THE EFFECT OF COLD STORAGE AND COOKING PROCEDURES ON THE LEVELS OF OXYTETRACYCLINE RESIDUES IN BEEF FROM DODOMA REGION, TANZANIA

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EXTENDED ABSTRACT

Worldwide, there is an increased use of antimicrobial drugs due to occurrence of diseases of human and animals. The general objective was to study the effect of cooking procedures and cold storage on the levels of Oxytetracycline (OTC) residues in beef in Tanzania. The study used a cross-sectional research design whereby both quantitative and qualitative data were collected from Dodoma region, Tanzania. The household survey was conducted to assess knowledge, attitude and practice on beef consumption among 254 residents. The results show that community based health education and promotion of proper antimicrobial use in animals and preventing drug residues is highly recommended to this population. Beef samples were also analyzed by using High Performance Liquid Chromatography Mass-Spectrometry (HPLC-MS). The quantitative data were analyzed using the IBM Statistical Package for Social Science (SPSS) software version 20 and Epi info version 7. A simple and sensitive method for the detection of OTC levels in ready-to-eat beef by HPLC-MS was modified and validated and used for beef analysis in this study. The advantages of the modified method were cleaning by Supelclean ENVI-carb active coal is cheaper compared to solid phase extraction and samples drying using a stream of liquid nitrogen is cheaper and more than six samples can be dried at a time. For the raw beef, the results indicate that the mean concentration level of OTC was very low (0.69 ± 0.09 ng/g). The boiled and barbecued beef, the mean concentration was 69.4 ±41.93 ng/g and 69.40±38.91 ng/g, respectively. The results indicate that one should not count on heat-treatment to eliminate residues of OTC from beef. The effect of the cold storage on the concentration of OTC residues in beef stored at -20 °C for 60 and 120 days showed that the mean concentration of OTC residues before freezing was 191.71 ± 90.21 ng/g. The mean concentration of OTC after freezing at -20 ºC for 60 and 120 days were 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g respectively. These results revealed a significant

($p<0.05$) reduction of OTC residues of 30% after 60 days and 65% after 120 days of freezing at -20 °C.

DECLARATION

I, FRIDA RICHARD MGONJA, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work done within a period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

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DEDICATION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
ADI	Acceptable daily intake
AMR	Antimicrobial resistance
AMRs	Antimicrobial Residues
Beef	Flesh of a cow, bull, or ox.
Butcher	Person who slaughters animals or dresses their flesh
CEC	Commission of European Communities
CEC	Codex Expert Committee
CTC	Chlortetracycline
CVMBS	College of Veterinary Medicine and Biomedical Sciences
DAD	Diode Array Detector
DNA	Deoxyribonucleic Acid,
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
G +ve	Gram-positive bacteria
G –ve	Gram-negative bacteria
GTFCh	Germany Society of Toxicology and Forensic Chemistry
h	hour
HPLC	High Perfomance Liquid Chromatography
HPLC- MS	High Performance Liquid chromatography – Mass Spectrometry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid Chromatography- Mass spectrometry
LMD	Limit of Detection

LOD	Limit of Detection
Meat	Flesh of an animal, mammal or bird.
MRL	Maximum residues limits
OTC	Oxytetracycline
pH	potential of hydrogen
ppb	parts per billion
R^2	correlation coefficient
SPSS	Statistical Package for Social Science
SUA	Sokoine University of Agriculture
TC	Tetracycline
TCs	Tetracyclines
TL	Tolerance level
TLC	Thin- layer chromatography
UN	United Nation
US	United States
WHO	World Health Organization
ZARI	Zambia Agriculture Research Institute

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Antibiotics are substances produced naturally by living organisms or synthetically in the laboratory and are capable of destroying or preventing the growth of microorganisms (Wageh *at el.,* 2013). These agents have been used for the treatment of diseases in animals, prevention of infection in animals and to improve feed utilization and production (Kabir *et al.,* 2003; Reig and Toldra, 2008; Heshmati *et al.,* 2013). They are also used in human health care to treat bacterial infections. Numerous agents including antimicrobial agents, pesticides, pathogenic microorganisms and aflatoxins endanger the security of human food. Population increase coupled with increase in incomes and changing life-styles have been associated with greater dependence on marketed foods, and concern about food safety especially with animal source food has been increasing in developing countries (Grace *et al.,* 2010).

Diseases in the livestock industry in sub -saharan Africa including Tanzania remain a challenge. In Tanzania and other African countries the challenges have been addressed mainly by the use of antimicrobials that include the oxytetracycline followed by beta lactam antibiotics like penicillins (Olufemi and Agboola, 2009; Katakweba *et al.,* 2012). Behind the success story associated with the use of antimicrobial, the issue of residues serves as a major concern to consumers` health. Due to the widespread use of antimicrobials in the treatment of diseases in cattle, much effort has been directed towards the proper management and monitoring of its usage in order to prevent contamination of raw milk and meat products (Alica *et al.,* 2003; Jahed, 2011).

The ingestion and administration of antimicrobials or feed additives to food producing animals may result in their accumulation in body tissues, organs and secretions (Muriuki *et al.,* 2001; Dipeolu and Alonge, 2002; Kabir *et al.,* 2004). Unacceptable levels of oxytetracycline residues in meat may result in gastrointestinal disturbances, hypersensitivity, bone and teeth problems in children and development of bacterial resistance to consumers (Larkin *et al.,* 2004; Shankar *et al.,* 2010; Goetting *et al.,* 2011). To obtain safe animal products such as milk and meat, animals have to be kept healthy and in good management including feeding, control of animal diseases and treatment provided when they are sick. Antimicrobial agents are essential drugs for both human and animal health and welfare (Olatoye and Ehinmowo, 2010).

Several studies on antimicrobial residues in foods of animal origin such as milk, beef and eggs have been carried out in Tanzania (Mmbando, 2004; Karimuribo *et al.,* 2005; Kurwijila *et al.,* 2006; Mdegela *et al.,* 2009; Gwandu, 2013). Despite the reports by these scholars so far, there is limited information on the effect of cooking procedures and cold storage on the levels of antimicrobial residues and this creates a scientific gap of knowledge which needs to be addressed. It is of importance to address how serious the antimicrobial residues are after cooking and storage and the levels be known to the public in Tanzania. It is known that antimicrobial residues can be destroyed by cooking procedures, cooking time and storage. For example, Gratacós-Cubarsí *et al.* (2007) reported that ordinary cooking procedure such as microwave and boiling reduced the initial concentrations of tetracycline (TC) residues by 56 to 82%, respectively. Also Salah *et al.* (2013) reported that sufficient cooking temperature and time can have significant effect on the losses of TC residues and provide an additional margin of safety for consumers. Loksuwan (2002), revealed that milk spiked with OTC, TC and chlorotetracycline (CTC) at 200, 200, and 400 ppb, respectively, and heated to 63 ºC for

30 minutes showed residue reduction. Another study by Dipeolu and Ayo-Adisa (2006), reported that conventional heat treatment such as cooking, does not eliminate most antimicrobial residues (AMRs) in meat but reduces the levels of AMRs in foods. In Tanzania, there is inadequate information on the effect of heat treatment on the stability of the residues in foods of animal origin.

To control occurrence of harmful effects of drug residues in humans and animals, various regulatory and control measures have been effected. These include the imposition of monitoring procedures, and setting maximum acceptable residue limits (MRL) in animal food products (FAO/WHO, 2014). In addition, countries have their own control regulations and MRL's based on the joint FAO/WHO Codex Expert committee or JECFA recommendations. Informed existence of antibiotics in human food vary widely between several countries and are recognized to be low or non-existent in places where quality assurance programmes are operative (Kurwijila *et al.,* 2006; Aning *et al.,* 2007; Henzelin *et al.,* 2007). Such projects comprise mainly learning programmes and extensive analysis of foodstuffs for antibiotic residues. Conducting quality assurance programmes to improve the health of the public to minimize the adverse effects of antimicrobial is a key task for developing countries especially where there is veterinary mismanagement of such drugs, and sales of animal source food are mainly informal (Drew, 2009).

Numerous guidelines are available in developed countries for cautious use of antimicrobials in food animals but very little has been done in most developing countries to reduce irrational practice of medicines in food animals (Byarugaba, 2004). In Tanzania, there is neither a national maximum acceptable residue level (MRL) for OTC levels in either milk, meat or eggs nor monitoring systems for controlling antimicrobial residues. These are needed to protect consumers from harmful effects of antimicrobial residues.

It is in this context that this study was carried out in order to investigate the effect of cold storage and cooking procedures on the levels of OTC residues in beef from Dodoma Region, Tanzania.

1.2 Antimicrobials Agents

1.2.1 History of antimicrobials

Antimicrobial agents are capable of inhibiting the growth of micro-organisms and are essential for both human and animal health, and welfare. The tetracyclines were discovered from a systematic analysis of about 100 000 soil samples worldwide in the 1940's and 1950's, and around 75 antimicrobial creating moulds were found (Nelson and Levy, 2011).

This was after penicillin discovery, a fungal metabolite, by Fleming in 1928 and its later development by Ernst Chain and Howard Florey during World War II that was central to the antimicrobial revolution (Guardabassi and Kruse, 2008). Penicillin came into clinical use in 1940s and it remains as an outstanding agent in terms of safety and efficacy. It led in the era of antimicrobial chemotherapy by saving the lives of many wounded soldiers during the World War II. Throughout the subsequent two decades, new classes of antimicrobial agents were developed one after another, leading to a golden age of antimicrobial chemotherapy. In 1944, streptomycin, an amino-glycoside antibiotic, was found from the soil bacterium *Streptomyces griseous*. The synthesized antimicrobial agent nalidixic acid, a quinolone antimicrobial, was obtained in 1962. Afterwards, chloramphenicol, tetracycline, macrolide, and glyco-peptide (e.g vancomycin) were discovered from soil bacteria.

1.2.2 Classification of antimicrobials

Antimicrobials used in animals are similar to antimicrobials used in humans. Antimicrobials can be classified based on the mechanisms of antimicrobial action which fall into four categories: inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, and inhibition of protein synthesis (Chambers and Deck, 2009; Wang, 2012). Another classification of antimicrobials is based on that of the United States' Pharmacopoeia (USP, 1999, 2000a–m) such as: Beta-Lactams [Penicillin G, Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin and cepholosporins], Tetracyclines [Chlortetracycline, Oxytetracycline and Tetracycline], Sulphonamides (trimethoprim): Sulphathiazole, Sulphamethazine, Sulphadoxine and Sulphasoxazole; Aminoglycosides [Neomycin, Streptomycin, Gentamycin, Tobramycin and Amikacin], Macrolides [Erythromycin and Tylosin], Quinolones [Ciprofloxacin, sarafloxacin and enrofloxacin], Cyclic Peptides [Vancomycin, Streptogramins and Polymyxins], Lincosamides [Clindamycin], Oxazolidinoes [Linezolid] and Miscellaneous Antibiotics [Chloramphenicol and Dapson].

1.2.3 Use of antimicrobials in food animals

Antimicrobial use in food animals commenced over 50 years ago in order to enhance animal health and performance (Phillips *et al.*, 2004). Use of antimicrobials in animals is generally similar to its use in humans (Olatoye and Ehinmowo, 2010). For this reason, many of the antimicrobials used in animals are also used in humans, leading to the development of the global problem of antimicrobial resistant pathogens (Doyle, 2006; Olatoye and Ehinmowo, 2010). Tetracyclines are common antimicrobials used in animals (Donoghue, 2003), followed by macrolides, lincosamides, penicillins, aminoglycosides, fluoroquinolones, cephalosporins and phenicols (Schwarz and Chaslus-Dancla, 2001). Oxytetracycline is one of the most commonly used antibiotics in livestock production in

Tanzania and other African countries (Olufemi and Agboola 2009; Katakweba *et al.*, 2012). In Nigeria, Kabir *et al*. (2003) and Ezenduka *et al*. (2011), also showed OTC as the commonly used antimicrobial in poultry management. Chlortetracycline is used by 87% of the farms in Trinidad and Tobago. It is used as a feed additive; for the therapeutic and prophylaxis purposes in poultry, pigs and cattle (Adesuyin *et al*., 2004).

Tetracyclines have stronger action on the Gram-positive bacteria and a weaker one on the gram-negative ones; they also have action on mycoplasmas, chlamydiae, rickettsias, spirochetes, actinomycetes, and some protozoa (Sundin, 2003). Tetracycline has bacteriostatic action. Adverse effects caused after the therapeutic use of tetracyclines are known. Tetracyclines should not be given to children of the age of 6–8 years or to pregnant women due to the risk of developing tooth discoloration. Other chronic effects include nephrotoxicity, hepatotoxicity, skin hyperpigmentation in the sun exposed areas and hypersensitivity reactions. Other effects of tetracyclines are hypouricemia, hypokalemia, proximal and distal renal tubular acidosis (Goldfrank *et al.*, 2002).

Tetracyclines, which are commonly used are oxytetracycline (OTC), chlortetracycline (CTC), tetracycline (TC), doxycycline and minocycline. Antimicrobial agents can be administered to animals for treatment (therapy) or prevention of the diseases (prophylaxis). They can also be used as feed additives in farm animals to improve growth rate or feed efficiency in poultry and cattle (Nisha, 2008). Numerous strategies are accessible in developed countries for cautious use of antimicrobials in foodstuff; nevertheless very little is being done in most developing countries to reduce unreasonable use of drugs in food animals (Byarugaba, 2004).

1.2.4 Antimicrobial residues in animals in the world

Antimicrobials were introduced to the veterinary field after the use of antibiotics for the treatment of bacterial diseases in humans. Antimicrobials are used for the management of diseases such as mastitis, arthritis, respiratory cases, gastrointestinal infections and other infectious bacterial diseases (Draisci *et al*., 2001; Donoghue, 2003; Doyle, 2006; Singer and Hofacre, 2006; Löhren *et al.,* 2009).

Like in other countries, antimicrobial use has emerged to be a problem in Tanzania, which need to be addressed accordingly. In Tanzania, regulations regarding antibiotic drug use in farm animals as well as observing and control of their residues are not sufficiently imposed (Nonga *et al.,* 2009). Because of this it may led to the high rate of antimicrobial residues in animals products.

In different countries number of antimicrobial residues have been reported from different researchers (Muriuk *et al.,* 2001; Phillips *et al.,* 2004; Olatoye and Ehinmowo, 2010; Doyle, 2006; Donoghue, 2003; Schwarz and Chaslus-Dancla, 2001; Olufemi and Agboola, 2009; Kabir *et al*.,2003; Adesuyin *et al*.,2004; Ezenduka *et al*., 2011; Nisha, 2008).

A number of studies also have been conducted to determine the levels of AMRs in foodstuffs of animal origin in Tanzania. Mmbando (2004); Karimuribo *et al.* (2005); Mdegela *et al.* (2006); Kurwijila *et al.* (2007); Zuhura *et al.* (2015) and Mgonja *et al.* (2016) reported antimicrobial residues prevalence of 41.2%, 70%, 4.5%, 36%, 70%, 71.1% and 73.6%, respectively, in cattle, chicken meat, milk and eggs in various regions of Tanzania. In addition, studies conducted in Morogoro and Dodoma regions showed high levels of antimicrobial residues in chicken meat, eggs and beef (Nonga *et al.,* 2013; Zuhura *et al.,* 2015; Mgonja *et al.,* 2016).

1.2.5 Resistance to Antimicrobial Agents

Health implication of the usage of antimicrobials in animals could be direct or indirect. The direct way is when humans are exposed to low doses of antimicrobials and following development of resistant strain of microorganisms (Nisha, 2007). Ingesting of meat containing antimicrobial residues over an extended period of time may cause to development of resistant gut flora and pathogens in human beings such as *E. coli* and *Samonella sp* (McEwen and Ferdork-Cray, 2002). Indirect exposure could be consumption foods contaminated with resistant microorganism originating from the use of antimicrobials in animals (McEwen and Ferdork- Cray, 2002). In developing and high income countries, withdrawal period and antimicrobial residues control are conducted in slaughterhouses to prevent harmful residues in food that humans consume (Olatoye and Ehinmowo, 2010).

1.2.6 National Action Plan on Antimicrobial resistance

Antimicrobial resistance (AMR) has been a problem which needs to be addressed accordingly. The National Action Plan addresses those actions needed in order to combat AMR in the country. Awareness and promotion of behavioral change through public communication on AMR, targets human, animal and plant health. AMR knowledge, surveillance and research should be strengthened by establishing national surveillance for AMR, and building capacity for a national reference laboratories for AMR surveillance. AMR national agenda should be established and developed in order to ensure harmonized AMR guidelines, data management and human, animal and plant health settings.

In order to overcome the development and the spread of AMR infections, better hygiene and infection prevention measures are essential. The effective National Action Plan

requires political support from government departments, Ministry of Health, community development and gender (National Action Plan on Antimicrobial resistance, 2017 – 2022)**.**

1.2.7 Tetracycline antibiotics

1.2.7.1 Introduction

Tetracyclines rank among the antimicrobial agents mostly used in the animal food production (Schmidt and Rodrick, 2003). Tetracyclines (TCs) are classified as antibiotics with broad antibacterial and bacteriostatic activity against Gram-positive and Gram-negative bacteria together with intracellular Mycoplasma and Chlamydia (Botsoglou and Fletouris, 2001; Cinquina *et al.,* 2003). Tetracyclines are formed naturally by Streptomyces of the genus Actinobacteria (Alekshun and Levy, 2007). The mode of action by which they work is by binding to the 30s ribosomal subunit and inhibiting protein synthesis. Other members of this class of Tetracyclines are CTC; OTC and doxycycline that are commonly used in food animals and veterinary practice worldwide (Stead *et al.,* 2004).

Nevertheless, the use of this class of antimicrobial in food animals might result in accumulation of its residues or metabolites in animal derived food products, particularly if the withdrawal period is not detected. These residues may pose health hazards to consumers, depending on the type of food and the amount of residue present. Human health problem that could arise from the ingesting of tetracycline residues in meat and other animal products include gastrointestinal disturbances (Ezenduka *et al.,* 2011), teratogenicity and allergic reactions (Akbar- Shahid *et al.,* 2007) development of resistant pathogens in animals and human (Navratilova *et al.,* 2009 and Mishra *et al.,* 2011). Tetracyclines have been reported to cause hypouricemia, hypokalemia, proximal and distal

renal tubular acidosis (Goldfrank *et al.,* 2002). A study by Aamer *et al.* (2000) indicated that ingestion of tetracycline cause teeth and bones effects in small animals.

1.2.8 Chemistry

1.2.8.1 Tetracyclines

The basic structure of TCs is a hydronaphthacene skeleton containing four fused rings. The various TCs mainly differ in their substitution patterns at the C5, C6 and C7 positions. TCs occur as tawny yellowish, odorless, slightly bitter and powdery crystalline bases. Chemically they are four ringed amphoteric compounds differentiated by radical substitutions on the rings. All tetracyclines form salts with both acids and bases (Riviere and Spoo, 2001). They are hygroscopic exhibiting an acid pH in aqueous solutions (Riviere and Spoo, 2001) a crucial factor in the preparation of their extraction buffers from various tissues for analytical purposes. For therapeutic use, they are mainly prepared as their hydrochloride salts (Riviere and Spoo, 2001) especially for parenteral injection. The names of the commonly used tetracyclines are shown in Table 1.1

Table 1.1: Physical and chemical properties of the commonly used tetracyclines

Drug	**Molecular weight**	**PK_a**
Chlortertracyline	478.88	3.3, 7.4, 9.3
Doxycycline	462.46	NA
Minocycline	457.48	2.8, 5.0, 7.8, 9
Oxytetracycline	460.44	NA
Tetracycline	444.43	8.3, 10.2

Source: Riviere and Spoo, 2001).

NA – information not available

1.2.8.1.1 *Oxytetracycline* (C_{22} H_{24} N_2 O_9)

Oxytetracycline occurs mainly in two forms, i.e. the dihydrate form OTC and the hydrochloride form (OTC salt) (Brander *et al.*, 1993). It is very stable compared to chlortetracycline (Brander *et al.*, 1993). This is an important property with respect to its extraction and analytical processes.

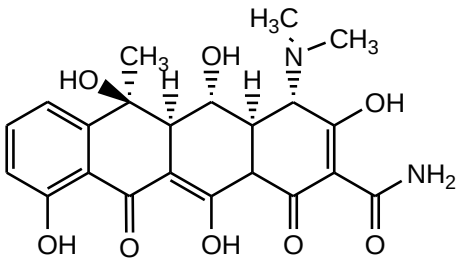

Figure 1.1: **Molecular structure of OTC**

1.2.8.1.2 *Oxytetracycline dihydrate* (C_{22} H_{24} N_2 O_9.$2H_2O$)

Synonyms: Oxytetracycline, Terrafungine

Chemical name: 4-Dimethylamino - 1, 4, 4a, 5, 5a, 6, 11, 12a - Octahydro - 3, 5, 6 10, 12, 12a - hexahydroxy - 6- methyl-l, 11-dioxo aphthadene-2-carboxyamide dihydrate. Its molecular weight (MW) is 496.5 while its molecular structure (Figure 1.2).

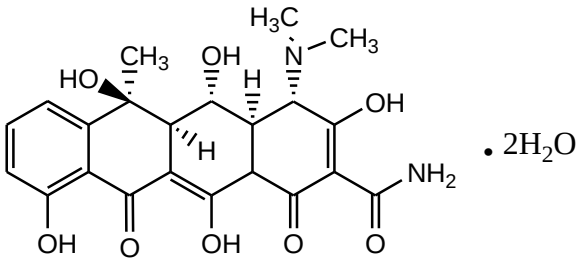

Figure 1.2: **Molecular structure of oxytetracycline dihydrate**

Physically it is a tawny crystalline powder with a specific rotation of –188 ºC to –120 ºC 0 of a 1.0% solution in 0.1N hydrochloric acid. It has a solubility of 1 in 2000 in water and 1 in 100 in ethanol. It deteriorates in solution with a pH < 2 and is rapidly destroyed by alkalis (Rach *et al.,* 2008).

1.2.8.1.3 *Oxytetracycline hydrochloride* (C_{22} H_{24} N_2 O_9. HCl)

This is the most common form of preparation for commercial use (Riviere and Spoo, 2001). Its chemical name is; 4-Dimethylamino- 1,4,4a, 5, 5a, 6,11,12a octahydro – 3,5,6,10,12,12a – hexahydroxy – 6- methy l–1, 11 - dioxonaphthacene –2 carboxyamide hydrochloride (Rach *et al.,* 2008). Physically it is a yellow hygroscopic crystalline powder. While its solubility is 1 in 2 of water and 1 in 45 of ethanol, it is insoluble in ether and chloroform (Rach *et al.,* 2008).

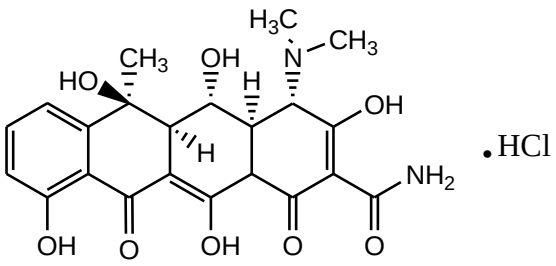

Figure 1.3: **Molecular structure of oxytetracycline hydrochloride**

1.2.9 Analysis of TCs

In most developed countries, consumer responsiveness of the established and potential public health effects of antimicrobial residues in foodstuff and the wish of producers to evade litigation has headed to the expansion of numerous qualitative tests that are accessible for qualitative analysis of tetracyclines, and have numerous sensitivities. For analysis, numerous techniques have been stated in the literature, mainly due to difficulties related to differences in physico-chemical properties between families of compounds (Kaufmann, 2009). The efficiency of this method is based on multi-detection on liquid chromatography attached with tandem mass spectrometry (Bohm *et al.,* 2009). Microbiological, competitive enzyme immunoassay method and bioassay methods have low cost and simplicity and are used for antibiotic qualitative screening purposes; though,

they lack sensitivity and specificity (Pastor-Navarro *et al.,* 2009). Competitive enzyme immunoassay technique has the advantage that it does not include cleanup and extraction procedure of sample as is needed in chromatography techniques (Abhishek *et al.,* 2014). The technique is time saving as it offers optimal Iimit of Detection (LOD) and reproducibility in a very short period of time (Abhishek *et al.,* 2014).

Consequently, chromatographic techniques, such as thin layer chromatography (TLC), and high performance liquid chromatography (HPLC), and capillary electrophoresis (CE), have been used in place of microbiological assays since they are quantitative, precise and give reliable measurements of antibiotic residues in animal tissues or muscles (Cinquina *et al.,* 2003; Zhao *et al.,* 2004; Posyniak *et al.,* 2005).

For the confirmation and quantification purpose of specific antimicrobial residues more sensitive chromatographic and/or immunochemical methods such as validated High Performance Liquid Chromatography (HPLC) (Cinquina *et al.,* 2003; Popelka *et al.,* 2005) and Enzyme Linked Immunosorbent assay (ELISA) are required.

1.2.10 Antimicrobial action

At therapeutic levels, the main OTC has bacteristatic action on sensitive microorganisms. At higher concentrations, it may be bacteriocidal (Brander *et al.,* 1993). A major differences among antibacterial agents is that of bactericidal vs bacteriostatic agents. Bactericidal drugs, cause death and disruption of the bacterial cell, and this include drugs that primarily act on the cell wall (eg, β-lactam antibiotics), cell membrane (eg, daptomycin), and bacterial DNA (eg, fluoroquinolones). Bacteriostatic agents, however, prevent bacterial replication without killing the organism. Most bacteriostatic drugs, such as TCs and macrolides act by inhibiting protein systhesis and sulfonamides act by inhibits

bacterial folic acid synthesis (Surbhi *et al.*, 2011). Oxytetracycline antimicrobial activity is effected by the binding of OTC to base number A 892 and protein S7 on the 30S ribosomal subunit of the 70S bacterial ribosome of susceptible organisms. It then interferes with the folding of the base A 892-1400 region on the r RNA (Chopra and Roberts, 2001). This OTC/30S ribosomal subunit binding effectively interferes with the binding of the amino-acyl-tRNA or activated amino acid to the mRNA/ribosome complex molecule. This action effectively blocks the elongation of a microbial protein peptide chain. This results in the inhibition of microbial protein synthesis of susceptible fast proliferating microorganisms such as bacteria (Chopra and Roberts, 2001; Riviere and Spoo, 2001).

1.2.11 Microbial resistance to TCs

The development of resistance to antimicrobial agents by consuming foodstuff of animal source has been receiving much attention as well as importance of reducing exposure to antibiotic residues in foodstuff's (European Commission of the Communities, 2005). Microbial resistance to tetracyclines is reported to occur. It is caused by the tetracycline - R factor or gene that acts in 3 mechanisms. The plasmid and transposon encoded tetracycline efflux systems is the first mechanism of resistance development to tetracyclines (Chopra and Roberts, 2001). The appearance of the plamid and transposon encoded efflux system on the bacterial membrane inhibits tetracycline buildup in the cell which stops tetracyclines from reaching and interacting with the ribosomes. These tetracyclines encoded efflux systems originate in both the G-ve and G+ve bacteria and contain several plasmid and transposon encoded membrane located resistance proteins that form a comprehensive network of tetracycline efflux proteins. The efflux of tetracyclines from bacterial cells is by active transport (Chopra and Roberts, 2001; Angulo *et al.*, 2004).

The development of plasmid and transposon coded ribosomal defense factors is the second mechanism of resistance to tetracycline (Chopra and Roberts, 2001). These defense factors act as alternate binding sites to TCs, inhibiting TC interaction with base A892 on the 30S subunit of 70S ribosome. A good example of these defense factors is the 'tetm' resistance system found naturally on Streptococci and 'tet O' system found on *Campylobacter jejuni* (Chopra and Roberts, 2001).

Plasmid-mediated detoxification of TCs is the third mechanism of microbial resistance to tetracycline as reported in *Escherichia coli* bacteria grown aerobically. Transposon coded resistance transmission is the more disreputable form because of the flexibility of its transmission. Different plasmids, transposons do not need sophisticated DNA binding mechanism (Chopra and Roberts, 2001).

1.2.12 Cooking effect on antimicrobial residues

In developed countries, several researchers have been interested in assessing whether antibiotic residues can be destroyed by cooking methods, pasteurization, or canning processes (Isidori *et al.,* 2005; Hassani *et al.,* 2008; Hsieh *et al.,* 2011). In Tanzania few studies have been conducted in order to assess heat stability of veterinary drug residues and the studies were carried out using HPLC (Mgonja *et al.,* 2016).

Previous studies have indicated that OTC and erythromycin are heat-labile (Hassani *et al.,* 2008), while chloramphenicol, aminoglycosides, quinolones, clindamycin are heat-stable (Papapanagiotou *et al.,* 2005). Same class of antibiotics were similarly stated to display different heat stability depending on different matrices and cooking procedures involved (Franje *et al.,* 2010). Most heat stability studies assessed the degradation of parent drugs

with a small number of studies agreeing on the possible production of toxic breakdown products (Gratacos-Cubarsi *et al.,* 2007; Franje *et al.,* 2010).

Since meat is always heated before consumption, few reports have been published in Tanzania about the effect of heating on the stability of TCs residues in meat. The destiny of drug residues during heat processing is nevertheless uncertain. There is scarcity of information on the effect of freezing with time on the concentration of antimicrobial residues in foodstuffs of animal origin.

1.2.13 Effect of boiling on antimicrobial residues

Studies regarding cooking process on antimicrobial residues by VanHue *et al.* (2013), showed that TC residues in muscles were reduced by 45.35 to 67.05% after boiling for 9 minutes, 38.17 to 65.74% after deep-frying for 9 minutes, and 38.17 to 48.47% after microwaving for 1 minute. Another study by Javadi *et al.* (2011), showed a reduction in the concentration of doxycline residues after different cooking processes such as boiling. Furthermore a study by Van Egmond *et al.* (2000), reported that mean biological activity of enrofloxacin in pork tissues only reduced to 68% after heat treatment at 134 ºC for 20 minutes. A study by Loksuwan *et al.* (2002), revealed that milk spiked with OTC, TC and CTC at 200, 200, and 400 ppb, respectively, and heated to 63 ºC for 30 minutes had residues reduced by 19.36 to 86.17%. Mishra *et al.* (2011,) stated that pasteurization of milk at 65 ºC for 30 minutes produced no significant reduction in cloxacillin residues in milk

1.2.14 Effect of freezing on antimicrobial residues

Freezing is a form of preservation process for meat by hindering the growth of microorganisms. The destiny of antimicrobial residue concentration when freezing meat

with time is unclear. A study by EI Atabani *et al.* (2014), revealed that out of 100 fresh broiler fillet, 34% were positive for antibiotic residues while only 8 % of frozen samples were positive for antibiotic residues. These findings indicated that freezing may be an important factor in reduction of antibiotic residues in the examined frozen samples.

Using microbiological method, Pavlov *et al.* (2005), found a decreasing level of tobramycin in poultry products stored at -18ºC over a period of 60th days. The residue levels were initially higher in the liver, followed by breast and thigh muscles. While the muscles had no drug residues on the 30th th day, the rate of residue decrease was slower in the liver, with 25% of the residue on the 30th day which subsequently decreased to 14% on the 60th day.

1.3 Research Statement and Justification

Control of diseases in the livestock industry in sub-saharan Africa inluding Tanzania remains to be a challenge. In Tanzania the challenges have been addressed mainly by the use of antimicrobials which include TCs, among the first antibiotics followed by beta lactam antibiotics like penicillins and cephalosporins**.** Behind the success story of these drugs the issue of drug residues emanates as major concern as far as consumers` health is concerned (Karimuribo *et al.,* 2005).

Different scholars in the country have reported the presence of drug residues in uncooked tissues (meat) and products (milk) ranging from 2.8% to 76% in different regions in Tanzania: Mmbando (2004); Kurwijila *et al.* (2006); Mdegela *et al.* (2006); Nonga *et al.* (2013). Despite the reports by these scholars so far, there is limited information on the effect of cooking procedures, cooking time and storage on the levels of TCs residues and this creates a scientific gap of knowledge which needs to be addressed. It is of importance

to address how serious the residues are after cooking and the levels be known to the public in Tanzania. Since it is evident that temperatures have effect on the levels of antimicrobial residues (Salah *et al.,* 2013) it is then important that this be evaluated at our context as cooking is practiced by many Tanzanias during preparation of meat. Practice on the effect of storage on different foods before cooking is also done

The study findings will provide essential information on the risk of antimicrobial residues in meat chain supplies in Tanzania. Consequently, the study findings will provide vital evidence-based information not only for essential production of high quality meat products, but will also significantly contribute to the development of evidence-informed food safety policies. Knowing levels of antimicrobial residues will help in alleviating antimicrobial resistance in Tanzania by sending the message to the livestock extension officers who will advise livestock owners on the seriousness of AMR and therefore raise awareness on AMR so that the livestock owners may change. Furthemore, the study findings will contribute to the antimicrobial residues surveillance initiatives to promote good dairy farming practices, including proper use of antimicrobials for better livestock keeping in Tanzania.

1.4 Objectives of the study

1.4.1 Overall objective

To study the effect of cold storage and cooking procedures on the levels of OTC residues in beef in Tanzania.

1.4.2 Specific objectives

i. To assess the knowledge, attitude and practice of beef consumers on OTC residues in Dodoma region.

ii. To determine the level of OTC residues in beef samples.

iii. To assess the influence of various cooking procedures on OTC residues in the beef samples.

iv. To evaluate the influence of cold storage on OTC residues in beef samples.

1.4.3 Research questions

i. What are the levels of antimicrobial residues in beef in Tanzania?

ii. Are antimicrobial residues in beef supplies a serious problem in Tanzania?

iii. Are the producers, people involved in the slaughter process, beef outlets, store food vendors and beef consumers aware of the risk of consuming food items with antimicrobial residues?

iv. What are the effects of different cooking methods (boiling and barbeque)?

v. Does cold storage affect the concentration of OTC residues?

1.5 Limitations of the Study

i. HPLC equipment was not working for 7 months, which caused beef samples to be stored for a long time before analysis. This might have affected the results (Tansel *et al.*, 2006).

ii. There were unanticipated delays for 4 months in delivering the HPLC reagents from the supplying company in Johannesburg South Africa.

1.6 Definitions of key terms

All over the study, a number of terminologies have been used which are used in Pharmacology and Toxicology and the following definitions as used by the WHO/FAO JECFA committee have been provided;

1.6.1 Antibiotic, drug or chemical residue

This is the deposition or accumulation of a parent compound or its metabolites or decomposition products within cells, tissues or organs following the use of antimicrobials for prophylaxis, treatment of disease or as a feed additive to promote growth and feed efficiency. These residues or accumulations have potential toxicological significance (Reig and Toldra, 2008; Hisham, 2013).

1.6.2 Maximum residue limits (MRLs)

This is the maximum permissible level or concentration of a drug or chemical in or on a feed or food at a specified time of slaughter and harvesting, processing, storage and marketing up to the time of consumption by animal or human (European Commission, 2001; Hisham, 2013). MRLs are established based on extensive toxicological studies of potential risks of ingesting to humans (Donoghue, 2003; Myllyniemi, 2004; Nisha, 2008).

1.6.3 Extra-label use of antimicrobial

Antimicrobial use without label specifications or manner, or ways not suggested by the company is referred to as extra -label use. The labels of antimicrobial must contain all necessary information concerning the use of the drug such as classes in which it may be used, illnesses for which it may be administered, quantity and course of administration as well as withdrawal time (Schwartz and Chaslus-Dancla, 2001).

1.6.4 Acceptable Daily Intake (ADI)

This is the maximum daily intake of a drug or chemical, which can be ingested in a lifetime without appreciable or deleterious effects to the health of the consumer considering the facts available at the time. It is expressed in mg of the drug (chemical) per kg body weight per day. A 60 kg per person is considered standard. The values for OTC and TC are 0.3 µg/kg bwt/day and 0.5 µg/kg bwt/day respectively (Hisham, 2013 and FAO/WHO, 2014).

1.6.5 Bioavailability of a drug

This refers to both the rate and extent of absorption of a drug. The extent of absorption means the fraction or percentage of the oral or parenteral dosage form, which reaches the systemic circulation intact.

1.6.6 Drug/Chemical withdrawal period

This is the time required for the depletion of a toxicologically potential residue to reach a safe concentration as defined by MRL (Vranic *et al.,* 2003; Hisham, 2013). It also refers to the time interval between termination of treatment to an animal and the time of consumption of its products such as milk and meat on slaughter. Food security is the only intention why both MRLs and withdrawal periods are recognized (Kaferstein, 2003).

1.6.7 Limits of detection (LOD)

The limit of detection refers to the minimum amount of residues or analytes needed to be present in the test samples in order to obtain a positive result. The LOD of most available test kits are usually set at or below the MRLs. When the amount of the analyte such as antimicrobial residue present in the test sample falls below the LOD of the test kit, the kit recognizes or interprets the result as a negative case but vice versa, if the concentration of the analyte equals the LOD is above it.

1.7 General Methodology

1.7.1 Study area

Figure 1.4: Map of Tanzania showing Dodoma Region

This study was carried out in Dodoma region in Tanzania (Figure 1). Dodoma Region lies at 4º to 7º latitude South and 35º – 37º longitude East. The region is centrally positioned in Tanzania and is bordered by four regions namely, Manyara in the North, Morogoro in the East, Iringa in the South and Singida in the West.

The highest part of the region is a plateau rising gradually from 830 metres in Bahi Swamps to 2000 metres above sea level in the highlands north of Kondoa District. Dodoma region comprises of seven districts namely Kongwa, Bahi, Kondoa, Mpwapwa, Chamwino, Dodoma Rural and Dodoma Urban.

1.7.2 Ethical considerations

This study was approved by the Directorate of Research and Postgraduate Studies Committee of Sokoine University of Agriculture. The study was performed in accordance with existing Tanzanian legislation and in line with good scientific conduct with respect to safety and legislative aspects. Participants of the study were informed orally about the study objectives and were given an informed consent form written in Swahili to sign. Participation was voluntary and participants were free to choose whether or not to participate in the study and were allowed to drop off at any point. All information collected was confidential and anonymities of respondents were observed.

1.7.3 Study design and Sample size determination

The study adopted a cross sectional design using purposively sampling technigue. The Districts in Dodoma region with slaugherhouses and butcher were involved in this study. The sample size was calculated according to the formula by Magaret, (2004):

$$\mathbf{N = Z^2 \times P\,(1\text{-}P)/d^2}$$

Where: Z = confidence level/ confidence interval (95% CI) 1.96,

P = Estimated prevalence/proportion, 1-P = the probability of having no hazards disease,

d = precision level 5% (0.05), N = sample size

For meat samples, the sample size was calculated based on the 21% prevalence reported by Muriuki *et al.* (2001)

1.7.4 Participants' interview

A questionnaire involved semi-structured questionnaires, with both open- and closed-ended questions were administered to participants living in Dodoma region, Tanzania. The study participants were identified by using a multi-stage stratified sampling procedure and

simple random sampling technique was applied to select the households; 254 informants were interviwed. Both quantitative and qualitative research methods were used to explore the adult residents insights.

1.7.5 Collection of meat samples

Beef samples were purposevely collected from slaughterhouses and butcheries. About 250 grams of each meat sample were tranfered into clean sterilized small polythene bags, placed in a cool box with ice packs and sent to the Zambia Agriculture Research Institute (ZARI) laboratory. The collected samples were kept at the -20°C until the time for carrying out extraction and analysis by HPLC.

1.7.6 Heat treatment of meat samples

1.7.6.1 Boiling procedure

One hundred gram (100 g) weighed sample was placed into a strainer, immersed in about one litre of boiling water. Water was added during boiling time to keep the volume of water for 30 minutes. It was then allowed to cool before extraction and analysis of OTC residues.

1.7.6.2 Barbecue preparation

One hundred gram (100 g) weighed sample was barbecued well for 20 minutes and allowed to cool before extraction and analysis of OTC residues.

1.7.7 Extraction of OTC

1.7.7.1 Samples extraction and clean up

Oxytetracycline was extracted by organic solvents from aqueous alkaline solutions method (Froehlich, 2013 and Mgonja *et al.*, 2016) as summarized (Figure 1.4).

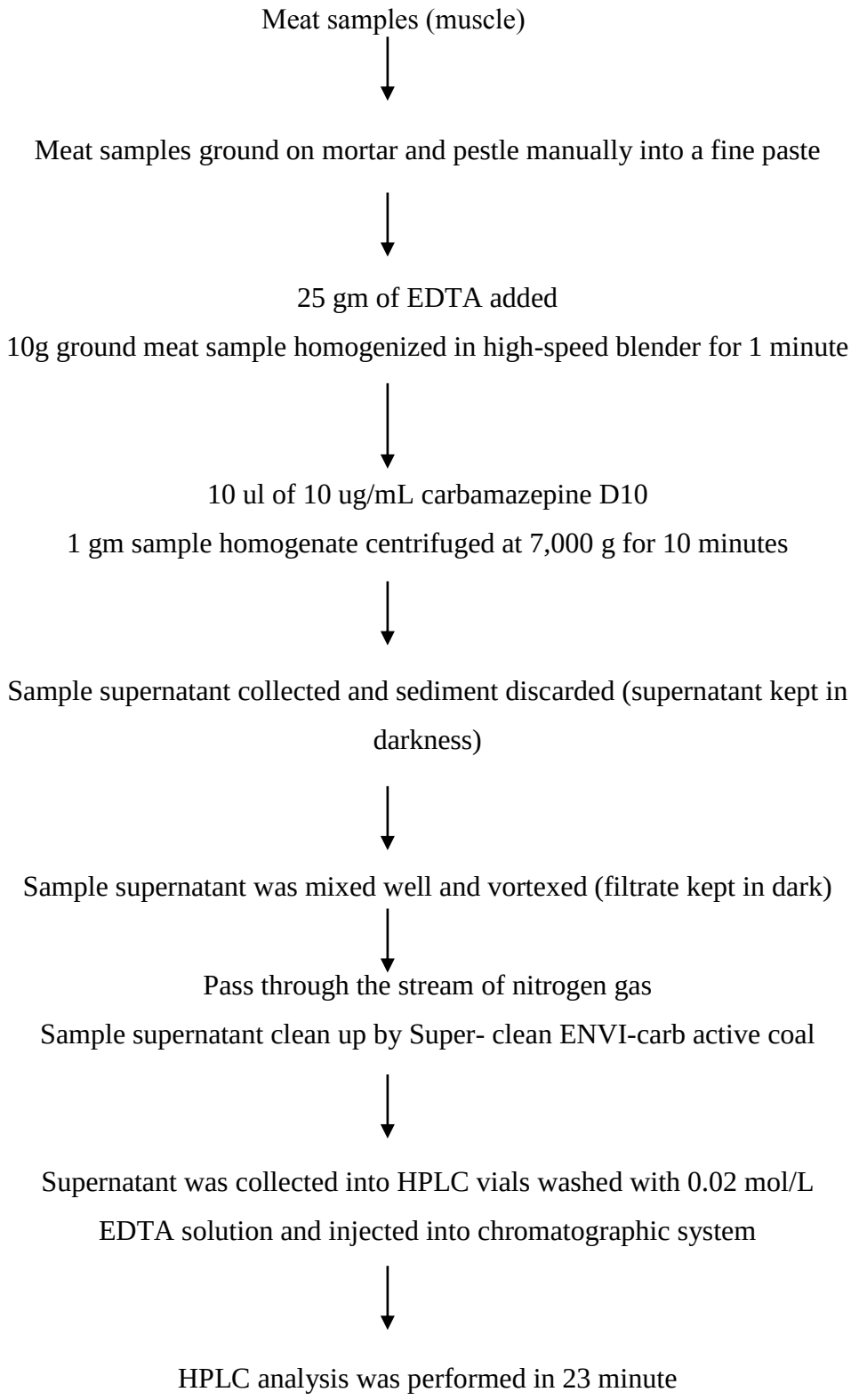

Figure 1.5: **Extraction and clean up procedure of OTC from meat samples**

1.7.8 Clean up and drying procedure of OTC from meat samples

Supernatant clean up was done by Superclean ENVI-carb active coal, then pass through the stream of nitrogen gas. This method of cleaning is cheap compared to Solid phase extraction and 6 - 8 samples can be dried simultaneously.

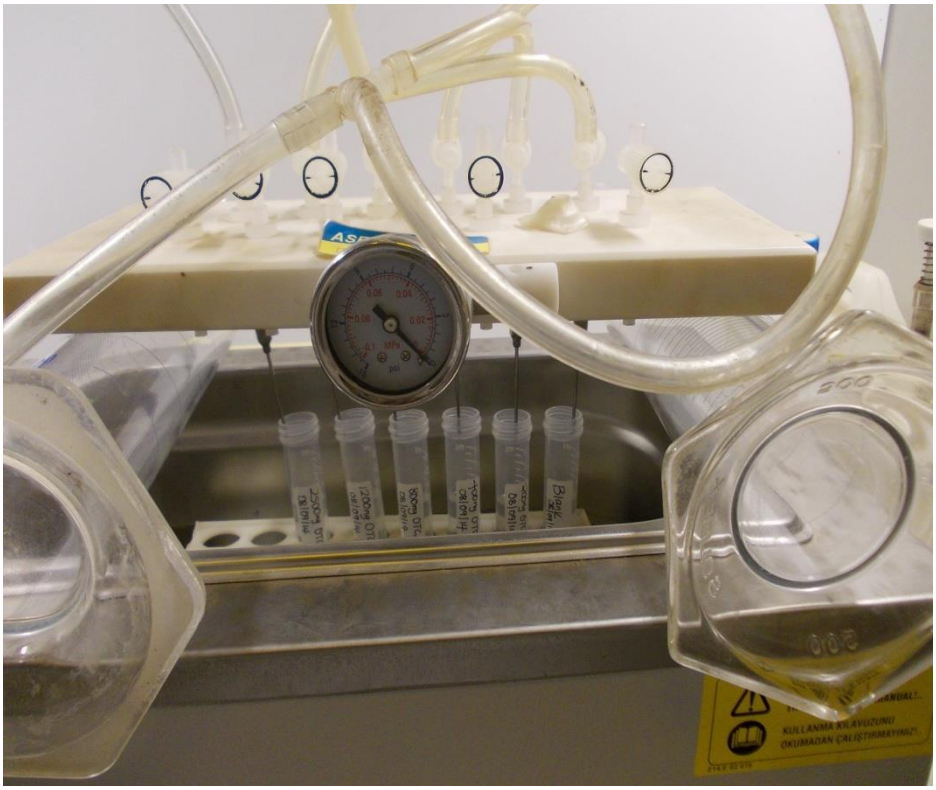

Figure 1.6: Set up apparatus for drying procedure

1.7.9 Recovery experiment

Sample recovery was determined with blank bovine muscle spiked at 200 ng/g. To test the recovery, 10 samples were prepared that contained 1 g of homogenized muscle tissue of the negative control. They were spiked with 20 µL of 10 ug/mL spiking solution equivalent to 200 ng/g of the analyte. Four samples were used to calculate the recovery mean and six samples were used to calculate the recovery-corrected content. The recovery percentage of OTC obtained in this study was 68%.

1.7.10 High performance liquid chromatography (HPLC-MS)

The HPLC was equipped with DAD detector and mass spectroscopy (Model Agilent Technologies 6130 Quadrupole LC/MS; Germany) to target the flowing parent ions using Single Ion Monitoring (SIM) mode 461 mass per charge ratio (m/z) for OTC. The analytical column was reversed-phase Eclipse XDB C-18. 4.6 x 150 mm set at a flow rate of 0.5 ml/min. The column temperature was 25°C. Mobile phase A was HPLC water with 0.1% formic acid and solvent C was Acetonitrile with 0.1% formic acid. The starting mobile phase composition at 0 min was 85% Water: 15% Acetonitrile at 0.5 ml/min. The wavelength of the DAD detector was set at 275 nm and 355 nm respectively. Internal calibration curves were prepared by spiking the blank matrix with pure chromatographic standard solutions in the range between 200 ng/g and 2500 ng/g injected for each compound and estimates of the amount of the analytes in samples were interpolated from these graphs (Froehlich, 2013; Mgonja *et al*., 2016).

Figure 1.7: HPLC equipment

1.8 Organization of the Thesis

The PhD thesis has been developed in "publishable manuscripts format of the Sokoine University of Agriculture". The first chapter addresses introduction, literature review, problem statement justification, objectives. Chapter two contains findings on the assessment on knowledge, attitude and practice in relation to beef consumption among residents living in Dodoma, Tanzania (Manuscript i). Chapter three contains findings on a validated and modified method that used in the present study. This article presents findings on "A simple and sensitive method for the detection of OTC level in ready-to-eat beef by liquid chromatography-mass spectrometry" (Paper 1). Chapter four contains findings of the study conducted in fulfilling specific objective ii resulting to a manuscript on OTC residue levels in beef in Dodoma region, Tanzania (Manuscript ii). Chapter five contains findings on effect of heat treatment on OTC residues in beef in Dodoma, Tanzania (Manuscript iii) and Chapter six contains findings of the study on effect of cold storage on OTC residues in beef in Dodoma, Tanzania (Manuscript iv). The format and writing style of the four manuscripts mentioned above follow the requirements of the targeted journals.

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CHAPTER TWO

MANUSCRIPT I

Knowledge, attitude and practice in relation to Antimicrobial residues in beef among residents in Dodoma Region.

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Knowledge, attitude and practice in relation to antimicrobial residues in beef among residents in Dodoma Region.

Abstract

The safety of food of animal origin is of concern in the developing countries. Some of the antimicrobial agents that are used for the treatment of animal diseases seems to occur in the animal products. The knowledge, attitude and practice in relation to OTC residues in beef among residents in Dodoma Region, Tanzania was evaluated. A cross sectional study included interviewing 254 randomly chosen respondents using questionnaires targeting adult residents living in and around the slaughterhouses since there is a potential for consumption of more meat. Fifty two percent of the respondents were not aware of drug residues, 57% never heard about drug residues in food of animal origin such as milk and meat, 35% know residues can be harmful to human and 61% did not know if animals are treated with antimicrobial drugs when they were sick. Only 27% of the respondents knew common antimicrobial agents that cause residues in animal meat and milk and were able to mention. Majority of respondents (74%) did not know any method for the prevention of antimicrobial residues. Fifty six percent of the age group of 20-35 years purchased meat from butcheries. Secondary school (68.4%) and College (52.9%) respondents purchased meat from butcheries compared to informal (23.8%) and primary (49.2%) respectively that purchased meat locally within the villages. Majority of informal (66.7%) and primary (47.6%) respondents purchased meat locally within the villages. The differences were strongly significant $p< 0.0001$. Women (57.1%) used one hour to prepare meat. Age group 20-35 years (88.1%) prepared meat by cooking. Age group of 36-45 years prepared meat for 1 hour and 2 hours. College respondents (68.8%) barbequing meat compared to smoking and freezing. The results in this study indicate that respondents had low

knowledge and awareness on antimicrobial use and drug residues. This might be due to low level of education of respondents as majority of them had informal and primary education. Many of the drug respondents were not aware of the drug residues and did not know antibiotic residues can have effects in human health. Community based health education and promotion on antimicrobial use and preventing drug residues is highly recommended to this population.

Key words: knowledge, attitude, practice, residues in beef, residents, Dodoma Region, Tanzania.

INTRODUCTION

Tanzania has one of the largest ruminant livestock populations in Africa. It is ranked as a second country with largest herd in Sub Saharan Africa: United Republic of Tanzania (URT, 1994). It has 21.3 million cattle of which about 680 000 are dairy cattle, which are mainly crosses of Friesian, Jersey, and Ayrshire breeds with the Tanzania Shorthorn Zebu (NSCA 2007/2008). Of the meat producing animals, cattle are the most important as they produce most of the red meat and contribute 53% of total meat production, whereas sheep and goats contribute about 22% while the remaining percentage is contributed by pigs, poultry and non-conventional animals (URT, 1994).

Control of diseases in the livestock industry in sub - sahan Africa including Tanzania remains to be a challenge**.** The treatment of animals due to the infectious diseases has become a problem due to indiscriminate and frequent use of antibiotics (Nisha, 2008). Antimicrobial agents are among the drugs for the treatment of diseases in livestock in developing countries (Karimuribo *et al.,* 2005; Nonga *et al.,* 2009). Oxytetracycline

(OTC) is the most commonly used antibiotic in livestock production in Tanzania and other African countries (Olufemi and Agboola, 2009; Katakweba *et al.,* 2012).

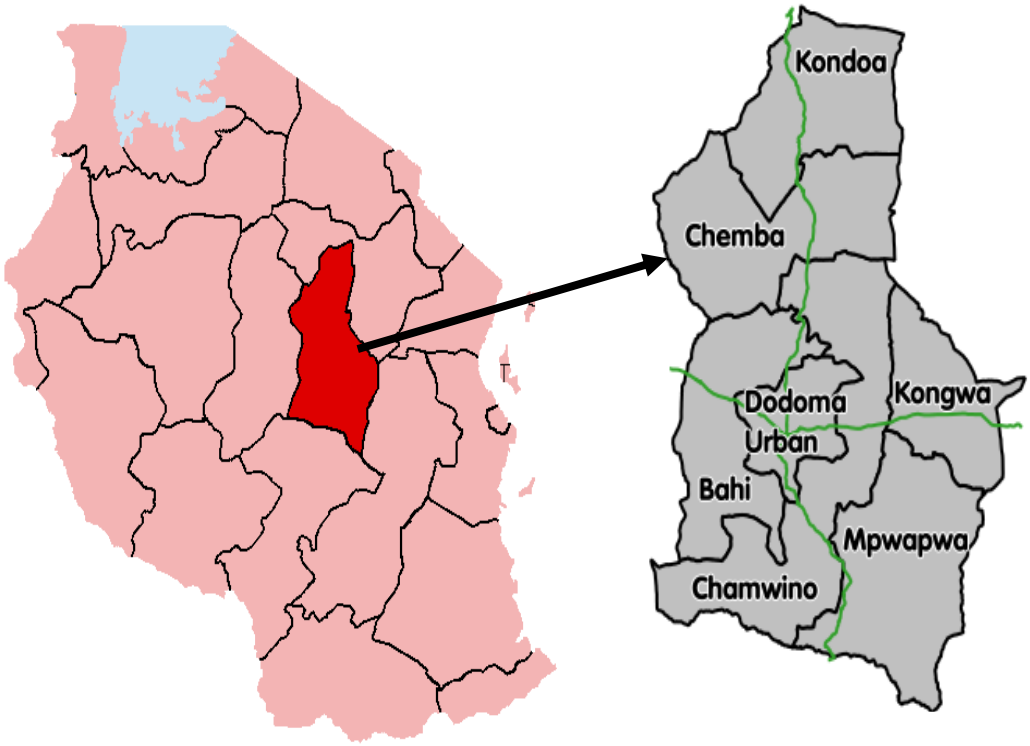
According to Aryal, (2001) the practice of using antimicrobials in animals is a worldwide problem owed to antimicrobial resistance; nearly all the antimicrobials used in animals are also used in human medicine. Some of drugs such as OTC, are used commonly to treat and protect cattle against several infections (Katakweba *et al.,* 2012). OTC is used in livestock for prophylactic, therapeutic treatment, and as a growth promoter due to its broad spectrum activity (Karimuribo *et al.,* 2005). The ingestion of unacceptable levels of OTC deposits in meat causes gastrointestinal disturbances, hypersensitivity, bone and teeth problems in children and development of bacterial resistance (Larkin *et al.,* 2004). Although the extent of antibiotic use in animals in developing countries is unknown, a study from Kenya reported that tetracyclines, sulfonamides and trimethoprim, nitrofurans aminoglycosides, beta-lactams and the quinolones are the most commonly used drugs in food-producing animals in Kenya (Mitema *et al.,* 2001). This study also revealed that the tetracyclines contributed approximately 55% of the total consumption.

Informal access to antimicrobial and absence of awareness may lead to mismanagement and overuse of the antimicrobial which result in the failure of observing withdrawal periods (Nisha, 2008). Cinquina *et al.* (2003), reported withdrawal period of 5–20 days before animals are slaughtered. Therefore, the aim of this study was to assess the knowledge, attitude and practice in relation to antimicrobial residues among beef consumers in Dodoma, Tanzania.

MATERIALS AND METHODS

This study was carried out in Kongwa, Kondoa, Chamwino, Dodoma Rural and Dodoma

Urban Districts in Dodoma region, Tanzania (Figure 2.1).



(a) Tanzania (b) Dodoma Region

Figure 2.1: Map of Tanzania (a) showing Dodoma Region(b)and its districts

Data collections

Data collection included individual interviews using questionnaires targeting 254 residents living in Dodoma Region. Both closed and open-ended questions were included in the questionnaires. The information included demographic characteristics (age, education, occupation and marital status), where they buy meat, how often they consume meat in their family, amount consumed per meal, how they prepare beef before consuming and how much time it takes to prepare), knowledge about antimicrobial residues in meat, effects of antimicrobial residues in human being, common antimicrobial agents which can cause residues and methods used to prevent antimicrobial residues.

Data analysis

Data obtained from questionnaires was entered into Excel database, analysed by using SPSS version 20 software, Chi square test and the P-value.

RESULTS

Respondent's demographic information

Demographic information on the 254 respondents regarding sex, age, education, marital status and occupation is summarized in Table1. The majority of the respondents (87%) were females and 13% were males (Table 2.1).

Table 2.1: **Demographic characteristics of respondents (n=254)**

Characteristic		Frequency (n)	Percent (%)
Sex	Male	33	13.0
	Female	221	87
Age group in years	20-35	168	66.1
	36-45	66	26
	46-55	16	7.1
	56-65	2	0.8
Education level	Informal	21	8.3
	Primary	63	24.8
	Secondary	136	53.5
	College	34	13.4
Marital status	Single	86	33.9
	Married	162	63.8
	Widow	3	1.2
	Divorced	3	1.2
Occupation	Peasant farmers	67	26.4
	Business	138	54.3
	Student	49	19.3

Respondents‘ practice about beef

Most of the respondents purchased meat from butcheries 58%, followed by 36% who purchased it locally within the village (Table 2.2). Fifty three percent of the respondents bought beef while 26% bought liver. Respondents were consuming beef 3.5 ± 1.3 times per month and majority of respondents (69%) were consuming meat three to five times per month. Cooking was the most common method (80%) of beef preparation and majority (51%) of respondents took an hour to prepare beef before consuming.

Table 2.2: Respondents' practice about beef (n=254)

	Characteristic	Frequency (n)	Percent
Source of meat	Supermaket	15	5.9
	Butcheries	147	57.9
	Locally	92	36.2
Meat intake per month	1 -2 times	60	23.6
	3 -5 times	174	68.5
	6 -8 times	20	7.9
Meat part	Liver	66	26.0
	Kidney	22	8.7
	Neck	32	12.6
	Muscle	134	52.8
Meat preparation	Eating raw	0	0
	Cooking	202	79.5
	Barbeque	27	10.7
	Smoking	25	9.9
	Freezing	0	0
Cooking time	15 mins	1	.4
	30 mins	69	27.2
	1 hour	120	51.2
	2 hours	54	21.3

Respondents‘ knowledge on antimicrobial use and drug residues.

Fifty two percent of respondents did not know drug residues and 57 % never heard about drug residues in food of animal origin such as milk and meat (Table 2.3). Majority of respondents 65% knew about the effects of residues in human, but only 39.% were aware that animals are treated with antimicrobials when they got sick. Only 31%) of respondents mentioned same antimicrobials they knew while only 26% were able to mentioned the methods for prevention of drug residues in animal meat and milk.

Table 2.3: Respondents' knowledge on antimicrobial use and drug residues

Characteristic		Frequency (n)	Percent
Awareness on drug residues	Yes	122	48
	No	132	52
Drug residues in food	Yes	110	43.3
	No	144	56.7
Effects of residues in human	Yes	89	35
	No	164	64.6
Animals treated with antimicrobial drugs	Yes	100	39.4
	No	154	60.6
Common antimicrobial drugs	Yes	68	26.8
	No	186	73.2
Mentioned any antimicrobial drug	Yes	78	30.7
	No	176	69.3
Prevent antimicrobial drug	Yes	67	26.4
	No	187	73.6

The study indicates that majority of respondents' purchases meat from butcheries. While 75.8% of the men purchased meat from the butcheries than from meat market and locally within the village, majority of women 53.4% purchased muscle tissue while 30.3% of men purchased liver. Fifty six percent of the age group 20-35 purchased the meat tissues. Secondary (68.4%) and College (52.9%) respondents purchased meat from butcheries

compared to informal (23.8%) and primary (49.2%) respectively. Majority of informal (66.7%) and primary (47.6%) respondents purchases meat locally within the villages. The differences were strongly significant $p< 0.0001$.

Majority of the respondents (Table 2.5) prepare meat by cooking. Women (57.1%) took one hour to prepare meat. Age group 20-35 (88.1%) prepared meat by cooking. Age group 36-45 prepared meat for 1 hour and 2 hours respectively. College (68.8%) respondents' preferred barbequing meat compared to smoking and freezing.

The age group (35-45) seemed to be more aware of drug residues compared to the other group (56.1%). The same age group had heard about drug residues in animal-origin (54.5%) and knew that residues are harmful to human (65.2%), Table 2.6.

Awareness on the drug residues seemed to be better based on the education levels. Knowledge on antimicrobial drugs increased as the education increased, informal<primary<secondary<college. The differences were strongly significant $p<0.0001$. The female participants seemed to be unware of the knowledge on antimicrobial drugs (62%) compared to men. Students had more knowledge on antimicrobial use (55%) compared to peasant and businesspersons. The differences between them were strongly significant $p<0.0001$.

Table 2.4: Relationship between source of meat and demographic characteristics of respondents (n=254)

Characteristic		Total n = 254	Source of meat n (%)				Part of meat n (%)				
			Meat market	Butcher	Buying locally	P value	Liver	Kidney	Neck	Muscle	P value
Sex	Male	33	1 (3.0)	25 (75.8)	7 (21.2)	> 0.05	10 (30.3)	4 (12.1)	3 (9.1)	16 (48.5)	> 0.05
	Female	221	14 (6.3)	122 (55.2)	85 (38.5)		56 (25.3)	18 (8.1)	29 (13.1)	118 (53.4)	
Mean age in years	32±15.56										
Age group in years	20-35	168	10 (6.0)	100 (59.5)	58 (34.5)		38 (22.6)	14 (8.3)	22 (13.1)	94 (56.0)	
	36-45	66	3 (4.5)	33 (50)	30 (45.5)	> 0.05	19 (28.8)	8 (12.1)	8 (12.1)	31 (47.0)	> 0.05
	46-55	18	2 (11.1)	12 (66.7)	4 (22.2)		9 (50.0)	0 (0)	1 (5.6)	8 (44.4)	
	56-65	2	0 (0)	2 (100)	0 (0)		0 (0)	0 (0)	1 (50.0)	1 (50.0)	
Education level	Informal	21	2 (9.5)	5 (23.8)	14 (66.7)		3 (14.3)	1 (4.8)	2 (9.5)	15 (71.4)	
	Primary	63	2 (3.2)	31 (49.2)	30 (47.6)	< 0.0001	14 (22.2)	8 (12.7)	10 (15.9)	31 (49.2)	> 0.05
	Secondary	136	5 (3.7)	92 (68.4)	38 (27.9)		33 (24.3)	13 (9.6)	17 (12.5)	73 (53.7)	
	College	34	6 (17.6)	18 (52.9)	10 (29.4)		16 (47.1)	0 (0)	3 (8.8)	15 (44.1)	
Marital status	Single	86	7 (8.1)	61 (70.9)	18 (20.9)		24 (27.9)	5 (5.8)	14 (16.3)	43 (50.0)	
	Married	162	6 (3.7)	85 (52.5)	71 (43.8)	< 0.0001	42 (25.9)	17 (10.5)	16 (9.9)	87 (53.7)	> 0.05
	Widow	3	2 (66.7)	0 (0)	1 (33.3)		0 (0)	0 (0)	1 (33.3)	2 (66.7)	
	Divorced	3	0 (0)	1 (33.3)	2 (66.7)		0 (0)	0 (0)	1 (33.3)	2 (66.7)	
Occupation	Peasant	67	6 (9.0)	31 (46.3)	30 (44.8)		16 (23.9)	8 (11.9)	10 (14.9)	33 (49.3)	
	Business	138	4 (2.9)	80 (58.0)	54 (39.1)	< 0.01	33 (23.9)	10 (7.2)	19 (13.8)	76 (55.1)	> 0.05
	Student	49	5 (10.2)	36 (73.5)	8 (16.3)		17 (34.7)	4 (8.2)	3 (6.1)	25 (51.0)	

Table 2.5: Relationship between meat preparation and demographic characteristics of respondents (n=254)

charactaristics		Total n = 254	Meat preparation n (%)						Duration of meat preparation n (%)				
			Eating raw	Coooking	Barbequing	Smooking	Freezing	P value	¼ hour	½ hour	1 hour	2 hour	P value
Sex	Male	33	0 (0)	32 (97.0)	0 (0)	1 (3.0)	0 (0)	> 0.05	0 (0)	7 (21.2)	18 (54.5)	8 (24.2)	> 0.05
	Female	221	2 (0.9)	17 (76.9)	25 (11.3)	22 (10.0)	2 (0.9)		1 (0.5)	62 (28.1)	112 (57.7)	46 (20.8)	
Mean age in years	32±15.56												
Age group in years	20-35	168	2 (1.2)	148 (88.1)	5 (3.0)	13 (7.7)	0 (0)		1 (0.6)	51 (30.4)	83 (49.4)	33 (19.6)	
	36-45	66	0 (0)	42 (63.6)	14 (21.2)	8 (12.1)	2 (3.0)	< 0.01	0 (0)	12 (18.2)	38 (57.6)	16 (24.2)	> 0.05
	46-55	18	0 (0)	10 (55.6)	6 (33.3)	2 (11.1)	0 (0)		0 (0)	6 (33.3)	8 (44.4)	4 (22.2)	
	56-65	2	0 (0)	2 (100)	0 (0)0	0 (0)	0 (0)		0 (0)	0 (0)	1 (50.0)	1 (50.0)	
Education level	Informal	21	2 (98)	15 (71.4)	0 (0)	4 (19)	0 (0)		0 (0)	5 (23.8)	10 (47.6)	6 (28.6)	
	Primary	63	0 (0)	60 (95.2)	3 (4.8)	0 (0)	0 (0)	<	0 (0)	25 (39.7)	34 (54.0)	4 (6.3)	< 0.05
	Secondary	136	0 (0)	102 (75.0)	19 (14.0)	13 (9.6)	2 (1.5)	0.0001	1 (0.7)	31 (22.8)	71 (52.2)	33 (24.3)	
	College	34	0 (0)	25 (73.5)	3 (68.8)	6 (17.6)	0 (0)		0 (0)	8 (23.5)	15 (41.1)	11 (32.4)	
Marital status	Single	86	2 (2.3)	78 (90.7)	3 (3.5)	3 (3.5)	0 (0)		0 (0)	30 (34.9)	34 (39.5)	22 (25.6)	
	Married	162	0 (0)	120 (74.1)	22 (13.6)	18 (11.1)	2 (1.2)	< 0.01	1 (0.6)	38 (25.3)	94 (58.0)	29 (17.5)	> 0.05
	Widow	3	0 (0)	1 (33.3)	0 (0)	2 66.7()	0 (0)		0 (0)	1 (33.3)	0 (0)	2 (66.7)	
	Divorced	3	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	2 (66.7)	1 (33.3)	
Occupation	Peasant	67	2 (3.0)	59 (88.1)	1 (1.5)	3 (4.5)	2 (3.0)		0 (0)	23 (34.3)	35 (52.2)	9 (13.4)	
	Business	138	0 (0)	106 (76.8)	19 (13.8)	13 (9.4)	0 (0)	< 0.01	1 (0.7)	33 (23.9	70 (50.7)	34 (24.6)	> 0.05
	Student	49	0 (0)	37 (75.5)	5 (10.2)	7 (14.3)	0 (0)		0 (0)	13 (26.5)	25 (51.0)	11 (22.4)	

Table 2.6: Relationship between respondents awareness on drug residues and demographic characteristics of (n=254)

Characteristic		Total n = 254	Awareness on drug residues			Ever heard about drug residues in animal-origin food			Drug residues can be harmful human		
			Aware	Unaware	P value	YES	NO	P value	Aware	Unaware	P value
S Sex	Male	33	20 (60.6)	13 (39.4)	> 0.05	15 (45.5)	18 (54.5)	> 0.05	24 (72.7)	9 (27.3)	> 0.05
	Female	221	102 (46.2)	119 (53.8)		95 (43.0)	128 (57.0)		141 (63.8)	80 (36.2)	
Mean age in years	32±15.56										
Age group in years	20-35	168	74 (44.0)	94 (56.0)		65 (38.7)	103 (61.3)		108 (64.3)	60 (35.7)	
	36-45	66	37 (56.1)	29 (43.9)	> 0.05	36 (54.5)	30 45.5)	< 0.05	43 (65.2)	23 (34.8)	> 0.05
	46-55	18	9 (50.0)	9 (50.0)		7 (38.9)	11 (61.1)		12 (66.7)	6 (33.3)	
	56-65	2	2 (100)	(0 (0)		2 (100)	0 (0)		2 (100)	0 (0)	
Education level	Informal	21	3 (14.3)	18 (85.7)		4 (19.0)	17 (81.0)		12 (57.1)	9 (42.9)	
	Primary	63	24 (38.1)	39 (61.9)		23 (36.5)	40 (63.5)	< 0.05	31 (49.2)	32 (50.8)	< 0.01
	Secondary	136	77 (56.6)	59 (43.4)	< 0.001	67 (49.3)	69 (50.7)		3 (68.4)	43 (31.6)	
	College	34	18 (52.9)	16 (47.1)		16 (47.1)	18 (52.9)		29 (85.3)	5 (14.7)	
Marital status	Single	86	43 (50.0)	43 (50.0)		35 (40.7)	51 (59.3)		53 (61.6)	33 (38.4)	
	Married	162	77 (47.5)	85 (52.5)	> 0.05	72 (44.4)	90 (55.6)	> 0.05	108 (66.7)	54 (33.3)	< 0.05
	Widow	3	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)		3 (100)	0 (0)	
	Divorced	3	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		1 (33.3)	2 (66.7)	
Occupation	Peasant	67	28 (41.8)	39 (58.2)		22 (32.8)	45 (67.2)		33 (49.3)	34 (50.7)	
	Business	138	72 (52.2)	66 (47.8)	> 0.05	68 (49.3)	70 (50.7)	> 0.05	98 (71.0)	40 (29.0)	< 0.01
	Student	49	22 (44.9)	27 (55.1)		20 (40.8)	29 (59.2)		34 (69.4)	15 (30.6)	

Table 2.7: Relationship between respondents 'awareness on antimicrobials and demographic characteristics (n=254)

Characteristic		Total n = 254	Animals are treated with antimicrobial drugs			Common antimicrobial agents causing drug residues			Method to prevent drug residues		
			Aware	Unaware	P value	YES	NO	P value	Aware	Unaware	P value
Sex	Male	33	16 (48.5)	17 (51.5)	> 0.05	13 (39.4)	20 (60.60	> 0.05	6 (18.2)	27(81.6)	> 0.05
	Female	221	84 (38.0)	137 (62.0)		55 (24.9)	166 (75.10		61 (27.6)	61 (72.4)	
Mean age in years	32±15.56										
Age group in years	20-35	168	60 (35.7)	108 (64.3)		39(23.2)	129 (76.8)		130 (77.4)	38 (22.6)	
	36-45	66	31 (47.0)	35 (53.0)	> 0.05	21 (31.8)	45 (68.2)	< 0.05	42 (63.6)	24 (36.4)	> 0.05
	46-55	18	8 (44.4)	10 (55.6)		7 (38.9)	11 (61.1)		14 (77.8)	4 (22.2)	
	56-65	2	1 (50.0)	1 (50.0)		1 (50)	1 (50)	>0.05	1 (50)	1 (50)	
Education level	Informal	21	3 (14.3)	18 (85.7)		6 (28.6)	15 (71.4)		13 (61.9)	8 (38.1)	
	Primary	63	14 (22.2)	49 (77.8)		12 (19)	51 (81)	< 0.05	51 (81.0)	12 (19.0)	< 0.0001
	Secondary	136	62 (45.6)	74 (54.4)	<	38 (27.9)	98 (72.1)		108 (79.4)	28 (20.6)	
	College	34	21 (61.8)	13 (38.2)	0.0001	12 (35.3)	22 (64.7)		15 (44.1)	19 (55.9)	
Marital status	Single	86	27 (31.4)	59 (68.6)		18 (20.9)	68 (79.1)		68 (79.1)	18 (20.9)	
	Married	162	71 (43.8)	91 (56.2)	> 0.05	47 (29.0)	115 (71.0)	> 0.05	116 (71.6)	46 (28.4)	< 0.05
	Widow	3	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)		1 (33.3)	2 (66.7)	
	Divorced	3	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)	
Occupation	Peasant	67	13 (19.4)	54 (80.6)		18 (26.9)	49 (73.1)		51 (76.1)	16 (23.9)	
	Business	138	60 (43.5)	78 56.5)	<	31 (22.5)	107 (77.5)	> 0.05	102 (73.9)	36 (26.1)	> 0.05
	Student	49	27 (55.1)	22 (44.9)	0.0001	19 (38.8)	30 (61.2)		34 (69.4)	15 (30.6)	

Discussion

In the present study we assess the knowledge, attitude and practice in relation to antimicrobial residues among beef consumers in Dodoma, Tanzania. The results obtained in this study indicate that the respondents interviewed had low knowledge and awareness on antimicrobial use and antimicrobial residues. Some of the respondents were aware that animals are treated with antimicrobials but (65%) they could not realise that the same antimicrobials can cause antimicrobial residues in animal meat and milk. The other reason which could be considered is lack of awareness to respondents on the possible side effects of antimicrobials and other drugs to humans. Furthermore, the study has demonstrated a relationship between education and beef purchasing. Most of secondary and college residents purchased beef from burcheries while infomal and primary residents purchases locally within village. Majority of female (76.9%) prefered cooking beef for one hour.

The current results confirm previous reporting from a rural District in China on lack of knowledge and practice on cautious use of antimicrobial and antimicrobial resistance in developing countries (Chenggang *et al.,* 2011; Katakweba *et al.,* 2012). This might be due to low levels of education of the respondents as majority of them had informal and primary education only.

This study showed that age also plays a role regarding knowledge of antimicrobials. The study is also in line with a socio-demographic analysis conducted by SPECIAL EUROBAROMETER 338 in 2010 within European countries (European Commission 2010) which revealed that women seem to be better informed than men on this topic and age also plays a role and as regards to knowledge of antimicrobials. Respondents with higher education are also more likely to have a more clear knowledge on the

antimicrobials effects. However, the respondents need to be educated on the possible effects associated with use of beef with antimicrobial residues.

This study is also in line with the study described by Bilashoboka *et al*. (2016) who accessed the level of knowledge, concerns and practices of animal keepers, consumers and extension agents in relation to antimicrobials withdrawal requirements and observed that most of animal keepers interviewed were ignorant of antimicrobial residues and withdrawal periods. The majority of respondents were not aware of the antimicrobial residues in beef whereas the businessmen and law enforcers were aware.

The factors that may contribute to antimicrobials and antimicrobial residues in food in developing countries, such as Tanzania include 1) lack of sufficient knowledge in use of antimicrobial for human and animal, 2) failure to observe withdrawal periods when antimicrobials are administered to animals, 3) lack of updated antimicrobial use and treatment guidelines and 4) Easy access to antimicrobials such as oxytetracycline (Nisha, 2008).

Conclusion

It is concluded that this study suggested that many of the respondents were aware of the antimicrobial residues but did not know that antimicrobial residues can have effects in human health..

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Ethical issues

Permission for this study was granted by the Executive Directors of the Dodoma Region Council and ethical approval for the study was obtained from the Ethical Committee of the Sokoine University of Agriculture. The university issued a research permit letter on behalf of the Tanzanian Commission for Science and Technology.

Competing interests

The authors declare no conflict of interests

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CHAPTER THREE

PAPER 1

A simple and sensitive method for the detection of Oxytetracyline levels in ready-to-eat beef by Liquid Chromatography-Mass Spectrometry

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Full Length Research Paper

A simple and sensitive method for the detection of "Oxytetracycine" levels in ready-to-eat beef by liquid chromatography-mass spectrometry

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Antimicrobial drug residues have emerged as one of the public health problems worldwide. In this study, a modified sensitive liquid chromatography mass spectrometry (LC-MS) method to detect the "Oxytetracycine" (OTC) levels in ready-to-eat beef meat in Tanzania was evaluated. Beef samples were extracted in acetonitrile in ethylenediaminetetraacetic acid (EDTA) buffer (pH 4), followed by cleaning up with Supelclean ENVI-carb active coal and a stream of nitrogen gas. The wavelength of the diode array detector (DAD) was set at 275 and 355 nm. The detection limit of the method was calculated as 18.2 ng/g and the recovery rate of OTC was 78.6%. A total of 45 ready-to-eat beef meat samples were analyzed, with 16 (35.5%) and 29 (64.5%) barbequed and boiled samples, respectively. Of the 45 samples, 35 (77.8%) samples had OTC residues while 9 (25.7%) samples had violative residue levels above the maximum residue limits recommended by the Food and Agriculture Organization and the World Health Organization. The highest concentration was 545.2 ng/g. Therefore, withdrawal period and proper use of antibiotics for animal production should be of concern as consumers are at risk of adverse effects due to consumption of unacceptable levels of drug residues and a risk of developing microbial resistance. To the best knowledge of the authors, this is the first study to evaluate LC-MS method to detect the OTC levels in ready-to-eat beef meat in Tanzania.

Key words: Oxytetracycline, high performance liquid chromatography, mass spectrometry, ready-to-eat beef meat, residues.

INTRODUCTION

Antimicrobial drug residue in animal products is an increasing public health problem worldwide. One of the major areas of interest is investigating the proper use and monitoring of antibiotics usage to prevent contamination (Alica et al., 2003). Questions have been raised about the drug label, discard times as several drugs are retained in

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animal bodies longer than indicated by the manufacturer (Seymour et al., 1988). Improper administration of antimicrobials by farmers and veterinarians without observing the withdrawal time for treated animals may not only result in antimicrobial residues in meat but may also contribute to the development of microbial drug resistance and spreading of drug resistant bacteria that may result in serious health consequences (Booth, 1988). Human health problems that could arise from the consumption of unacceptable levels of OTC residues in meat include gastrointestinal disturbances, hypersensitivity, bone and teeth problems in children and development of bacterial resistance (Larkin et al., 2004; Shankar et al., 2010).

The problem regarding tetracycline residues is very common and has to be addressed accordingly, since tetracyclines are the commonly used antimicrobial drugs. With this regard the Food and the Agriculture Organization (FAO) and the World Health Organization (WHO), 2004 recommended the maximum residue limits (MRLs) to be 200, 600 and 1200 µg/kg in muscles, livers and kidneys, respectively. For the analysis of tetracyclines levels, various methods have been reported in the literature mainly due to difficulties related to differences in physico-chemical properties between families of compounds (Kaufmann, 2009). Methods for the detection of tetracyclines are many but a more specific method such as HPLC is the efficient technique (Loksuwan 2002; Cinquina et al., 2003). The method efficiency is based on multi-detection on liquid chromatography coupled with tandem mass spectrometry (Bohm et al., 2009).

Residues are ordinarily measured on uncooked tissues. It is also important to monitor the levels of drug residues in both raw and ready-to-eat foodstuffs. Studies have shown that temperatures have effect on the levels of drug residues (Salah and Ali, 2013). It is even more important to analyse the levels of OTC residues and to evaluate if residues levels can be reduced by cooking procedures (Ibrahim and Moats, 1994). So far, there is limited literature about the effect of cooking on levels of residues and this creates a scientific gap of knowledge which needs to be addressed in Tanzania. Therefore, the objective of the present work was to modify and validate a simple and sensitive LC-MS method for analyzing Oxytetracycine (OTC) residues (Froehlich, 2013). The validated method was applied to determine the levels of OTC in ready-to-eat beef meat samples.

MATERIALS AND METHODS

Samples

A total of 45 ready-to-eat beef meat samples were randomly collected from different areas in Dodoma, Tanzania (Majengo Sokoni, Mnadani, Chakonichako, Rozi Garden and Bahama Mama). The samples collected were already prepared as barbequed "nyama choma" or boiled. These two methods of preparation were selected because they are most practiced in Tanzania. Antibiotics-free meat samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The blank matrix samples were barbaqued or boiled before extraction.

Sample pretreatment and extraction

The samples were kept at -20°C until analysis and were allowed to defrost at room temperature. A representative portion of the defrosted sample (10 g) was weighed and mixed with 25 mg of EDTA per gram sample. The sample and the EDTA were homogenized for 1 min using a blender. The blended sample was further ground using a mortar and pestle. One gram of homogenized sample was accurately weighed into 15 ml polypropylene centrifuge tubes. To the sample, 10 µl of 10 µg/ml carbamazepine D10 internal standard solution equivalent to 100 ng/g concentration was added.

Five milliliters acetonitrile were added to the sample and vortexed for 1 min. Each sample was centrifuged for 10 min at 7000 rpm and the supernatant was collected into a separate 15 ml centrifuge tube by decantation. 5 ml acetonitrile were again added to the residue and vortexed for 1 min. The samples were then centrifuged for 10 min at 7000 rpm. Both supernatants were combined in a 15 ml centrifuge tube bringing the total volume to 10 ml. All samples were briefly mixed using a vortex and dried under a stream of nitrogen gas to 2 ml, according to Froehlich's HPLC method (Froehlich, 2013).

Sample clean-up by Supelclean ENVI-carb active coal

After drying each sample to 2 ml, 0.5 ml of HPLC grade water and 30 µl of formic acid were added, making the mixture 1.2% acid. Then 15 mg of Supelclean ENVI-carb active coal was added to all the samples and mixed for 30 s using a vortex and centrifuged for 10 min at 7000 rpm. The supernatants were collected into separate 15 ml centrifuge tubes and dried to 0.5 ml. The dried samples were then transferred into HPLC vials washed with 0.02 mol/L EDTA solutions and injected into chromatographic system (Froehlich, 2013). The HPLC analysis was performed in 23 min.

Sample analysis by LC-MS method

The HPLC was equipped with DAD detector and mass spectroscopy (Model Agilent Technologies 6130 Quadrupole LC/MS) to target the flowing parent ions using Single Ion Monitoring (SIM) mode 461 mass per charge ratio (m/z) for OTC. The analytical column was reversed-phase Eclipse XDB C-18. 4.6 × 150 mm set at a flow rate of 0.5 ml/min. The column temperature was 25°C. Mobile phase A was HPLC water with 0.1% formic acid and solvent C was Acetonitrile with 0.1% formic acid. The starting mobile phase composition at 0 min was 85% Water: 15% Acetonitrile at 0.5 ml/min. The wavelength of the DAD detector was set at 275 and 355 nm, respectively. Internal calibration curves were prepared by spiking the blank matrix with pure chromatographic standard solutions in the range between 200 and 2500 ng/g injected for each compound and estimates of the amount of the analytes in samples were interpolated from these graphs.

Validation

To test the analytical method trueness, 14 samples were prepared. Each contained 1 g of homogenized muscle tissue of the negative control sample (blank matrix). Seven samples were spiked with 20

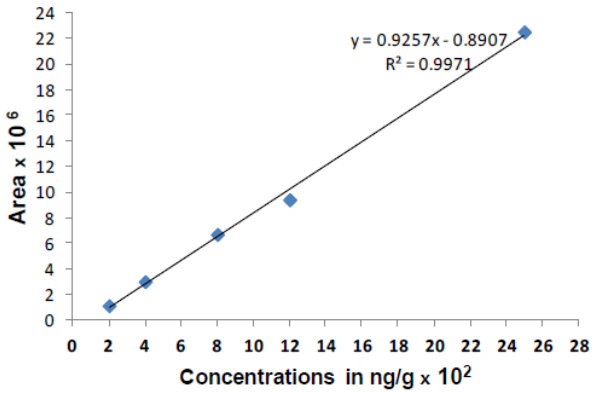


Figure 1. Calibration curve of oxtetracycline standard.

µl of 10 ng/ml solutions, equivalent to 200 ng/g of analyte. Seven samples were spiked with 250 µl equivalent to 2500 ng/g of the analyte. All samples were processed using the described LC-MS method.

Preparation of standard stock and working solution

A stock standard solution of OTC compound was prepared by dissolving 10 mg of the compound in 10 ml of methanol to obtain a final concentration of 1 mg/ml. The stock standard solution was then put in amber glasses to prevent photo-degradation and stored at -20°C and left to stabilize for at least 4 weeks. They were then diluted with 95% water: 5% acetonitrile to give a series of working standard solution of 200, 400, 800, 1200, and 2500 ng/g.

Recovery experiment

Samples recovery was determined with blank bovine muscle spiked at 200 ng/g. To test the recovery, 10 samples were prepared that contained 1 g of homogenized muscle tissue of the negative control. They were spiked with 20 µl of 10 ug/ml spiking solution equivalent to 200 ng/g of the analyte. Four samples were used to calculate the recovery mean and six samples were used to calculate the recovery-corrected content.

Data analysis

The data were analyzed using Epi Info (version 7) (Centre for Disease Control, Atlanta, USA). The association between different categorical and continuous variables was determined by the Fisher's exact test. One-way analysis of variance (ANOVA) test statistic was used to determine any significant differences in the mean residue levels of oxytetracycline; a probability of $P < 0.5$ was considered statistically significant.

RESULTS AND DISCUSSION

Calibration of OTC standard

OTC standard powder was accurately weighed and dissolved in methanol to make the stock solution and several serial dilutions of the stock solution were made and injected to the LC-MS to plot the standard curve of linear R^2 value = 0.9971 within the range of 200 to 2500 ng/g (Figure 1).

Samples recovery

The recovery rate of OTC was 68% (Table 1), while the recovery-corrected rate for the samples were 78.6% ranging from 64.8 to 86.9% (Table 2). For repeatability and reproducibility, data were obtained by extracting 7 replicates on three successive days at two concentrations of 200 and 2500 ng/g; with coefficients of variation of 6.60 to 10.60% and 6.30 to 10.60% for OTC, respectively. Results of this study revealed that the repeatability and reproducibility were corresponding to the validation methods done by Biswas et al. (2007).

LC-MS technique was employed to determine the levels of OTC in ready-to-eat beef meat samples in Dodoma, Tanzania. In this method, carbamazepine D10 was used as internal standard to correct internal and external error. The detection of OTC residues levels was done by using LC method with MS detector. This is because OTC can be successfully determined using LC with MS detector in various matrices. Adequate treatment of samples during extraction was done in order to obtain maximum sensitivity of OTC and to reduce matrix interference. The samples were considered positive for OTC if their retention time and peak corresponded to that of the reference standard. The retention time of the standard was at 3.624 min. The chromatographic peak increased with increase in concentration of the standard

The limit of detection (LOD) is the lowest concentration which can be qualitatively measured, and is defined as the concentration at which the signal-to-noise ratio of the corresponding signal is 3-to-1. In this study, the LOD

Table 1. Certified reference materials for OTC in bovine muscle

Recovery of OTC from meat spiked at 200 ng/g of the analyte (ng/g)	
Recovery 1	129.0
Recovery 2	145.6
Recovery 3	134.6
Recovery 4	137.8
Mean recovery	136.8 (68%)

Table 2. Recovery-corrected contents

Analysis of certified reference material	Measured content (ng/g)	Recovery-corrected contents (%) Y=B10/B15*100
Replicate 1	184.6	74.1
Replicate 2	174.3	78.5
Replicate 3	163.4	83.7
Replicate 4	157.4	86.9
Replicate 5	198.9	64.8
Replicate 6	163.4	83.7
Mean	-	78.6±3.3
Standard deviation	-	8.1
Coefficients of variation	-	10.3
Recovery rate	-	78.6%

B10 is the mean recovery. B15 is the replicate 1.

Table 3. Number and percentage in parentheses of beef samples barbequed and boiled with and without oxytetracycline (OTC) residues

Cooking types	OTC residues (%)	No OTC residues (%)	Total (%)
Barbequed	12 (75)	4 (25)	16 (35.5)
Boiled	23 (79.3)	6 (20.7)	29 (64.5)
Total	35 (77.7)	10 (22.2)	45 (100)

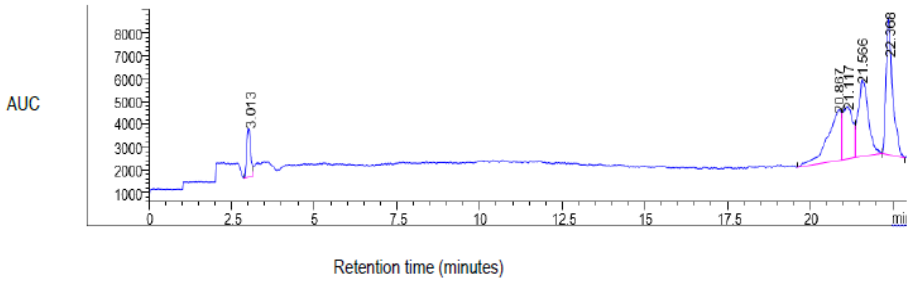
Fisher exact test 0.73, P = 0.74

was 18.2 ng/g, corresponding to the LOD obtained by Hassani et al. (2008). The limit of quantification (LOQ) is the lowest concentration of analyte which can be quantitatively measured and was 54.6 ng/g.

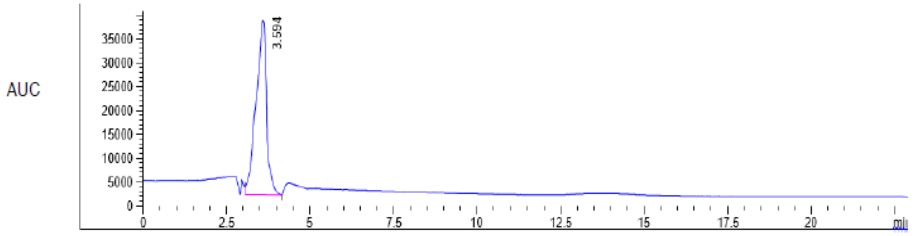
Figure 2 shows LC-MS profiles of the OTC obtained from the blank beef meat samples, blank beef samples spiked with 400ng OTC, standard solution and spiked beef meat samples.

Results indicate that of the 45 beef meat samples analyzed 16 (35.5%) were barbequed samples and 29 (64.5%) boiled samples. The observed differences are statistically insignificant (P > 0.05) as shown in Table 3. Thirty five samples (77.8%) had OTC residues with 26 (74.3%) samples having residues below the FAO/WHO (2004) recommended MRLs. Nine (25.7%) samples had OTC at violative levels above the recommended MRLs. Of the 9 samples with detectable violative OTC levels, 2 (22.2%) and 7 (77.8%) samples were barbequed and boiled meat samples, respectively. However, the observed differences were statistically insignificant (P > 0.05) as shown in Table 4. The study findings indicate the need for one health strategy to enhance the optimal health for humans, animals and the environment.

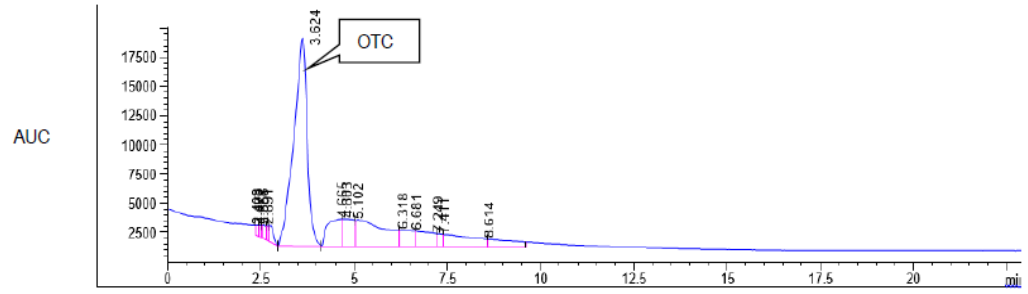
Mean concentration of OTC residues in barbequed and boiled samples were 130.67 ± 96.6 and 361.96 ± 69.40 µg/kg, respectively. The concentration of OTC residues from each sample is shown in Table 5. This study shows higher proportions of oxytetracycline–positive samples than those reported in other studies (Addisalem et al., 2012) and Bedada and Zewde (2012). Studies have reported varied drug residues in raw meat samples, 41.2% (Mmbando, 2004) and 76.4% (Nonga et al., 2013)



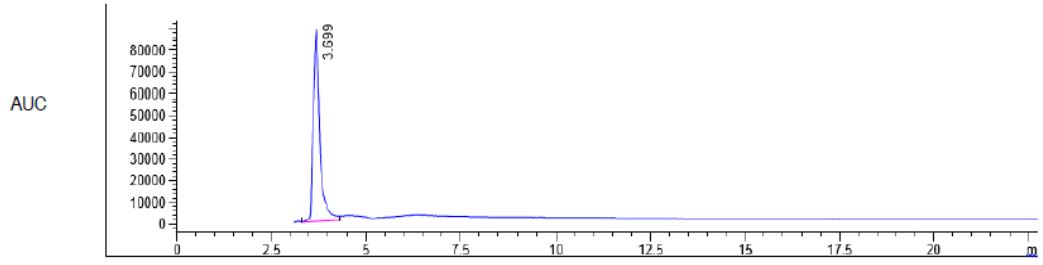
a) Chromatogram of blank beef meat sample



b) Chromatogram of blank beef samples spiked with 400ng OTC.



c) Chromatographic standard solution



d) Chromatogram of spiked beef meat sample of positive OTC thermally treated.

Figure 2. LC-MS profiles of OTC. (AUC = Area under the curve). (a) Chromatogram of blank beef meat sample. (b) Chromatogram of blank beef samples spiked with 400ng OTC. (c) Chromatographic standard solution. (d) Chromatogram of spiked beef meat sample of positive OTC thermally treated.

Table 4. Number and percentage in parentheses of beef samples with OTC residues

Cooking types	<MRLs of 200 µg/kg	>MRLs of 200 µg/kg	Total
Barbequed	10 (38.5%)	2 (22.2%)	12 (34.3%)
Boiled	16 (61.5%)	7 (77.8%)	23 (65.7%)
Total	26 (74.3%)	9 (25.7%)	35 (100%)

Fisher exact test 0.45, P =0.38

Table 5. OTC concentrations levels in ready-to-eat beef meat samples.

Cooking types	Sample code	Concentration OTC in ng/g			Total
		<MRL	>MRL	No-residues(ND)	
Boiled	SAMPLE 1C	-	-	0	-
	SAMPLE 2C	119.32	-	-	-
	SAMPLE 3C	184.11	-	-	-
	SAMPLE 4C	119.96	-	-	-
	SAMPLE 5C	-	-	0	-
	SAMPLE 6C	74.65	-	-	-
	SAMPLE 7C	-	440.11	-	-
	SAMPLE 8C	72.91	-	-	-
	SAMPLE 9C	25.92	-	-	-
	SAMPLE 10C	47.56	-	-	-
	SAMPLE 11C	103.28	-	-	-
	SAMPLE 12C	95.33	-	-	-
	SAMPLE 13C	-	288.75	-	-
	SAMPLE 14C	200.01	-	-	-
	SAMPLE 15C	-	-	0	-
	SAMPLE 16C	-	545.20	-	-
	SAMPLE 17C	183.74	-	-	-
	SAMPLE 18C	89.11		-	-
	SAMPLE 19C	-	326.46	-	-
	SAMPLE 20C	190.44	-	-	-
	SAMPLE 21C	-	295.36	-	-
	SAMPLE 22C	-	-	0	-
Boiled	SAMPLE 23C	-	417.54	-	-
	SAMPLE 24C	79.07	-	-	-
	SAMPLE 25C	190.96	-	-	-
	SAMPLE 26C	-	444.70	-	-
Barbequed	SAMPLE 27B	134.09	-		-
	SAMPLE 28B	-	-	0	-
	SAMPLE 29B	-	-	0	29
	SAMPLE 30B	142.54	-	-	-
	SAMPLE 31B	-	-	0	-
	SAMPLE 32B	-	-	0	-
	SAMPLE 33B	-	-	0	-
	SAMPLE 34B	77.95	-	-	-
	SAMPLE 35B	104.19	-	-	-
	SAMPLE 36B	81.53	-	-	-
	SAMPLE 37B	105.16	-	-	-
	SAMPLE 38B	-	-	0	-

Table 5. Contd

	SAMPLE 39B	132.12	-	-	-
	SAMPLE 40B	71.71		-	-
	SAMPLE 41B	-	395.09	-	-
	SAMPLE 42B	-	287.64	-	-
	SAMPLE 43B	182.88	-	-	-
	SAMPLE 44B	120.22	-	-	-
	SAMPLE 45B	52.17	-	-	16
Total		**26**	**9**	**10**	**45**

in Tanzania. Nevertheless, the study conducted by Mmbando (2004) from muscle tissue in the Morogoro and Dodoma municipalities, Tanzania, indicate that only 41.2% of samples were positive for oxytetracycline residues. Drug residues in raw meat have also been reported in other countries, 44% in Nigeria (Stolker and Brinkman, 2005), 50% in Iraq (Tajick and shohreh, 2006), 21% in Ghana (Donkor et al., 2011) and 71.3% in Ethiopia (Addisalem et al., 2012). From Ghana, Donkor et al. (2011) and Mmbando (2004) reported 21 and 41.2% oxytetracycline residues in muscle tissue were relatively low compared to levels seen in the current study. These results reported here are consistent with those previously reported by Nonga et al. (2013) from Tanzania and those by Addisalem et al., (2012) from Ethiopia of 76.4 and 71.3%, respectively.

The presence of OTC residues in the ready-to-eat meat observed in the present study is a clear indication that drug residues are not destroyed by heating/cooking. The reasons might be due to the method used, time of cooking and type of tetracycline (TC) used. Several studies reported the effect of heat on foodstuffs. Nguyen et al. (2013) have reported that heat treatments were shown to reduce the concentration of drug residues level in foodstuffs, therefore decreasing the toxic effects to consumers. Javadi (2011) and Gratacós-Cubarsí et al. (2007) showed reductions in the concentration of doxycline (DOC) and OTC residues level after different cooking processes. A study by Al-Ghamdi et al. (2000) also indicated that cooking by boiling decreased OTC, Chlortetracycline (CTC) and DOC levels in meat and liver.

Conclusion

A simple, rapid and sensitive LC-MS method for the detection of OTC levels in beef meat samples was evaluated. The method was capable of detecting residue and non-residue meat samples. A significant proportion of ready-to-eat beef meat samples (25.7%) had OTC level above the FAO/WHO MRLs of 200 µg/kg. This indicates that animals are slaughtered without giving adequate withdrawal period or misuse of antibiotics for animal production in Dodoma region, Tanzania. The consumers of ready-to-eat beef meat are at risk of adverse effects due to consumption of unacceptable levels of drug residues and a risk of developing microbial resistance.

The study findings signify the need for the One Health approach for effective surveillance of drug residues in foodstuffs. Therefore, withdrawal period and proper use of antibiotics for animal production should be a public health concern given that the One Health approach aims to attain the optimal health for humans, animals and the environment. To the best knowledge of authors, this is the first study to evaluate LC-MS method to detect the OTC levels in ready-to-eat beef meat in Tanzania.

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Conflict of interests

The authors have declared that they have no conflict of interests.

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CHAPTER FOUR

MANUSCRIPT 1I

Oxytetracycline residue levels in beef in Dodoma region, Tanzania

Status: Submitted in the *Journal of Food Science*

Oxytetracycline residue levels in beef in Dodoma region, Tanzania

Abstract

Residues of antibiotics in meat pose a threat to human health due to the potential development of a resistance to the antibiotic drugs. Oxytetracycline (OTC) residues in beef were determined in a cross-sectional study. The study aim was to determine the OTC levels in beef using Liquid Chromatography - Mass Spectrometry. Sixty beef samples were purposively collected from slaughterhouses and butcheries in different Districts in Dodoma region, Tanzania, and OTC levels were determined. Twenty-one out of 60 samples (35%) had OTC residues but none of these samples had OTC levels above the maximum allowed residue limit (200 µg/kg). The highest oxytetracycline concentration was 4.95 ng/g and the mean concentration was 0.69 ± 0.09 ng/g. The results indicate that the mean concentration level was very low. Even though these levels may not induce adverse effects, from a food safety viewpoint, high-level occurrence of OTC should however be of concern.

Keywords: Liquid Chromatography–Mass Spectrometry, Residue levels, raw beef, OTC.

Introduction

To obtain the animal products like milk and meat, animals have to be kept healthy. The care includes feeding, management, control of animal diseases and treatment when they are sick. Some of the drugs used for treatment of animal diseases in Tanzania include antibacterials such as tetracyclines, beta lactams like penicillins and cephalosporins (Katakweba *et al.,* 2012). The TCs, among the first antibiotics have bacteriostatic activity against both Gram-positive and Gram-negative bacteria and are widely used for the treatment of bovine mastitis among many other diseases (Uekane *et al*., 2011). Beta lactam antibiotics like penicillins and cephalosporins have been used in veterinary medicine practices for prophylaxis and as growth promoters (Mehtabuddin *et al*., 2012). The most widely used class of antibiotics for the treatment of bacterial infections in animal production are the beta-lactam antibiotics, this is according to Stolker and Brinkman, (2005).

Veterinary drug residues in meat have been reported to cause toxic or allergic reactions in humans (Martinez, 2005). The presence of OTC residue (Figure 3.1) in raw beef may cause a health problem to consumers. Accoding to Bilatu (2012), if antibiotics are not used in a responsible and appropriate manner, there is a probability of losing the efficiency for treatment of diseases in human and animal due to the development of antibiotic resistance. Due to the harmful effects the veterinary medicine residues can cause in humans, there is a need for countries to establish surveillance systems for antimicrobial usage (Martinez, 2005). Preventing drug or antimicrobial residues in meat is the responsibility of livestock officers, ministry and every farmer, therefore a well-planned drug use programme (Breton *et al*., 2007) can avoid drug residues. Extra measures have to be taken in order to protect humans from desirable effects of veterinary drug residues derived food sources. This is the reason why the Food and the Agriculture Organization (FAO) and the World Health

Organization (WHO), 2014 recommended the maximum residue limits (MRLs) to be 200 µg/kg, 600 µg/kg and 1200 µg/kg in muscles, livers and kidneys, respectively.

Residue levels in animal products depend on the initial dosage and the duration between the drug administration and animal product collection. This timeframe is called the withdrawal or washout period (Botsoglou and Fletouris, 2001). The antibiotic residues can remain in an animal's body after slaughtering if withdrawal period is insufficient. Therefore, withdrawal periods of 5–20 days are recommended before animals are slaughtered (Cinquina *et al.*, 2003). Therefore, the aim of this study was to determine the OTC residues level in raw beef collected from Dodoma region, Tanzania by a method described by Mgonja *et al.* (2016).

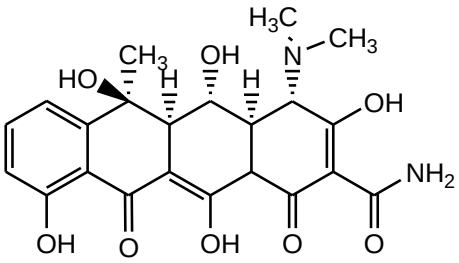

Figure 4.1: The molecular structure of OTC

Materials and methods

Samples

A total of 60 raw beef samples were purposively collected from different Dictricts in Bahi, Mpwapwa, Kongwa, Dodoma Urban and Rural and Kondoa. Slaughterhouses and butcher shops were selected using a simple random sampling technique. Each sample was transferred in separate sterile and labeled plastic bags in an icebox and transported to Zambia Agricutural Research Institute (ZARI) laboratory. All samples were analyzed for determination of OTC residues. The control and test samples were stored in a freezer at

−20 °C for approximately 1 week. Both control and test samples were thawed at room temperature for four hours before extraction and analysis of OTC residues. Antibiotic-free meat control samples (blank matrix) was collected from the Central Veterinary Research Institute of Zambia.

Analytical method validation

The following definitions and procedures for measuring the validation parameter were taken from the guidelines for the Germany Society of Toxicology and Forensic Chemistry GTFCh (2009).

Sample Pretreatment and Extraction

The samples were kept at -20 ºC until analysis and were allowed to defrost at room temperature. A representative portion of the defrosted sample (10 g) was weighed and mixed with 25 mg of EDTA per gram sample. The sample and the EDTA were homogenized for 1 minute using a blender. The blended sample was further ground using a mortar and pestle. One gram of homogenized sample was accurately weighed into 15 mL polypropylene centrifuge tubes. To the sample, 10 µL of 10 µg/mL carbamazepine D10 internal standard solution equivalent to 100 ng/g concentration was added.

Five mL acetonitrile were added to the sample and vortexed for 1 minute. Each sample was centrifuged for 10 minutes at 7 000 rpm and the supernatant was collected into a separate 15 mL centrifuge tube by decantation. Five mL acetonitrile were again added to the residue and vortexed for 1 minute. The samples were then centrifuged for 10 minutes at 7000 rpm. Both supernatants were combined in a 15 mL centrifuge tube bringing the total volume to 10 mL. All samples were briefly mixed using a vortex and dried under a

stream of nitrogen gas to 2 mL, then sample clean up was done by Supelclean ENVI-carb active coal (Mgonja *et al.,* 2016).

Sample analysis by LC-MS method

The reference standard for OTC and Ethylenediaminetetraacetic acid (EDTA) was supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were of high performance liquid chromatography (HPLC) grade (Merck Company, Germany).

The determination of OTC residues was carried out using HPLC with a diode array detector (DAD) as described by Mgonja *et al.,* (2016). The HPLC was equipped with DAD detector and mass spectroscopy (Model Agilent Technologies 6130 Quadrupole LC/MS) to target the flowing parent ions using Single Ion Monitoring (SIM) mode 461 mass per charge ratio (m/z) for OTC.

Validation

To test the analytical method trueness, 14 samples were prepared. Each contained 1 g of homogenized muscle tissue of the negative control sample (blank matrix). Seven samples were spiked with 20 µL of 10 ng/mL solutions, equivalent to 200 ng/g of analyte. Seven samples were spiked with 250-µL equivalent to 2500 ng/g of the analyte. All samples were processed using the described LC-MS method (Froehlich, 2013).

Recovery experiment

Samples recovery were determined with blank bovine muscle spiked at 200 ng/g. To test recovery, 10 samples were prepared and they contained 1 g of homogenized muscle tissue of the negative control. They were spiked with 20 µL of 10 ug/mL spiking solution

equivalent to 200 ng/g of the analyte. Four samples were used to calculate the recovery mean and six samples were used to calculate the recovery-corrected content.

Robustness

The robustness describes the sensitivity of a method towards changes to the analytical frame conditions like temperature, different matrices and variations of the pH. The effect of different matrices were tested. Two beef samples were tested by weighing in 1 g of beef of the negative control sample. One was spiked with 40 µL equivalent to of 400 ng of OTC. The other one was not spiked. The sample was then processed using the method described above.

Data analysis

The data were analysed using Epi Info (version 7) (Centre for Disease Control, Atlanta, USA). The Chi-square statistic and confidence intervals were used to compare proportions; a probability of $P < 0.05$ was considered statistically significant. Descriptive statistics were used to compute means, standard deviations and range.

Results:

Beef samples from Dodoma region were analyzed for OTC residues. The concentration of residue levels in each sample was calculated (in ng/g) sample. The obtained mean concentration was then compared to the maximum residues limits (MRLs) set by the World Health Organization (WHO) of (200 µg/kg). Of the 60 beef sampled 35% tested positive for OTC residues and 65% with no residues. Howeover, none of them had residue concentrations above the acceptable levels for muscle as per (Food and Agriculture Organization/World Health Organization 2014), Table 4.1.

Table 4.1: Number and percentage in parentheses of raw beef samples with and without OTC residues

Sample types	With OTC residues	Without OTC residues	Total
Raw beef	21 (35%)	39 (65%)	60 (100%)

The mean concentration of OTC residues was 0.69 ± 0.09 ng/g. The retention time of the standard stood at 5 minutes. The chromatographic peak increased with increase in concentration of the standard. The recovery percentage was 68%. The limit of detection and the limit of quantification were 18.2 ng/g and 54.6 ng/g, respectively. Therefore, the 0.69 ± 0.09 ng/g mean concentration of OTC residual detected was above this limit of quantification. The correlation coefficients associated with the linear regression for the analytical OTC standard (Figure 3.2) was = 0.9816.

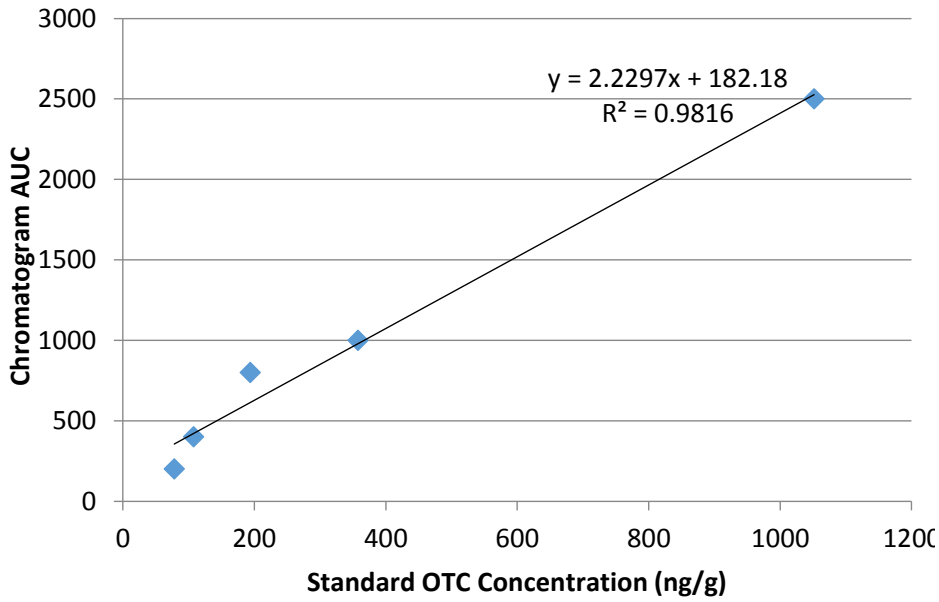

Figure 4.2: Calibration curve for oxytetracycline

Discussion

Tetracyclines are important class of antibiotics in food animal health and production. These antibiotics have been used for many decades in the treatment of diseases, promote

growth and to maintain animals health. Oxytetracycline has bacteriostatic activity and is widely used for the treatment of bovine mastitis among many other diseases (Uekane *et al.,* 2011). Katakweba *et al.* (2012) reported that oxytetracycline is one of the most commonly used antibiotics in livestock production in Tanzania and other countries. The easy use and access to the antibiotics and lack of awareness may lead to misuse of these drugs.

The method involved was described by (Mgonja *et al.,* 2016) which was capable of detecting beef samples with and without residues. The results indicate a presence of OTC residues in (35%) of the samples, with no samples being above the acceptable maximum residue levels recommended by the WHO and FAO. The OTC level in this study seems to be lower than that reported in other studies (Muriuki *et al.,* 2001) even though (Donkor *et al.,* 2011) reported a comparable proportion of OTC levels in beef samples 21% from cattle in Ghana. On the other hand a study conducted in beef from Morogoro and Dodoma municipalities, Tanzania shows only 41.2% of the samples tested positive for OTC residues (Mmbando, 2004). The reasons for this differences might be due to the method used and type of Tetracycline (TC) used.

OTC residues in beef samples were also reported 71.3% and 71.0% in studies conducted from Ethiopia (Addisalem *et al.,* 2012) and Goulette (2007) from USA, which are both relatively higher compared to the levels observed in current study. Nisha (2008) reported the presence of high levels of antibiotic residues in meat, is the results of misuse and overuse of the drug which may call for microbial resistance. Studies in Tanzania have also reported the presence of drugs residues in milk ; Kaale *et al.* (2007): Mdegela *et al.* (2006) and Kurwijila *et al.* (2006) of 2.8%, 4.5% and 36% respectively. Another study in milk by Zhang *et al.* (2014) from China and Goulette (2007) from USA showed that 7.7% and 71

% of test samples were positive for antibiotic residues. These findings are also in line with (Donkor *et al.,* 2011) and Mmbando (2004) who found comparable proportion of tetracylines in animal source food. A study of meat samples by Biswas also revealed the presence of OTC residues up to 13.3% samples, but no sample had residue concentration above MRL as indicated in this study.

The study findings signify the need for the One Health approach for effective surveillance of drug residues in foodstuffs. Therefore, withdrawal period and proper use of antibiotics for animal production should be a Public health concern given that the One Health approach aims to attain the optimal health for humans, animals and the environment.

Conclusion

The results indicate the mean concentration level was very low. Even though these levels were not supposed to induce adverse effects, from a food safety viewpoint, high-level occurrence of OTC should however be of concern.

Acknowledgments

The authors are grateful to the INTRA ACP MOBILITY Project for the financial support of this study, Zambia Agriculture Research Institute laboratory and livestock keepers.

Ethical issues

Permission for this study was granted by the Executive Directors of the Dodoma Region Council and ethical approval for the study was obtained from the Ethical Committee of the Sokoine University of Agriculture. The university issued a research permit letter on behalf of the Tanzanian Commission for Science and Technology.

Competing interests

The authors declare no conflict of interests.

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CHAPTER FIVE

MANUSCRIPT III

Effect of heat treatment on Oxytetracycline residues in beef

Status: Submitted in the *Journal of Veterinary Research*

Effect of heat treatment on Oxytetracycline residues in beef

Abstract

Literature about drug residues is mainly related to their concentrations in uncooked and cooked food. The aim of this study was to assess the effects of barbecuing and boiling treatments on the concentration of oxytetracycline (OTC) in beef samples collected from different Districts in Dodoma region, Tanzania. The beef samples were part boiled for 30 minutes and other barbecued for 20 minutes. The OTC content was measured in raw and heated samples by using high performance liquid chromatography (HPLC). The mean concentration of OTC for boiled and barbecued beef samples was 69.45 ± 41.93 ng/g and 69.40 ± 38.91 ng/g, respectively. Both the boiling and barbecuing procedures significantly decreased the OTC levels in beef ($p<0.05$), and the boiling procedure had the highest influence on reducing OTC concentration. The OTC concentrations after the heating treatments were below the maximum acceptable residue limits (MRL). In conclusion, heat treatment, such as cooking may be useful in reducing the amount of some antimicrobial residues (AMRs) in meats but effort should be geared towards total elimination of antimicrobial residues in foods of animal origin. Proper usage and withdrawal period of OTC should always be observed.

Keywords: HPLC, OTC**,** boiling, barbecuing, time, beef, Tanzania

Introduction

Antimicrobial agents are essential drugs for both human and animal health and welfare. These agents have been used for the treatment of diseases in animals, prevention of infection in animals and to improve feed utilization and production (Heshmati *et al.*, 2013). Effective treatment of diseases in the livestock industry in sub- saharan Africa including Tanzania continues to be a challenge. The challenges have been addressed mainly by the use of antimicrobials that include the tetracyclines, beta lactam antibiotics like penicillins and cephalosporins (Olufemi and Agboola, 2009; Katakweba *et al.,* 2012). The lack of restriction on antimicrobial drug availability, insufficient knowledge on drug use as well as failure to observe withdrawal period can contribute to the presence of high levels of antimicrobial residues in meat (Nisha, 2008). The effects caused by antimicrobial residues in foodstuff comprise carcinogenicity, bone marrow toxicity, mutagenicity, autoimmunity, (Nisha, 2008; Pavlov *et al.,* 2008). It is also important for oxytetracycline residues in meat to be controlled to the acceptable levels since it may result into allergic reactions and other hazardous effects (Shankar *et al.,* 2010; Abbasi *et al*., 2011).

To control occurrence of harmful effects of drug residues in humans and animals, various regulatory and control measures have been established. These include the setting of maximum acceptable residue limits (MRL) in animal food products (FAO/WHO, 2014); for (OTC), Chlortetracycline (CTC) and Tetracycline (TC) to be 0.2 mg/kg for muscle tissue in cattle and pigs, and 1.2 mg/kg for kidney tissue in cattle and pigs.

The drug residues in food of animal origin is generally connected to the concentration of the drugs in raw samples. Meanwhile, most of these foodstuffs are heated before ingestion. Data on the effect of heat is essential to provide a more precise evaluation on the concentration of these deposits the users may be exposed to. For example, Javadi

(2011) showed a reduction in the concentration of doxycycline residues after boiling. Gratacós-Cubarsí *et al.* (2007), stated that ordinary cooking procedure reduced the initial concentrations of TC residues by 56 to 82% using microwave and boiling, respectively. Another study conducted by Loksuwan *et al.* (2002), revealed that antibiotic residues were reduced when heated to 63 ºC for 30 minutes. The effects of heat treatment on residues of antimicrobials have also been researched in other studies (Botsoglou and Fletouris, 2001; Hassani *et al.*, 2008; Hsieh *et al*., 2011). However, these studies often used matrices other than milk (e.g. meat, aqueous solution, buffer solution) and they also used different temperatures and different methods of thermostability evaluation. This study sought to assess the the effects of barbecuing and boiling treatments on the concentration of OTC since these methods are applied to beef during household preparation in Tanzania.

The issue of drug residues poses a major problem as far as consumers`health is concerned. Due to the widespread use of antimicrobials for treatment of diseases in cattle, much effort has been directed towards the proper management and monitoring of antimicrobial usage in treatments in order to prevent contamination of raw milk and meat products (Alica *et al.,* 2003; Jahed, 2007). Several studies have been conducted on antimicrobial usage and residues in foods of animal origin such as milk, beef and eggs in Tanzania (Mmbando, 2004; Karimuribo *et al.,* 2005; Kivaria *et al.,* 2006; Mdegela *et al.,* 2009). Despite the reports by these scholars so far, there is limited information of the effect of cooking procedures on the levels of residues and this creates a scientific gap of knowledge which needs be addressed. It is of importance to address how serious the residues are affected after cooking and the levels be known to the Public especially in Tanzania.

Materials and methods

Study site

This study was carried out in Dodoma region in Tanzania. Dodoma Region lies at a latitude 4º to 7º South and longitude 35º to 37º East. The region is centrally positioned in Tanzania and is bordered by four regions namely, Manyara in the North, Morogoro in the East, Iringa in the South and Singida in the West. Purposive sampling technique was used to obtain beef samples from slaughterhouses and butcheries at Bahi, Kongwa, Dodoma Urban and Rural.

Chemicals and Reagents

Standard of OTC and Ethylenediaminetetraacetic acid (EDTA) were supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and Methanol were of HPLC grade (Merck Campany, Germany).

Analytical method validation

The following definitions and procedures for measuring the validation parameter were taken from the guidelines for the Germany Society of Toxicology and Forensic Chemistry GTFCh (2009).

Sample extraction and analysis

Sixty beef samples of 250 g each were collected in separate polythene bags and transported on ice bags to the University of Zambia for extraction and analysis. Antibiotic-free meat control samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The control and test samples were stored in a freezer at −20 °C for approximately 1 week and thawed at room temperature for eight hours before extraction and analysis of OTC residues. Beef samples that were positive for OTC

residues, were subjected to different cooking procedures; boiling and barbecue, similar to procedures applied to beef under household conditions.

Heat treatment of beef samples

1. **Boiling procedure:**

One hundred gram (100 g) sample was placed into a strainer, immersed in about one liter of boiling water. Water was added during boiling time to keep the volume of water for 30 minutes. It was then allowed to cool before extraction and analysis of OTC residues.

11. **Barbecue preparation:**

One hundred gram (100 g) sample was barbecued well for 20 minutes and allowed to cool before extraction and analysis of OTC residues.

Samples extraction

The extraction procedures were similar for spiked blank samples, test samples and those which were heat-treated. Samples were removed from the -20 ^{o}C freezer and were thawed. Approximately 10 g of muscle was weighed and mixed with 25 mg (EDTA) per gram sample.

The sample and the EDTA were homogenized using a blender for one minute. The blended sample was further ground using a mortar and pestle.

One gram (1g) of the homogenized sample was accurately weighed into a 15 mL polypropylene centrifuge tube. To the sample, 50 µL of 50 µg/mL caffeine solution, equivalent to 2500 ng caffeine, were added. Five millilitres (5 mL) acetonitrile was added using a 5 mL volumetric pipette and the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube by decantation. Five millilitres (5 mL) acetonitrile were added to

the residue, the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. Both supernatants were combined into a 15 mL centrifuge tube, briefly mixed using a vortex and gently dried under a stream of nitrogen to 2 mL. After drying, 0.5 mL of HPLC grade water and 30 µL of formic acid were added, making the mixture 1.2 % acidic. Fifteen milligrams (15 mg) of Supelclean ENVI-carb active coal were added and sample was mixed for 30 seconds using a vortex and centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube and dried to 0.5 mL.

Sample analysis

The determination of OTC residues was carried out according to the method of Mgonja *et al.* (2016) using HPLC- DAD. The HPLC apparatus was equipped with a constant flow quad pump at a flow rate of 0.5 mL/min. Elution of OTC from the analyte was done on an Eclipse XDB C-18 column 4.6 x 150 mm, 5µm I.D with HPLC grade water-acetonitrile containing 0.1% formic acid. A 100 µl injection volume of the analyte from each sample was injected in order to obtain average.

Peak areas of positive samples corresponded to retention time of 5.9 minutes of the reference standard for OTC. The concentrations of OTC residues in the samples were calculated from the linear equations obtained from the standard curves (Figure 5.1).

The Limit of Detection (LOD) documented for both boiled and barbecued beef samples were similar (Table 5.1). The relative standard deviations (RSD) was 8.9% that complied with the requirement of the Codex Alimentarius Commission of lower than 10%.

Table 5.1: Characteristics of the analytical method for cooked beef samples

Analyte	Matrix	LOD (ng/g)	LOQ (ng/g)	S.L (ng/g)	R (%)	RSD (%)
OTC	Boiled	18.2	54.6	100	66.6-75.9	8.9
OTC	Barbecued	18.2	54.6	100	66.6-75.9	8.9

LOD: limit of Detection

LOQ: limit of Quantification

S.L: spiked level

R: recovery

RSD: relative standard deviations

Data Analysis

The data was analyzed by a computer Programs (SPSS version 20) using t-test. A probability of $p < 0.05$ was considered statistically significant.

Results

The effect of different heat treatments on the concentration of OTC residues in beef samples are shown in Tables 5.2 and 5.3. The results revealed a reduction of OTC with boiling by 9.1-90.9.% in 30 minutes and barbecued resulted in 26.1 – 87.8% in 20 minutes. The mean concentration of OTC was significantly lower for boiled beef samples than for barbecued beef samples (69.45 ± 41.93 ng/g versus 69.40 ± 38.91 ng/g; $p < 0.05$). The reduction percentage was lower for the boiled beef than for the barbequed beef. The different levels of OTC residues between the raw, boiled and barbecued beef samples (Table 5.2) were statistically significant ($p<0.05$) .

Table 5.2: Percentage reduction of OTC residues in beef before and after cooking process

PLACE COLLECTED	RAW (Conc.ng/g)	RAW %	BARBECUED (Conc.ng/g)	BARBECUED % Reduction	BOILING (Conc.ng/g)	BOILING % Reduction
Dodoma Urban	322.28	100	93.77	70.9	71.10	77.9
Kongwa	25.06	100	14.69	41.3	22.48	10.3
Dodoma Urban	370.42	100	45.21	87.8	33.37	90.9
Dodoma Rural	167.32	100	45.46	72.8	32.37	80.6
Chamwino	262.16	100	134.07	48.5	134.07	48.5
Dodoma Rural	121.19	100	70.68	41.7	92.01	24.1
Kongwa	110.83	100	81.93	26.1	100.77	9.1
	P = 0.04					

Table 5. 3: Effect of cooking methods on OTC residues in beef

Treatment	Time	Raw samples (ng/g)	OTC Levels After heat-treatment (ng/g)	% Reduction
Barbecued	30 minutes	196.75 ± 124.75	69.45 ± 41.93	(26.1-87.8)%
Boiling	20 minutes	196.75 ± 124.75	69.40 ± 38.91	(9.1- 90.9)%
	P = 0.7			

The higher concentration of OTC was associated with a higher peak. The correlation coefficient associated with the linear regression for the OTC standard, is represented by R^2 = 0.94 (Figure 5.1).

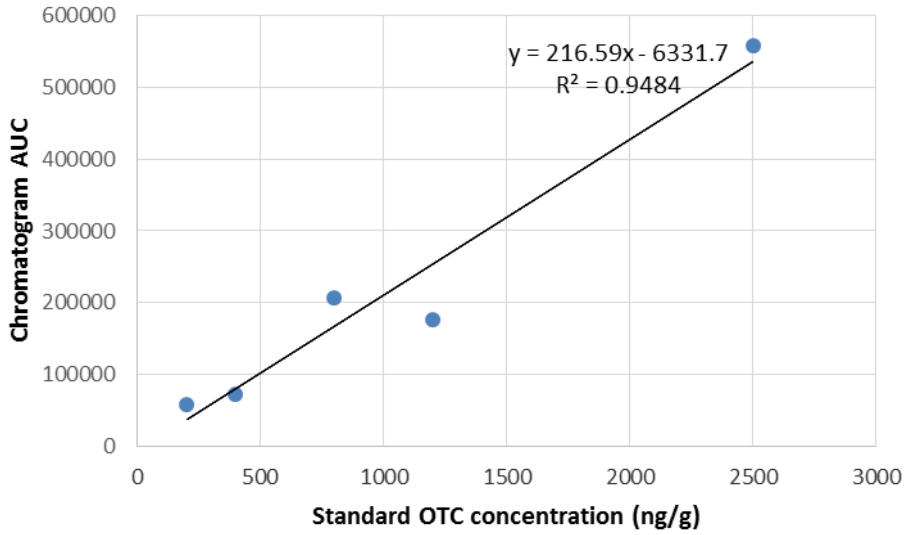

Figure 5.1: **Calibration curve of Oxytetracycline standard**

Discussion

Studies have indicated that cooking reduces the levels of antimicrobial residues in foods of animal origin but conventional heat treatment such as cooking, do not eliminate most AMRs in meat (Dipeolu and Ayo-Adisa, 2006). A study conducted by Van Egmond *et al.* (2000), reported the mean biological activity of enrofloxacin in pork tissues reduced to 68% after heat treatment at 134℃ for 20 minutes. In the present study both boiling and barbecuing cooking procedures significantly reduced OTC concentration in beef. The data revealed that the highest reduction in the OTC content of beef boiled for 30 mins was 87.8% while for beef barbecued for 20 minutes, the highest reduction was 90.9%. Al-Mustafa and Al-Ghamdi (2000) in another study involving norfloxacin reported that 40.5% and 72.1% of muscle and liver tissues of cattle retained AMRs above MRL after cooking at 100℃ for 20 minutes. Mishra *et al.* (2011) reported that pasteurization of milk at 65℃ for 30 minutes produced no significant reduction in cloxacillin residues in milk. Javadi, (2011) pointed that cooking process cannot eliminate AMRs present in meat because the temperature and time duration required are not attainable during normal cooking process and residues are excreted from tissue to cooking fluid during cooking process.

The decrease in OTC concentration observed in this study is consistent with earlier studies in which heat treatments decreased the concentration of antimicrobial residues in foodstuffs. For example, Rose *et al.* (1996), investigated the effect of cooking procedures including microwaving, boiling, roasting, grilling, braising and frying on OTC residues in animal tissues and observed 94% net reduction in OTC. Another study by Ibrahim and Moats (1994), reported that OTC levels were reduced by 95% when meat was boiled for 30 minutes. The results from the study are in agreement with Salah *et al.* (2013), who found 73.6% OTC reduction in meat by boiling for 30 minutes.

The decrease of OTC measured concentrations in beef samples might be attributed to the binding of OTC with proteins, which was caused by the various heat treatments. Furthermore, the increase in temperature above 80 °C results in the denaturation of proteins, which facilitates the unfolding of the polypeptide (Damodaran, 1996; Rose *et al.*, 1996). The latter condition gives rise to new binding sites becoming available, which were previously hidden, where the free OTC molecule might be bound to new binding sites in polypeptides, which in turn could explain the decreased OTC measured concentrations (Damodaran, 1996 ; Rose *et al.*, 1996).

It was also observed that none of beef samples had the OTC residues above the MRLs (200 ng/g) after the barbecuing and boiling processes. These results are in line with a study conducted by AI-Ghamdii *et al.* (2000); Nguyen *et al*. (2013) who found a decrease of OTC residues below MRLs after boiling for 20 minutes. There was however, no difference in OTC concentration in boiled and barbecued beef. The decrease in OTC concentrations during the boiling process was due to migration of the OTC from the meat to the cooking medium (water) while the decrease during the barbecuing process was due to juice oozing out from the meat (Rose *et al.,* 1995; Rose *et al.*,1996). The soup for OTC was not analysed in this study. The general loss of OTC residues was due to degradation of TC compounds (Rose *et al.,* 1995; Rose *et al.,*1996; Javadi *et al.,* 2011). These findings demonstrate an extra benefit of cooking as a food processing method.

Comparizon was made between urban and rural sources. It was found that urban beef seem to have higher concentrations of OTC residues than rural. Studies have shown that in Tanzania urban and periurban, livestock keepers are using more antimicrobials than in rural areas due to the need to capture the market in animal products (eggs, meat, milk), (Aiello and Moses, 2010).

Cooking and barbecue time of 30 and 20 minutes respectively, was due to the study conducted in different Districts in Dodoma region based on the knowledge, attitude and practice in relation to OTC in beef among adult resident in Dodoma Region. A study described by Christine, W (2017) who observed differences in the timing and duration of cooking between rural and urban areas. This study is against our study where by cooking types from urban were not differ from that of rural and majority of them prefers boiling, barbecue and smoking.

Availability of antibiotics such as OTC, lack of awareness and knowledge on proper use of guidelines from manufacturers may lead to mismanagement and overuse of the antibiotics. This may result to the failure to observe withdrawal periods and contribute to the high levels of antibiotic residues in meat (Nisha, 2008). The variation in OTC concentrations observed in the current study may be due to different types of beef samples and local animal farming practices. The results are similar to the findings by Muriuki *et al.* (2001) who reported residue level variations even from the same District which indicates the variation in animal husbandry practices.

Conclusion

In conclusion, heat treatment, such as cooking, may be useful in reducing the amount of some AMRs in meats but effort should be geared towards total elimination of drug residues in foods of animal origin. To ensure that residues of OTC in beef are below the (FAO/WHO, 2014) set a MRL of 0.2 mg/kg the withdrawal period of OTC should always be observed.

Acknowledgments

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Ethical issues

Permission for this study was granted by the Executive Directors of the Dodoma Region Council and ethical approval for the study was obtained from the Ethical Committee of the Sokoine University of Agriculture. The university issued a research permit letter on behalf of the Tanzanian Commission for Science and Technology.

Competing interests

The authors declare no conflict of interests.

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CHAPTER SIX

MANUSCRIPT IV

Effect of freezing on stability of oxytetracycline residues in beef from Dodoma Region, Tanzania

Status: Submitted in the *Journal of Food and Drug Analysis*

Effect of freezing on stability of oxytetracycline residues in beef from Dodoma Region, Tanzania

Abstract

The aim of this study was to determine the effect of freezing on the concentration of oxytetracycline (OTC) residues in beef samples stored at -20 °C (core beef temperature -12 °C and below) for 60 and 120 days. A total of 60 fresh beef samples were purposively collected from slaughterhouses and butcheries from Districts in Dodoma Region, Tanzania. The OTC residues were determined using high performance liquid chromatography (HPLC) with a diode array detector (DAD). Out of 60 beef samples analysed 16 beef samples had OTC. Results showed that the mean concentration of OTC residues in 16 positive samples before freezing was 191.71 ± 90.21 ng/g. The mean concentration of OTC after freezing at -20 ºC for 60 and 120 days were 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g respectively. These results revealed a significant ($p<0.05$) reduction of OTC residues of 30% after 60 days and 65% after 120 days of freezing. The percentage reduction of OTC residues was not dependent on the initial concentration or the freezing process but was rather due to unknown time dependent individual beef sample factors. It is concluded that, despite OTC levels in beef decreasing due to non-freezing factors, any residues significantly above Maximum Residues Level (MRL) may not be expected to reduce to acceptable levels as a result of freezing.

Key words: Oxytetracycline, cold storage, HPLC, beef.

Introduction

The presence of antimicrobial residues (AMRs), in food is a Public health concern. The availability of antimicrobial residues in some countries without effective regulations and

with inadequate awareness on appropriate drug use among livestock keepers results in the occurrence of high levels of antimicrobial residues in meat (Nisha, 2008). Some of the effects caused by antimicrobial residues in food include autoimmunity, carcinogenicity, mutagenicity and bone marrow toxicity (Pavlov *et al.,* 2008; Nisha, 2008). Furthermore, AMRs present in meat, milk and other foodstuff can initiate the development of resistant strains of bacteria due to the consumption of sub-therapeutic doses of antimicrobial (Mateu and Martin, 2001; Teale, 2002; Wilson *et al.,* 2003; Hardman and Limbird, 2007).

Several studies have been conducted to determine the levels of AMRs in food products of animal–origin in Tanzania. The prevalence of antimicrobial residues ranges from 2.8% to 100% in beef, chicken meat, milk and eggs in various areas in Tanzania (Mmbando, (2004); Karimuribo *et al.* (2005); Kurwijila *et al.* (2006). Nonga *et al.* (2009); Nonga *et al.* (2010); Nonga *et al.* (2013) and Mgonja *et al.* (2016) reported antimicrobial residues prevalence of 70%, 100%, 76.4%, and 71% respectively in cattle, meat, chicken meat, milk and eggs.

The destiny of antimicrobial residues during heat-treating is still uncertain. Many scientists have been concerned whether antimicrobial residues can be destroyed by cooking procedures, pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose *et al.,* 1995; Isidori *et al.,* 2005; Hassani *et al.,* 2008; Hsieh *et al.,* 2011; Mgonja *et al.,* 2016). A study described by EI Atabani *et al.* (2014) reported that out of one hundred local liver samples examined by microbial inhibition test for OTC residues, 5 samples (5%) reacted positive while all the 20 imported frozen liver samples examined were free from OTC residues.

Although freezing is a form of preservation of meat by hindering the development of microorganisms, various researchers have reported many variations in the reduction of antimicrobial residue concentrations with time in frozen meat making the reason for any reported reductions unclear. The stability of antimicrobials is generally expected to be higher during storage at –20 °C in comparison to storage at 4 °C (Honikel *et al.,* 1978; O Brien *et al.,* 1981; Pavlor *et al.,* 2005). However, O'Brien *et al.* (1981), reported that the concentration of oxytetracycline decreased by 7.4% and sulphadimidine by 20.1% in meat stored at 4 °C for 6 weeks. Gehad (2002), reported that there was no antibiotic residue detected in cattle muscle and organs after freezing for three months at -20 °C. It is therefore, hypothesized that cold storage could reduce OTC residues in beef from unacceptable levels to acceptable values with passing time. The aim of this study was therefore to investigate the effect of freezing on OTC residues in beef from abattoirs and butcheries.

Materials and methods

Study site

This study was carried out in Dodoma region in Tanzania. Dodoma Region is centrally positioned in Tanzania, lying at latitudes 4º to 7º South and longitudes 35º to 37º East, and is bordered by four regions namely, Manyara in the North, Morogoro in the East, Iringa in the South and Singida in the West.

Sample size and collection

A total of 60 beef samples were obtained from cattle slaughterhouses and butcheries in Bahi, Kongwa, and Dodoma Urban and Rural Districts. Slaughterhouses and butcheries were selected using purposively sampling technique. Each sample was transferred in a separate sterile and labeled plastic bags in an ice-box and transported to Zambia

Agricultural Reseach Institute (ZARI) laboratory. All samples were analyzed for determination of OTC residues. The control and test samples were stored in a freezer at −20 °C for approximately 1 week. Both control and test samples were thawed at room temperature for four hours before extraction and analysis of OTC residues. Antibiotic-free meat control samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The sixty samples were analyzed by HPLC and found that only 16 samples were positive for oxytetracycline. Sixteen beef samples, which were positive for OTC residues were subjected to cold storage at −20 °C for 60 and 120 days.

Analytical method validation

The procedures for validation parameter were taken from the guidelines for the Germany Society of Toxicology and Forensic Chemistry GTFCh (2009).

Samples extraction

The extraction procedures were similar for spiked blank samples and test samples. Samples were removed from the -20 ºC freezer and were thawed. Approximately 10 g of muscle was weighed and mixed with 25 mg (EDTA) per gram sample. The sample and the EDTA were homogenized using a blender for one minute. The blended sample was then further ground using a mortar and pestle.

One gram (1g) of the homogenized sample was accurately weighed into a 15 mL polypropylene centrifuge tube. To the sample, 50 µL of 50 µg/mL caffeine solution, equivalent to 2500 ng caffeine, were added. Five millilitres (5 mL) acetonitrile was added using a 5 mL volumetric pipette and the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube by decantation. Five millilitres (5 mL) acetonitrile was added to the

residue, the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. Both supernatants were combined into a 15 mL centrifuge tube, briefly mixed using a vortex and gently dried under a stream of nitrogen to 2 mL. After drying, 0.5 mL of HPLC grade water and 30 µL of formic acid were added, making the mixture 1.2% acidic. Fifteen milligrams (15 mg) of Supelclean ENVI-carb active coal were added; the sample was mixed for 30 seconds using a vortex and centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube and dried to 0.5 mL.

Sample analysis

The reference standard for OTC and Ethylenediaminetetraacetic acid (EDTA) was supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were of high performance liquid chromatography (HPLC) grade (Merck Company, Germany).

The determination of OTC residues was carried out using HPLC with a diode array detector (DAD) as describe by Mgonja *et al*. (2016). The HPLC apparatus was equipped with a constant flow quad pump at a flow rate of 0.5 mL/min. Elution of OTC from the analyte was done on an Eclipse XDB C-18 column 4.6 x 150 mm, 5µm I.D with HPLC grade water-acetonitrile containing 0.1% formic acid. A 100 µl of the analyte from each sample was injected to obtain average peak areas of positive samples corresponding to retention times of 5.0 minutes of the reference standard for OTC. The concentrations of OTC residues in the samples were calculated from the linear equation, $Y = 614.8x + 428699$ (where, Y = AUC for sample OTC chromatogram peak, x = concentration of OTC in sample) obtained from the standard curve (Figure 5.1). The Limit of Detection (LOD) was 18.2 ng/g and the Limit of Quantification (LOQ) value was 54.6 ng/g.

Data Analysis

The data were analysed using SPSS version 20. A probability of $p<0.05$ was considered statistically significant.

Results

The results revealed that there was reduction in concentration of OTC residues in beef after storage at -20 ºC for 60 and 120 days by 2%-30% and 11%-65% (Table 6.1). The mean concentration of OTC after the cold storage days was significantly lower than the mean concentration of OTC before the cold storage (166.40 ± 86.49 ng/g (60 days) and 133.50 ± 83.24 ng/g (120 days) versus 191.71 ± 90.21. ng/g; before sorage $p<0.05$). Only two samples with OTC levels marginally above Codex Alimantarious MRL of 200 ng/g before freezing had their concentration reduced to levels below the MRL during the freezing period. The higher concentration of OTC was associated with a higher peak. The correlation coefficient associated with the linear regression for the OTC standard concentration with AUC is represented by = R^2 0.99 (Figure 6.1).

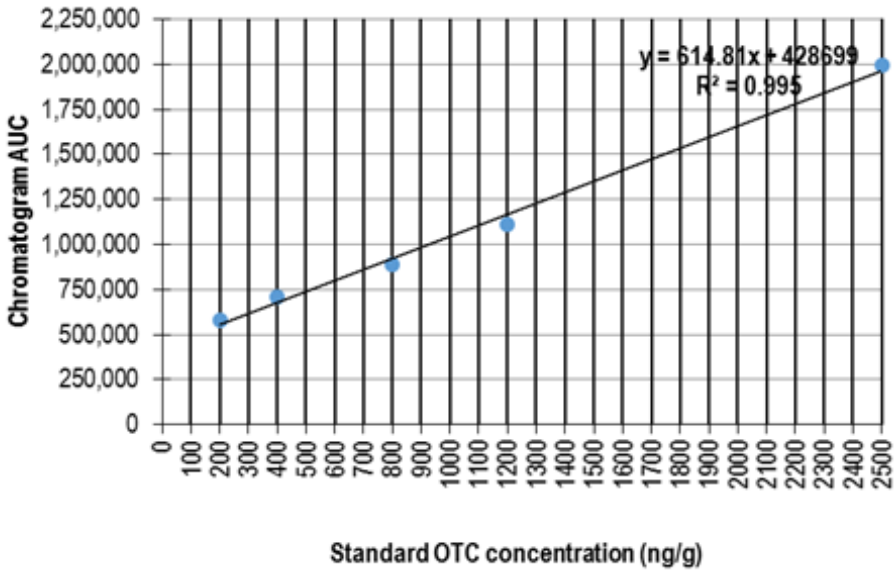

Figure 6.1: **Calibration curve of OTC standard**

Table 6.1: Concentration of OTC and percentage reduction after freezing at -20 ºC for 60 and 120 days

SAMPLE NO.	OTC CONCENTRATION IN BEEF (ng/g)			OTC CONCENTRATION REDUCTION (%)	
	Before storage	After 60 days at -20ºC	After 120 days at -20ºC	After 60 days at -20ºC	After 120 days at -20ºC
1	188.20	131.06	66.20	30	65
2	92.09	82.46	74.35	10	19
3	70.68	57.61	42.02	18	41
4	370.42*	357.42	315.66	3	15
5	167.32	145.34	114.00	13	32
6	262.16*	233.45	212.07	11	19
7	134.07	128.75	114.73	4	18
8	**221.37***	197.27	170.67	11	23
9	318.22*	229.7	146.15	28	54
10	228.62*	224.01	201.97	2	12
11	143.57	120.71	90.25	28	54
12	322.28*	314.33	284.63	2	12
13	132.11	126.6	96.51	30	65
14	108.14	75.25	38.13	4	26
15	**207.39***	154.81	108.95	25	47
16	100.77	82.66	59.79	18	41
RANGE	70.68 -370.42	57.61 – 357.42	42.02 – 315.66	2 - 30	11 - 65
MEAN	191.71 ± 90.21	166.40 ± 86.49	133.50 ± 83.24	13 ± 10	33 ± 22

P = 0.03

*Samples with OTC levels above Codex Alimantarious MRL of 200 ng/g in muscle before freezing. Shaded rows shows samples with concentrations that reduced to levels below the MRL during the freezing period.

Figure 6.2 below shows that the percentage reduction of OTC residues in frozen beef samples was not dependent on the levels of OTC in the sample before storage but rather on individal field sample factors that were not investigated in this study. Levels in all samples continued decreasing despite all of them being subjected to the same freezing conditions.

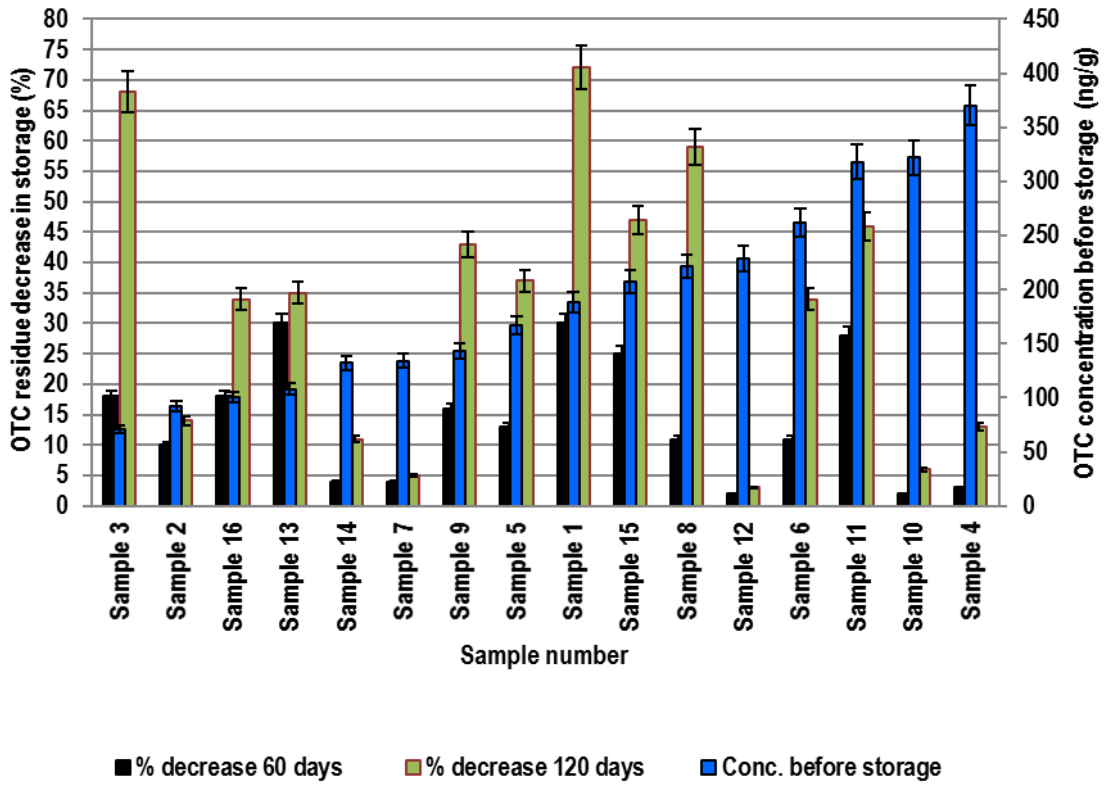

Figure 6.2: The OTC concentration percentage decrease in relation to its increasing concentration before storage

Discussion

Presence of antimicrobial residues in beef can pose hazards to human health. Among them are allergic reaction and imbalance of intestinal microflora, bacterial resistance to antibiotics in microorganisms and losses in the food industry through growth inhibition of food processing microorganisms (Cunha, 2001). Although drug manufactures always recommend withdrawal periods for drugs used in food producing animals, it is common to find OTC residues at concentrations above Codex Alimentarius Commission MRL in beef readily sold for human consumption (Kaneene and Miller, 1997). Various factors contribute to the presence drug residues in beef including; failure to observe withdrawal periods, age of the animal, disease status (Kaneene and Miller, 1997). This means that sometimes drug residues may be present in beef even when withdrawal period has been

observed. Therefore, it is important to understand local factors that may lead to the presence of antimicrobial residues in beef, as well as factors that break down these residues to acceptable levels. Pavlov *et al.* (2005), found a decreasing level of tobramycin sulphate from chicken breast and thigh muscle during the period of cold storage. The drug showed initial higher levels in the liver, followed by breast and thigh muscles, with no residues in the muscles on the 30th day. A study by Pavlov *et al.* (1993), showed that freezing at -20 ºC caused a lower degradation than that caused by boiling. So neither boiling nor freezing could be used as reliable methods to get rid of Amoxycline residues in meats.

In this study, HPLC was used to determine the concentration of oxytetracycline (OTC) in beef samples in order to determine whether cold storage has an effect on OTC residues. (Table 6.1). These results are consistent with a number of other studies which reported reductions in antimicrobial residue concentrations in meat following cold storage. O'Brien *et al.* (1981) used the diameter of growth inhibition zone to establish antimicrobial concentrations and observed that the concentration of OTC decreased by 7.4%, sulphadimidine by 20.1% and ampicillin by 76.05%-100% in meat samples stored at 4 ºC for 6 weeks.

It was also observed that freezing reduces residues depending of the type of antimicrobial in test (O'Brien *et al.* 1981). Usually, the stability of antimicrobials is far higher during storage at –20°C in comparison with 4°C (Honikel *et al.,* 1978; O Brien *et al.,* 1981; Pavlor *et al.,* 2005).

A study conducted in Turkey by Ayhan *et al.* (2015) indicated that residue levels decreased within days in drugs such as Florfenicol (a fluorinated synthetic analog of

thiamphenicol and Florfenicol amine (major metabolite of the antibiotic florfenicol, a fluorinated derivative of chloramphenicol) without any significant difference between storage conditions at -20°C and +4 ºC . This study is also in line with Tansel *et al.* (2006), that showed concentrations of gentamicin residues were retained for fourteen days at both refrigerated (+4°C) and room temperatures (15-20 °C), then started to lose strength on day 21 of storage. Another study by Papapanagiotou *et al.* (2005), reported that sulphamethazine (SMZ) residues were stable at -20 °C and −75 °C in all piglets muscle tissue examined for at least 3 and 5 months, respectively. A study by Alfredsson and Ohlsson (1998) reported that levels of sulphamethazine spiked in beef and frozen at -20 °C for 3 months decreased by 35%.

The level of penicillin G kept in a deep-freezer for 10 days decreased by half in the gluteal muscles, and by 20% in the kidneys (Boison *et al.*, 1992). Findings from other studies have shown that freezing of penicillin G, ampicillin, OTC, sulfonamide, quinolones and gentamicin have minor or no effect on the residues levels (Nouws and Ziv, 1976; Boison *et al.*, 1992; Verdon *et al.*, 2000; Baydan *et al.*, 2002; Sireli *et al.*, 2006). The decrease in the quinolones activity in frozen stock solutions stored at –20 °C did not exceed 10%, whereas the levels of β-lactams did not change during 3 months of storage (Okerman *et al.*, 2007).

Although many studies have demonstrated a general decrease of OTC levels during cold storage of beef, this study shows that the decrease was not a result of the process of freezing, but was rather due to individual sample factors prevalent at the initial stage of freezing (Lagerstedt *et al.*, 2008). This is supported by the fact that despite all samples being stored under the same conditions, OTC residues continued degrading at different rates during the whole study period. Only two samples with OTC levels marginally above

Codex Alimantarious MRL of 200 ng/g before freezing had their concentration reduce to levels below the MRL during the freezing period. Immediately after slaughter and before grading or freezing, beef must undergo the process of chilling (cold storage at 0 – 4 °C to achieve core beef temperature of 7 °C and below) in order to stop the growth of spoilage microorganism and improve the quality of meat (FAO, 1991). However, variations in the speed of the chilling process can produce meat with varying quality factors such as colour, pH and microbial growth (Aalhus *et al.,* 2001) which may have an effect on the stability of drug residues in meat. Therefore, more research is required in order to determine the effects of pre-slaughter and meat chilling factors on OTC residues in meat.

Conclusion

The results of this study show that OTC residues were detectable in frozen beef up to 120 days although on average, there was a significant decrease in concentration. The reduction of OTC residues was not dependent on the freezing process or the initial concentration but was rather due to unknown time dependent individual beef sample factors. Although OTC levels in beef decreased due to non-freezing factors, any residues above MRL may not be expected to reduce to acceptable levels as a result of freezing.

Acknowledgment

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Ethical issues

Permission for this study was granted by the Executive Directors of the Dodoma Region Council and ethical approval for the study was obtained from the Ethical Committee of the Sokoine University of Agriculture. The university issued a research permit letter on behalf of the Tanzanian Commission for Science and Technology.

Competing interest

The authors declare no conflict of interests.

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CHAPTER SEVEN

7.0 SUMMARY OF MAIN FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

7.1 Summary of the Main Findings

The main findings emanating from this study were as follows;

i. A simple and sensitive method for the detection of oxytetracyline levels in ready-to-eat beef by liquid chromatography-Mass Spectrometry was modified and validated.
ii. Cooking procedures and cold storage reduced antimicrobial residue levels.
iii. There were significant low levels of residues in beef in general.
iv. No proper monitoring of residues in beef in Tanzania.

7.1.1 Manuscript 1: The knowledge, attitude and practice in relation to beef consumption among residents living in Dodoma region.

Manuscript one on ‘evaluation of knowledge, attitude and practice in relation to beef consumption by residents in Dodoma’ will be submitted in the *Journal of Food Protection* (Elsevier). The objective of the study was to evaluate the knowledge, attitude and practice in relation to beef consumption among adult residents living in Dodoma region.

The results showed that Fifty two percent of the respondents were not aware of drug residues, 57% ever heard about drug residues in food of animal origin such as milk and meat, 35% did not know residues can be harmful to human and 61% did not know if animals are treated with antimicrobial drugs when they were sick. Only 27% of the respondents knew common antimicrobial agents that can cause drug residues in animal meat and milk and 31% were able to mention them. Majority of respondents (74%) did not

know any method to prevent drug residues. Fifty six percent of the age group 20-35 purchased the meat tissues. Secondary (68.4%) and College (52.9%) respondents purchased meat from butcheries compared to informal (23.8%) and primary (49.2%) respectively. Majority of informal (66.7%) and primary (47.6%) respondents purchased meat locally within the villages. The differences were strongly significant $p< 0.0001$. Women (57.1%) used one hour to prepare meat. Age group 20-35 (88.1%) prepared meat by cooking. Age group 36-45 years mention that meat preparation takes 1 hour and 2 hours respectively. College (68.8%) respondents'were barbecuing meat compared to smoking and freezing. The results in this study indicate that respondents had low knowledge and awareness on antimicrobial use and drug residues. This might be due to low level of education of respondents as majority of them had informal and primary education. Many of the drug respondents were not aware of the drug residues and did not know antibiotic residues can have effects in human health. Community based health education and education on antimicrobial use and preventing drug residues is highly recommended to this population**.**

7.1.2 Paper One: A simple and sensitive method for the detection of Oxytetracycline levels in ready-to-eat beef by liquid chromatography-Mass Spectrometry.

Paper one, 'A simple and sensitive method for the detection of "Oxytetracycline" levels in ready-to-eat beef by liquid chromatography-Mass Spectrometry' was published in the year 2016 in an *African Journal of Pharmacy and Pharmacology* (*Academic Journal*). The paper validated a simple and sensitive method for the detection of "Oxytetracycline" levels in ready-to-eat beef by liquid chromatography-Mass Spectrometry'. Beef samples were extracted in acetonitrile in ethylenediaminetetraacetic acid (EDTA) buffer (pH 4), followed by cleaning up with Supelclean ENVI-carb active coal and a stream of nitrogen gas. The wavelength of the diode array detector (DAD) was set at 275 nm and 355 nm.

The detection limit of the method was calculated as 18.2 ng/g and the recovery rate of OTC was 78.6%. To test the method 45 ready-to-eat beef meat samples were analyzed, 16 (35.5%) and 29 (64.5%) barbequed and boiled samples, respectively.

The findings showed that out of 45 samples, 35 (77.8%) samples had OTC residues while 9 (25.7%) samples had violative residue levels above the maximum residue limit recommended by the Food and Agriculture Organization and the World Health Organization. The highest concentration was 545.2 ng/g. Therefore, withdrawal period and proper use of antibiotics for animal production should be of concern as consumers are at risk of adverse effects due to consumption of unacceptable levels of drug residues and a risk of developing microbial resistance. To the best knowledge of authors, this was the first study to evaluate LC-MS method to detect the OTC levels in ready-to-eat beef meat in Tanzania.

The reasons for the modification were

i. Cleaning and drying by solid phase extraction is very expensive for researchers.

Advantages of the modified method

ii. Cleaning by Supelclean ENVI-carb active coal is cheaper compared to solid phase extraction.

iii. Samples drying using a stream of liquid nitrogen is cheaper and more than six samples can be dried at a time.

iv. More researchers can use this technique since it is cheaper and more samples can be cleaned and dried at the same time.

7.1.3 Manuscript two: Oxytetracycline residue levels in beef in Dodoma region, Tanzania

Manuscript two on, 'Oxytetracycline residue levels in beef in Dodoma region, Tanzania' will be submitted in the *Journal of Food Science.* The manuscript determined the residues levels in the beef consumed by the people living in Dodoma. The OTC levels were determined by using Liquid Chromatography - Mass Spectrometry (LC- MS). A total of 60 beef samples were collected from various slaughterhouses and butcheries and analysed.

The findings showed that twenty-one out of 60 samples (35%) had OTC residues and no samples had OTC levels above the maximum allowed residues limit (200 µg/kg). The highest oxytetracycline concentration was 4.95 ng/g and the mean concentration was 0.69 ± 0.09 ng/g. The results indicate that the mean concentration level was very low. Even though these levels were not expected to induce adverse effects, from a food safety viewpoint, high-level occurrence of OTC should however be of concern.

7.1.4 Manuscript three: Effect of heat treatment on oxytetracycline residues in beef.

Manuscript three on, **'**Effect of heat treatment on oxytetracycline residues in beef' submitted in the *Journal of Veterinary Research.* The manuscript determined the effect of heat treatment on oxytetracycline residues in beef consumed by the people living in Dodoma. The beef samples were boiled for 30 minutes or barbecued for 20 minutes. The OTC content was measured in raw and heated samples by using high performance liquid chromatography (HPLC).

The mean concentrations of OTC for boiled and barbecued beef samples were 69.45 ± 41.93 ng/g and 69.40 ± 38.91 ng/g, respectively. Both the boiling and barbecuing

procedures significantly decreased the OTC levels in beef ($p<0.05$), and the boiling procedure had the highest influence on reducing OTC concentration. The OTC concentrations after the heating treatments were below the maximum acceptable residue limit (MRL). In conclusion, heat treatment, such as cooking may be useful in reducing the amount of some Antimicrobial Residues (AMRs) in meats but effort should be geared towards total elimination of drug residues in foods of animal origin. Proper usage and withdrawal period of OTC should always be observed.

7.1.5 Manuscript four: Effect of freezing on stability of oxytetracycline residues in beef from Dodoma region, Tanzania.

Manuscript four on 'Effect of freezing on stability of oxytetracycline residues in beef from Dodoma region, Tanzania' will be submitted in the *Journal of Food and Drug Analysis* (Elsevier). This study sought to examine the effect of the cold storage on the concentration of oxytetracycline (OTC) residues in beef samples stored at -20 °C for 60 days. Beef samples were randomly obtained from cattle slaughterhouses and butcheries in districts in Dodoma region in Tanzania. The OTC residues were determined using high performance liquid chromatography (HPLC) with a diode array detector (DAD). Results showed that the mean concentration of OTC residues in 16 positive samples before freezing was 191.71 ± 90.21 ng/g. The mean concentration of OTC after freezing at -20 ºC for 60 and 120 days were 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g respectively.

These results revealed a significant ($p<0.05$) reduction of OTC residues of 30% after 60 days and 65% after 120 days of freezing at -20 °C. The percentage reduction of OTC residues was not dependent on the initial concentration or the freezing process but was rather due to unknown time dependent individual beef sample factors. It is concluded that, although OTC levels in beef decreased due to non-freezing factors, any residues

significantly above Maximum Residues Level (MRL) may not be expected to reduce to acceptable levels as a result of freezing.

7.2 Conclusions

These results may call for a proper management of antimicrobial use for animal's production as an added advantage to consumers. The results indicate that one should not count on heat-treatment or cold storage to eliminate residues of OTC from beef since the methods do not eliminate the antimicrobial residues completely. Community-centred health education and promotion on antimicrobial use and preventing antimicrobial residues is highly recommended to this population.

7.3 Recommendations

i. To confirm that residues of OTC in beef are below the (FAO/WHO, 2014) set a MRL of 0.2 mg/kg, the withdrawal period of OTC should always be observed.
ii. Even though residue levels may not induce adverse effects, from a food safety viewpoint, high-level occurrence of OTC should however be of concern.

7.4 Areas for Further Research

i. Effect of water on OTC during cold storage should be studied since water is a known participant in the degradative reactions of OTC
ii. During the HPLC analysis, many peaks are formed together with the OTC peak. These peaks have to be identified since they might be other compounds that cause residues reduction.
iii. It was observed that some of antimicrobial residues from boiling method enter into soup that is consumed by humans. Detection of antimicrobials in soup must be carried out.

APPENDIX

Appendix 1: Questionnaire

I am Frida Mgonja from Sokoine University of Agriculture. I am doing a survey about beef residues. The survey is completely voluntary and your answers will be kept strictly confidential and only used for the purpose of this study.

Study site – Dodoma Region

Date of interview -

Name of interviewee -

Language used to interview- Swahili

Section 1: Demographic data

No	Question	Coding category
1.	Sex	1) male 2) female
2.	How old are you?	1) 15-20 2) 21-20 3) 31-40 4) 40 and above
3.	What is the highest level of education you have attended?	1) none 2) primary 3) secondary 4) high school 5) college 6) vocational 7) others specify
4.	What is your religion?	1) Christian 2) Muslim 3) traditional 4) others specify
5.	What is your current occupation?	1) peasant 2) business 3) student
6.	What is your marital status?	1) single 2) married 3) widow/ widowed 4) divorced

Section 2: Information about meat

No	Question	Coding category
7.	Where do you buy meat?	1) Meat market 2) Butcher 3) Buying locally within the village 4) Any other places
8.	How often do you consume meat in your family?	State
9.	Total number of the family member. What is the amount of cattle meat consumed per meal per person in your family (approximate average)?	State
10.	Who consume large share of the meat meal in your family?	1) Children 2) Young women 3) Young men 4) Married woman 5) Married Men 6) Older women and men (above 50 years) 7) Any other (please mention)
11.	How do you prepare cattle meat before consumption?	1) Eating raw meat (1) Yes (2) No 2) Cooking (1) Yes (2) No 3) Barbeque (1) Yes (2) No 4) Smoking (1) Yes (2) No 5) Freezing (1) Yes (2) No
12.	How much time does it take to prepare cattle meat before consumption?	1) ¼ hour 2) ½ hour 3) 1 hour 4) 2 hour 5) Others specify.....
13.	Which part of meat your mostly used?	1) Liver 2) Kidney 3) Neck part 4) Muscles 5) Others specify.....

Section 3: Drug Residues

No	Question	Coding category
14.	Do you know drug residues?	1) Yes 2) No
15	Have you ever heard about drug residues in food of animal origin such as milk, meat etc.?	1) Yes 2) No
16.	Do you think residues can be harmful to human?	1) Yes 2) No
17.	Do you know that animals are treated with antimicrobial drugs when are sick?	1) Yes 2) No
18.	Do you know common antimicrobial agents which can cause drug residues in animal meat and milk?	1) Yes 2) No
19.	Can you mention any antimicrobial drug you know?	1) Name them
20.	Do you know any method to prevent drug residues?	1) Mention them

21. Those are all of the questions I had, do you have any comments that we have not discussed?

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Thank you for your cooperation