	2	2520
0.05	2	1253

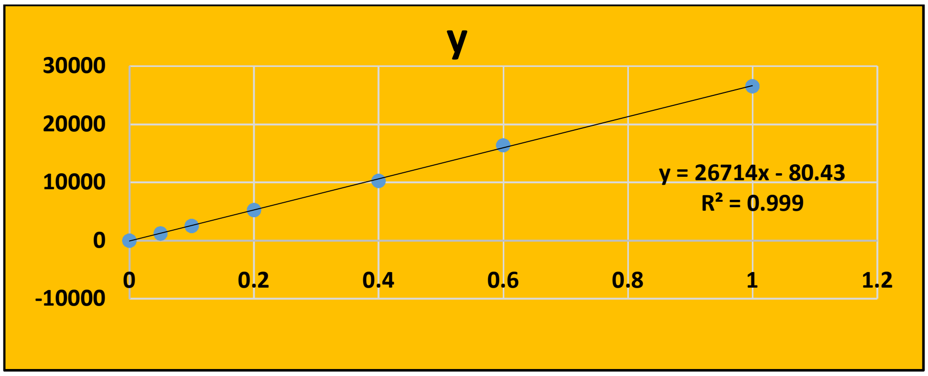

Fig.4.3: Standard calibration curve for OTC.

The R^2 (coefficient of determination) is very important criteria used to evaluate fitness of the linearity of the data determined using linear regression line. As the data shown in the Fig. 4.3, the coefficient of determination (R^2) is 0.999 which indicates that there is strong linear causation between concentration (amount) of OTC and the absorbance of detector (peak area).

LOQ and LOD

Both LOQ and LOD were determined by injecting 0.04, 0.05, 0.06 and 0.08 ppm in triplicate and standard deviation (SD) of the response is shown in Table 4.7 and slope of calibration curve was calculated as shown in Figure 4.4.

Table 4.7. The LOQ and LOD of the HPLC instrument with method used

OTC conc. level (in ppm)	No. of replicate	Mean of response	SD
0.04	3	1047	53.27
0.05	3	1218	111.14
0.06	3	1507	59.16
0.08	3	1743	323.19
Average SD			**136.69**

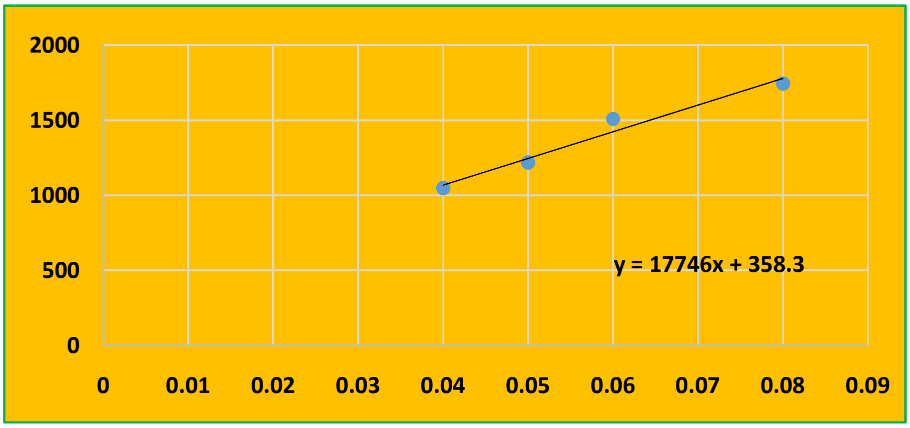

Fig.4.4. Calibration curve for LOQ and LOD

Standard deviation (SD) is 136.69 as shown in Table 4.7 and slope (S) of calibration curve was calculated to be 17746 as shown in Fig. 4.4. Then for the method used in this study, LOD was determined as 3(SD/S) = 3(136.69/17746) = 0.023ppm and LOQ was determined as 10(SD/S) = 10(136.69/17746) = 0.077ppm.

4.2.2. Oxytetracycline level in chicken meat sample

A total of 30 chickens from which three meat samples i.e. breast, leg and gizzard were analyzed from each for the presence of oxytetracycline. A total of 90 samples (thirty breast meat samples, thirty leg meat samples and thirty gizzard meat samples) were analyzed for the determination of oxytetracycline. Each sample was analyzed in duplicate. The result obtained from analysis shows that all the chicken meat samples (90 meat samples from the 30 chickens) were at non-detectable level of oxytetracycline residue.

All the chicken meat samples analysed for the level of OTC had no detectable oxytetracycline residue. The samples were checked in a different laboratory by different methods to confirm the findings. The SPE (solid phase extraction) was also done for 10 samples of the 90 samples, but the result was the same to that previously obtained. The result was also verified by changing column type, detector type (DAD and florescence instead of UV-detector) with different extraction chemicals and mobile phase adapted from different published literature to confirm the finding.

To verify the results some samples were anlysed in another laboratory with different HPLC and chromatograph condition in 'Kality Animal Products Veterinary Drugs and Feed Quality Assessment Center', but the same finding was obtained.

The finding of this study show that oxytetracyline residue in chicken meat in this farm is at non-detectable level. Different researchers reported the lower incidence OTC in their studies such as Alla *et al*., (2014) which has the same control and analysis condition as this study. Moreover, there are some studies which reported that there are no antibiotic residues detected in chicken meat and organs after freezing for a long period (Mahmoud and Mohsen, 2008). The sample used in this study was stored in deep freeze for a month prior to analysis.

The research done by Cetinkaya *et al*., (2012) using LCM-SMS on the detection of oxytetracycline residue in 60 samples show that there is no residue in samples analysed, however, the number of the sample size included in the survey was relatively small compared to the total number sold in the market and the slaughterhouses. The sample used in this study was taken from one farm slaughterhouse (because of time constraint and cost) which may not fully represent all poultry farms / slaughterhouses management and practices in the country.

Even though different studies show that the withdrawal time for oxytetracycline is from 5-7 days in chicken meat, oxytetracycline residues were not-detectable after 12 hours in the case of the birds treated with 211mg/L or 211mg/kg (the maximum approved dose of the oxytetracycline (FOI, 2005)) and below (Reyes, 2010). The poultry farm from which the samples for this study were taken does not know the exact amount of OTC added to water and feed. It may be lower than the above indicated concentrations.

One of the characteristics of tetracyclines is their ability to form complexes with polyvalent cations of metals; a property which was observed soon after these substances had been discovered. According to the research results by Ziołkowski *et al*., (2016) on high concentrations of calcium (Ca2+), magnesium (Mg2+), and iron (Fe3+) ions in feeds and water in which OTC is provided to chickens are able to decrease the absorption of OTC from gastrointestinal tract in chickens which may cause a decrease of the residue in meat. In the poultry farm from which the samples of this study were taken, the ground animal bone is a major ingredient of the chicken feed. It is known that bone is rich in calcium which might be one of the factors that decrease the level of oxytetracycline residue to non-detectable level in these samples.

Limitation of the study

The sample used in this study was taken only from one farm slaughterhouse (because of time constraint and cost) which may not fully represent all poultry farms / slaughterhouses management and practices in the country. Almost all poultry farms are not willing to give sample for analysis of antibiotics residue as they think it has some implications on their products because they are not sure about the status of antibiotics residue in chicken meat of their farm. So, it was difficult to get samples from different poultry farms or slaughterhouses. It also difficult to assess the truth knowledge and attitude of professional workers because they respond as they know and say ‘yes’ for question asked.

Chapter five

5. Conclusion and Recommendations

5.1. Conclusion

Knowledge, attitude and practice survey of the workers of the farm indicates that there is knowledge gap about antibiotics residue, the withdrawal time of the antibiotics and the factors that cause the residue. Workers are not even aware that OTC residue in meat can affect human health. Managers and professionals know about residues and its risk on the human health but they have not put in place protocols so the feeders to prevent OTC residue in chicken meat.

The farm does not have a précised amount of each antibiotic to be added per kg of feed or per liter of water. So, amount of OTC and other antibiotics added to feed and water is different in different days.

The poultry farm has no equipped laboratory, technician and methods to check for residue in chicken meat before delivery to market and has no withdrawal time for OTC.

Data obtained from the HPLC-UV analysis of the collected samples indicate that the analysed chicken meat samples do not have detectable oxytetracycline residues.

The possible reasons for non-detectable levels of OTC residue in the samples may be due to different factors such as the ingredient of the feed they use (*i.e.* ground bone which is rich in Ca^{2+}) which can decrease the bioavailability of the antibiotics, the amount of antibiotics (i.e. low amount) used in feed and water as the farm has no regular amount per kg of feed or per liter of water.

The finding of the study shows that the chicken meat samples collected for the analysis from the poultry farm do not pose risk to consumers. However, unless corrective measures on KAP are put in place, there is a potential risk that OTC residue may reach consumers.

6.2. Recommendations

- According to KAP survey result, awareness creation and training program is required for the feeder and other workers on feed and antibiotics residue, risk factor, withdrawal time of each antibiotics and effect of residue on consumers.
- It is advisable for the poultry farm to be equipped with laboratory, technicians and methods to check antibiotics residue in sampled chicken meat before mass slaughtering.
- Setting up policy and regulation is required such as adapting MRL from FDA or WHO to monitor and control OTC residues in chicken meat.
- Establishment of additional chicken meat and feed analysis centers such 'Kality Animal Products Veterinary Drugs and Feed Quality Assessment Center' is required in the country.

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Appendix 1

Questionnaires

These questions are used to collect some information about the poultry farms in Bishoftu town. These questionnaires contain feeds and feeding mechanisms of poultry, ingredient of the feeds, antibiotics they used, route antibiotic supplies (oral with feed or with water, by injection), Withdrawal time before slaughtering and other knowledge assessment of the respondents

Respondent background information

Sex____ Qualification (field and certificate)____________________________

Age____ work and responsibility_____________________________________

1. **Workers and management knowledge survey response of the poultry farm**
   Put the mark (√) in the box of your response

No.	Questions	Yes	No
1	Do you know about antibiotics residues in meat?		
2	Do you know that withdrawal time is needed for antibiotics used for chicken?		
3	Do you know the factors that increase antibiotics residue in chicken meat?		
4	Do you know the risk of antibiotics residue on human?		

2. Practice survey workers and manager's response in the farm about the feed and antibiotics of the chicken

❖ Write the practice you know in the farm in the provided space

No.	Questions	Responses
1	What are the ingredients of the chicken feeds?	
2	What are the types antibiotics used in the farm?	
3	What mechanisms of supplying the antibiotics to chicken?	
4	What is the withdrawal time of each antibiotic used in the farm?	

Appendix 2

መጠይቅ

ይህ መጠይቅ በብሾፍቱ ከተማ ውስጥ ከምገኙት የዶሮ እርባታ ፈርም እንፎርሜን ለመሰብሰብ ይውላል።

መጠይቁ ስለፋርሙ የምግብ አዘገጃጀት፤ የአመጋገብ ዘዴንፒ የምግብ ይዘትፒ የምጠቀሙት የመድሐኒት ዓይነትፒ የመድሓኒት አሰጣጥዘዴፒ የመድሓኒ የማቆረጫ ጊዜ ሌሎችየተሣታፊዎችንእውቀት እ አመለካከት ለመዳሰስ ነው።

1. <u>የተሰተፊዎች ግለታሪክ</u>

ፆታ -- የት/ት ደረጃ---

ዕድሜ-- ሥራ ሓላፍነት---

2. **የተቐሙ አስተዳደሮች ሠረተኞች እውቀት ዳሳሳ**

የመረጥከው ባዶ ሰጥን ውስጥ (√) ምልክት አስቀምጥ

ተ.ቁ	መጠይቅ	አዎ	አለቅም
1	መድሓኒት ሥጋ ውስጥ ዘቅጦ እንደምቀር ተቀለ(ሽ)		
2	የእያንደንዱን ለደሮ የምጠቀሙትን መድሓኒት የማቆረጫ ጊዜ እንደምያስፈልጋቸው ተቀለ(ሽ)		
3	በሥጋ ውስጥ የመድሓኒት መዝቀጥ መንሥኤዎች ልሆኑ የምችሉትን ተቀለ (ሽ)		
4	የመድሓኒት ዝቃጭ ያለው ሥጋ በሰው ላይ የምያደርሰውን ጉዳት ተቀላ (ሽ)		

3. **የተቋሙ አስተዳደሮች ሠረተኞች ተግበር አመላካከት ዳሳሳ**

በተሰጠወ ባዶቦታ ውስጥ የምተውቀውን በተቐሙ ውስጥ የምከነውን ተግባር ዘርዝር

ተ.ቁ	ጥያቄ	መልስ
1	የተቐሙ የዶሮ መ ይዘት ዘርዝር (ሪ)	
2	በተቐሙ ውሰጥ የምጠቀሙትን የመድሓኒት ዓይነት ምን ምንድ ቸው	
3	መድሓኒቶ ለዶሮች የምሰጣቸው እንዴት ነው	
4	የእያንዳንዱን በተቐሙ ውስጥ ጥቅምለይ የምውሉ መድሓኒቶች የመቐረጫ ጊዜ ፃፍ (ፊ)	