ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

DETECTION AND DETERMINATION OF OXYTETRACYCLINE AND PENICILLIN G ANTIBIOTIC RESIDUE LEVELS IN BOVINE BULK MILK FROM DEBREZEIT AND NAZARETH DAIRY FARMS

BY
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JUNE, 2008
DEBRE ZEIT, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Veterinary Science in Tropical Veterinary Public Health

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DEDICATION

This thesis is presented to be a remembrance to Prof. Dr. Gisela Kittel for her unreserved help and concern she has provided me to promote my life in dead.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Mosses Kyule for his excellent comments, for patiently and skillfully going through the manuscript. I would like to forward my acknowledgment to my co-advisors Dr. Kelay Belihu, Dr. Girma Zewda and Dr.Tadelle Dessie for their encouragement and sharing their time and knowledge to guide, correct and makes this manuscript harmonious.

I express my thanks to International Livestock Research Institute for sponsoring my research. Much of the acknowledgement goes to Faculty of Veterinary Medicine, Addis Ababa University for funding the research project.

Sincere thanks are due to the staffs of department of microbiology in Faculty of Veterinary Medicine for their inestimable value during my research period. I am highly indepted to staffs of Chemistry Department in Science Faculty and to staffs of Drug Quality Control and Toxicology Laboratory for all support and encouragement I received.

I am extremely grateful to Prof Dr. Gisela Kittel for her incalculable financial help and kindness in the successes of my life.

Much of the acknowledgment goes to my lovely friends Dr. Gebreyohanes, T., Dr. Fiseha, A., Dr. Molalegne, B., Dr. Gashaw, M., Dr. Akafeta, T., Dr. Yenahiwot, B., Dr. Shiferaw, G., and Ato Mulate, P., for sweety moments we shared and lovely moral during my stay with them.

Last but not least special thanks be reserved to my family whose dream is always the success of their children for whom they devoted greater part of their life.

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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
ANOVA	Analysis of Variance
CCPM	Critical Control Point Management
CSA	Central Statistical Authority
DACA	Drug Administration and Control Authority
DNA	Deoxyribo Nueclic Acid
EC	European Commission
EEC	European Economic Commission
EMEA	European Agency for Evaluation of Medical products
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FVM	Faculty of Veterinary Medicine
g	gram
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IDF	International Dairy Federation
Ig	Immunoglobulin
IOM	Institute of Medicine
IU	International Unit
JECFA	Joint Expert Committee on Food Additives
Km	Kilometer
LC	Liquid Chromatography
µg	micro gram
mm	Millimeter
MRL	Maximum Residue Limit
MS	Mass Spectrometry
nm	Nano Meter
NMSA	National Metrological Center Agency
NOEL	No Observed Effect Level
pH	Power of hydrogen

PPM	Parts Per Million
rpm	Revolution per minute
SPSS	Statistical Package of Social Science
STAR	Screening test for antibiotic residue
TLC	Thin Layer Chromatography
US	United State
USDA	United State Department of Agriculture
UV	Ultra Violet
VCPR	Veterinary Client Patient Relationship
WHO	World Health Organization
CEC	Commission of the European Communities
IOM	Institute of Medicine

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ABSTRACT

A cross-sectional study was conducted between October 2007 and May 2008 to detect and determine oxytetracycline and penicillin G residue levels in bulk milk of cows in Debre Zeit and Nazareth dairy farms. A total of 400 bulk milk samples were randomly collected from the respective study dairy farms. A questionnaire survey was carried out by personal interviews with dairy farm owners in Delvotest positive farms (cases) and Delvotest negative farms (controls) to identify various risk factors and to determine associations among the occurrence of antibiotic residues in milk. All samples were qualitatively screened for antibiotic residues by Delvotest SP assay. Concentration of the positive samples was determined by high performance liquid chromatography. Concentration was established using linear calibration reference curves. Out of 400 samples analyzed for antibiotic residues, 46 (11.50%) had detectable antibiotic residues. In Debre Zeit dairy farms, 34 (12.19%) and in Nazareth, 12 (9.92%) milk samples were positive for antibiotic residues. The mean residue level of oxytetracycline was 142.00µg/l and 125.25µg/l in Debre Zeit and Nazareth dairy farms and that of penicillin G was 4.77µg/l and 4.52µg/l in the two dairy farms, respectively. Oxytetracycline concentratyions in all samples in Debre Zeit and Nazareth dairy farms ranged at a concentration between 27-251µg/l and 45-192 µg/l, respectively. The antibiotic residues positive samples which showed residue of oxytetracycline above the WTO/FAO/CAC established maximum residue limit of 100gµ/l were 24 (70.58%) and 10 (83.33%) in Debre Zeit and in Nazareth dairy farms, respectively. For penicillin G with maximum residue limit of 4µg/l, they were 7 (20.58%) and 2 (16.66%) in Debre Zeit and Nazareth dairy farms, respectively. Penicillin G was found in some milk samples in Debre Zeit 19(58.8%) and Nazareth 5(41.7%) dairy farms. The results obtained confirmed that oxytetracycline and penicillin G were imprudently used in dairy farms. Results of the present study serve as preliminary base-line information for veterinary authorities, drug administration and quality control, other concerned organizations and professionals to take necessary measures in controlling and preventing of occurrence of drug residues in milk and other food products.

Keywords: Debre Zeit , Delvotest SP, High performance liquid chromatography, Milk, Nazareth, Oxytetracyclne, Penicillin G, Residue

1. INTRODUCTION

Milk is a lacteal secretion of the mammary glands of a mammal. As it is well known, milk is the first natural food that all young mammals ingest immediately after birth (Teka, 1997). Man has consumed milk and milk products even before the dawn of civilization. Because of its high nutritive value, milk is considered as one of the most important diet items of many people (Mehari, 1988). Nutritionally, milk has been defined as "the most nearly perfect food". It provides more essential nutrients in significant amounts than any other single food (O'Mahony, 1988).

Antibiotics have been used in the dairy industry for more than five decades. They are used in dairy cattle production primarily to treat or prevent disease and to a lesser extent to increase milk production or improve feed efficiency. Antibiotics used as growth promoters are administered at low doses for extended periods. As prophylactics, antibiotics are used at low doses to prevent disease. Although the duration of antibiotic use differs for growth promotion and prophylaxis, the dosage for both is typically less than 200 g/ton, and is considered subtherapeutic (IOM, 1989).

The therapeutic regimen is dictated by label instructions by the manufacturer or in accordance with extra-label instructions by a veterinarian. Antibiotics are administered to animals through injections (e.g., intramuscular, intravenous, or subcutaneous), orally, topically, or via intramammary or intrauterine infusion. Several types of antibiotics are commonly used in food animals (Mitchell *et al.,* 1998). Antibiotic classes include beta-lactams (e.g., penicillin, ampicillin, and cephalosporin), tetracyclines (e.g., oxytetracycline, tetracycline, and chlortetracycline), aminoglycosides (e.g., streptomycin, neomycin, and gentamycin), macrolides (e.g., erythromycin), lincosamides (e.g. lincomycin and pirlimycin), and sulfonamides (e.g., sulfamethazine and others) (Hoeben *et al*., 1998).

The use of antibiotics therapy to treat and prevent udder infections in cows is a key component of mastitis control in many countries. Due to the widespread use of antibiotic for treatment of mastitis in dairy cows, much effort and concerns have been directed towards the proper

management and monitoring of antibiotics usage in treatments in order to prevent contamination of raw milk. However, widespread use of antibiotics has created potential residue problems in milk and milk products that are consumed by the general public. Because of the public health significance, milk and milk products contaminated with antibiotics beyond a given residue levels, are considered unfit for human consumption (Hillerton *et al.*, 1999).

Antibiotic residues are small amounts of drugs or their active metabolites which remain in milk after treating the cows (CAC, 1998). Problems associated with antibiotic residues in milk include the risk of allergic reactions after consumption by penicillin-sensitized persons, increased resistance of pathogens towards antibiotics, and inhibition of bacterial starter cultures used in dairy production. The concerns arise mainly from the possibility that antibiotic -resistant bacteria may be transferred from animals to humans, through contact, through the environment (e.g., water, manure) or through contaminated milk products (CAC, 1998). Residues are illegal and milk supplies containing detectable concentrations are not acceptable. It has been estimated that antibiotic contaminated milk costs the US dairy industry $50 million annually (Rice *et al*., 1984).

Levels of the drug and their metabolites may persist at unacceptable levels and consumers can be exposed to them. The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage levels. Unauthorized antibiotic use may result in residues of these substances in milk and tissues (Ivona and Mate, 2002). Furthermore, many antibiotics used in animal agriculture are poorly absorbed in the animal gut. It is estimated that 25% to 75% of the antibiotics administered to feedlot animals could be excreted unaltered in feces (Elmund *et al*., 1971; Feinman and Matheson, 1978) and can persist in soil after application on land (Donoho, 1978; Gavalchin and Katz, 1994). There is little information available concerning the fate of antibiotics in the environment and their link to the emergence of resistant genotypes found there. The annual production of livestock and poultry waste in the United States is nearly 180 million tons (dry weight basis) (Hagedorn *et al*., 1999) and coupled with antibiotic usage, this waste is a potentially large source of both antibiotics and antibiotic-resistant bacteria released into the environment.

Antibacterial drugs such as oxytetracycline and penicillin G are routinely used in veterinary medicine for prevention and control of disease. Oxytetracycline is applied for the purpose of prevention or treatment of diseases such as bronchopneumonia, mastitis and metritis in cows. As a result, there is concern that residues of these compounds may be presented in the milk and milk products. The penicillins are widely used to treat or prevent local and systemic infections of farm animals. The use of penicillins as intramammary infusions or formulations to treat or prevent bovine mastitis is widespread (Haapapuro, 1997).

To detect antibiotic residues, different kinds of methods were developed. These consist of screening methods and chromatographic techniques to detect as many antibiotics as possible. The screening method is generally performed by microbiological, enzymatic and immunological methods. The screening methods are based on the various susceptibility of bacteria to different antibiotics. The antibiotic residue detection assays that are currently available use different methods and test micro-organisms (Mitchell *et al*., 1998). Microbiological assays for the detection of antibiotic residues utilize bacteria such as *Bacillus stearothermophilus* because of its high sensitivity to the majority of antibiotics. Both microbiological and chromatographic methods have been described for monitoring tetracyclines and penicillins in milk and animal tissues. Although the microbiological assay techniques have been recommended as official and conventional methods because of their simplicity, the bioassay methods lack specificity and provide only semi-quantitative measurements of residues detected and sometimes produce false positives (Kurittu, Lonnberg, Virta and Karp 2000). Therefore, chromatographic techniques, such as TLC, and HPLC, and capillary electrophoresis (CE), have been developed to replace microbiological assays (Chen and Gu, 1995; Cinquina *et al*., 2003; Ding and Mou, 2000; Furasawa, 2003; Huang *et al.,* 1997; Petkovska *et al.,* 2006; Posyniak *et al*., 2005; Zhao *et al.*, 2004).

Residues of antibiotic agents may be of toxicological significance for the consumer and may influence the technological properties of milk used for manufacturing fermented products. To guarantee consumers safe and high quality dairy products, raw milk is regularly analyzed for the presence of antibiotic residues. If the milk from a single cow undergoing treatment accidentally

enters the herd bulk milk, this may be sufficient to make the content of a tanker unsuitable for human consumption (McEwen *et al.,* 1991).

In order to safeguard human health, the World Health Organization (WHO) and the Food Agriculture Organization (FAO) have set standards for acceptable daily intake and maximum residue limits in foods (FAO and WHO, 1995). Regulatory limits for antibiotic residues have been imposed on the dairy industry in many countries (FDA, 1996; Folly and Machado, 2001). However, Ethiopia has not yet adapted international standards or established specifications for residue limits in the milk. The Ethiopian dairy industry has not adopted any control programs to ensure the safety of the milk. The drug residue limits, which apply to both the parent drug and its metabolites, need to be enforced at all levels in Ethiopia dairy industry in order to protect the health of consumers.

In Ethiopia, no studies have been conducted on oxytetracycline and penicillin G residue levels in milk and as such there are no data on the residual levels of these drugs in milk. Therefore, this study aims:

- To determine the prevalence of oxytetracycline and penicillin G residues in milk samples destined for consumption in Debre Zeit and Nazareth.

- To quantitatively determine concentration of oxytetracycline and penicillin G residues in milk samples with qualitatively positive results.

- To identify probable risk factors and to compare and contrast their associations with the occurrence of penicillin G and oxytetracycline residues.

- To assess the knowledge of the dairy farm owners about antibiotic residues in milk.

2. LITRATURE REVIEW

2. 1. Antimicrobial in dairy production

There are currently a number of antimicrobials approved for intramammary use in lactating cows (Table 1). Each of these antimicrobials has a prescribed withdrawal period. After a cow has been milked a full lactation, she is dried off to prepare her for calving in the next lactation. Although they are no longer milking, dry cows are still susceptible to mastitis. For this reason, nearly all cows are treated with a long acting intramammary antimicrobial (More, 2004).

Table 1: Examples of common antimicrobial agents administered to dairy cattle

Antibiotic family	Examples
Aminoglycosides	Gentamycine
Cephalosporins	Cephaprin
Ionophores	Monensin
Macrolids	Ertthromycin, Tylosin
Penicillins	Ampicillin, Penicillin G, Cloaxacillin
Tetracyclines	Oxytetracycline

Source: More (2004)

2. 2. Benefit of antimicrobial

The first recorded use of antimicrobial in dairy cattle was for the treatment of mastitis and this disease still accounts for the majority of antimicrobial use in dairy production. Antimicrobial are a valuable tool for controlling the expression of disease. Antimicrobial use in food animal production is a necessary and integral part of assuring an abundant, wholesome supply of food, protecting animal health and relieving animal from suffering (Mulamattathil *et al*., 2000; Moore, 2004).

2. 2. 1. Disease treatment (therapeutic)

On dairy farms, most antimicrobial are used therapeutically to treat individual animals affected by disease. Antimicrobial used in known clinically diseased animals for the purpose of restoring health and recovery from disease (Moore, 2004).

2. 2. 2. Disease control (metaphylactic)

The most common disease control use of antimicrobial in dairy is intramammary dry cow treatment. Dry cow antimicrobial treatment is an integral part of a good mastitis management and milk quality program. Antimicrobial used in groups of animals in which some individuals are clinically diseased for the purpose of reducing the spread of the disease to other group members (Moore, 2004).

2. 2. 3. Disease prevention (prophylactic)

Antimicrobials are given to animals at high risk of developing a disease with the purpose of keeping them free of that disease. There are instances in which antimicrobials are administered prophylactically (e.g., dry cow infusion, medicated milk replacer) to dairy cattle. Milk replacer typically contains an antimicrobial for prevention of gastroenteritis. A combination of oxytetracycline and neomycin are commonly used, while chlortetracycline is available for use in milk replacers as well (Moore, 2004).

2. 2. 4. Agricultural uses

Antibiotics are used for the principal purpose of improving production or metabolic efficiency of the animal. Example: Monensin is approved for feeding of cattle to improve feed efficiency and rate of gain. Monensin alters the microbial populations within the cow's rumen and allows the cow to gain more energy from the same amount of feed. About 90% of the antibiotics used in agriculture are given as growth-promoting and prophylactic agents, rather than to treat infection The recommended level of antibiotics for feeds were just 5 to 10 ppm in the 1950s but have been increased by 10 to 20 fold since then (Feinmen, 1998).

2. 3. Antimicrobial administration and residue

Administration of antimicrobial to dairy cattle is usually therapeutic, that is, in response to development of symptoms of disease. This type of chemotherapy shortens the period of antimicrobial administration and usually reduces the amount of antimicrobial employed. The use of feed and water-grade antimicrobial is prohibited in milking cows, so most antimicrobial are administered orally or given by infusion or injection. When a milking dairy cow is treated with an antimicrobial, the cow's milk must be withheld for a certain period. The producer must throw away this milk and receive no payment for it. All loads of milk are testing for antimicrobial residues to ensure that milk-containing residue does not inadvertently enter the food supply (Wray and Gnanou, 2000).

Dry-off antibiotic treatments can contaminate hundreds of thousands of litres of milk. When drying-off, each cow is injected with 4 syringes (one per quarter) containing a very high concentration of antibiotics. Antibiotics used in drying-off can remain in the udder for at least for 4 weeks and sometimes up to 10 weeks. Therefore, when calving the milk can contain antibiotic levels superior to the MRL and so return a positive result when tested. The length of time the antibiotics remain in the udder depends on the molecule and the excipient. As a result, in cases of premature calving, it is recommended that the milk is tested before delivery.

Whatever the method of administration, antibiotics can still be found in the milk. The presence of antibiotics in milk depends on three factors (Kanneene and Miller, 1997): Metabolism-the medicine can be changed and broken down within the body; the molecule's ability to pass through the membranes e.g. blood vessels and cell walls; elimination- urine, faeces, saliva and milk

2. 4. Common reasons for the occurrence of antimicrobial residues in milk

The most likely cause of violative drug residues is the failure to observe withdrawal times (Paige and Kent, 1987; Van Dresser and Wilcke, 1989; Guest and Paige, 1991; Paige, 1994). Improper maintenance of treatment records or failure to identify treated animals adequately may

lead to their omission (Sundlof, 1990).Violative drug residues can also occur as a result of improper use of licensed product through the illegal use of unlicensed substances. Extra label dosages and use of drugs which have not been approved for the species in question may lead to violative residues (Papich *et al*., 1993; Kanneen and Miller, 1997; Higgins *et al*., 1999).

The disease status of an animal and the way in which drugs are administered influence the potential for residues. Disease may affect the pharmacokinetics of the drug, metabolism, or the presence of infection and/or inflammation may cause the drug to accumulate in affected tissues. Contamination of animal feeding stuffs with a variety of compounds also occurs. The significance of this contamination depends on the pharmacodynamics of the compound and the species affected (McEvoy, 2002).

The main reasons for the occurrence of antimicrobial contamination in milk are:If milk from a treated cow is accidentally routed into the pipeline, an antibiotic treated dry cow is unintentionally milked, the same milking unit is used to milk an antibiotic treated cow before milking untreated cows, the milking unit is not cleaned and sanitized between uses, lactating cows are purchased and the new owner is unaware of recent antibiotic treatments prior to sale, one quarter of a cow is treated for mastitis and withheld from the bulk tank, equipment used to milk treated cows is handled carelessly and all antimicrobial treated dry cows are milked last but the milk line was not diverted from the bulk tank (Wallace, 2007).

Antimicrobials residues occur when employees fail to follow the specific label instructions when treating cows. They may also occur when treated cows are accidentally milked into the bulk tank before the withdrawal period is completed. Residue may also occur when employees fail to clearly identify treated cows with chalk marks, leg bands or neck chains. They may also occur when written records of treatments are not kept or are not checked prior to returning the treated cow to the milking herd. Treated cows should be housed and milked separately from main milking herd (Wallace, 2007).

Milking employee influence on antimicrobial residues: residue may occur when employers milk treated cows that have been identified as treated. Residue may also occur when employees treat cows and fail to properly identify the cows and separated from the milking herd. Management

along with the dairy veterinarian should develop written protocols for use of antibiotics and records systems to properly document antimicrobial use as a mean to prevent residues (Wallace, 2007).

2. 5. Significance of antimicrobial residues in milk

2. 5. 1. Public health significance

The non-restrictive usage of antimicrobial in animal rearing may lead to problems due to the presence of harmful residues in foods and raw materials of animal origin. Human health can either be affected through residues of drugs in food of animal origin, which may cause direct side effects, or indirectly, through selection of antimicrobial resistance determinant that may spread human pathogen (Peter and John, 2001). Human health problems that may result from intake of subchronic exposure levels include allergic reactions in sensitive people, toxicity and carcinogenic effects. Penicillins especially, as well as other ß-lactam antibiotics such as cephalosporins could cause allergies if high levels of residues persist in milk consumed by penicillin-allergic persons. Tetracyclines residues also have the potential to stain teeth of young children (Phillips *et al.*, 2000).

Antimicrobial resistance

The use of antimicrobials in food animals can result in antimicrobial resistance bacteria reaching the human population through variety of routes. Antimicrobial resistant bacteria such as E. coli can colonize intestine of people. Heavily exposed humans (farmers, who use food containing antimicrobials, slaughter house workers, cookers and other food handlers) often have a higher incidence of resistant E.coli in their feces than the general population. While many bacteria are non pathogenic, some pathogenic bacterial species from the intestines of animals cause zoonotic infection to human such as Salmonella species, Campylobacter species and these infection may be harder to treat because of acquired by humans are a potential sources of resistance plasmids for human pathogenic bacteria other than the zoonotic infection. Development and spread of

antimicrobial resistance represents a serious threat with potential public health implications (WHO, 2000). Obviously, bacteria are masters at developing antimicrobial resistance.

There are several factors which are thought to influence the development of resistance and these include drug concentration, long-term exposure, organism type, antimicrobial type and host immune status. Low-level, longterm exposure to antimicrobials may in particular have a greater selective potential than short-term, full-dose therapeutic use. One type of Salmonella (i.e., *S. typhimurium* DT104) is of particular concern to human health because this strain already exhibits resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, and there is evidence that antimicrobial resistant *S. typhimurium* can be passed from cattle to people (Lee *et al.,* 2000). The consumption of antimicrobial residues in food would lead to the development of resistance. This is because antimicrobial residue levels in food are very low and are likely to be further reduced by cooking and other food processing and also by metabolism in the gut. On this basis, it is very unlikely that a dose high enough to inhibit sensitive bacteria, and thus encourage the growth of resistant bacteria, would be achieved.

Toxicity

Immunopathological aspects (Hypersensitivity Reaction)

It is an immune-mediated response to a drug agent in a sensitized patient, and drug allergy is restricted to a reaction mediated by IgE. Drugs are foreign molecules, but their molecular weight is usually too small to be immunogenic, they must act as haptens, which must combine with carrier proteins to be immunogenic and elicit antibody formation (Riedl and Casillas, 2003). Immunogenic reactions may manifest from life-threatening anaphylactic reactions to milder reactions, such as rashes. In anaphylaxis exposure rapidly leads to severe acute bronchoconstriction, often risking a degree of asphyxia, marked hypotension, possibly edema at the site of challenges, and severe general illness. Antimicrobial residues in a previously sensitized individual may trigger an allergic reaction. Notwithstanding their non-toxic nature, β-lactams appear to be responsible for most of the reported human allergic reactions to

antimicrobials. Aminoglycosides, sulphonamides and tetracyclines may also cause allergic reactions (WHO, 1991).

Carcinogenic effects

Carcinogenic effects refer to an effect produced by a drug having carcinogenic or cancer producing activity. Among the carcinogenic veterinary drugs in current use in many countries are nitrofurans, nitroimidazols and grisofulvin. These drugs are acquired via food of animal origin as antimicrobial residues. The potential hazard of carcinogenic residues is related to their interaction or covalent binding with various intracellular compounds such as proteins, ribonucleic acid, glycogen, phospholipids and glutathione. This leads to damage of cellular components such as DNA (Booth and McDonald, 1988).

Mutagenic effects

The term mutagen is used to describe chemical agents that damage the genetic components of a cell or an organism. Several chemicals including alkalyzing agents and analogous of DNA bases have been shown to elicit mutagenic activities. There has been an increasing concern that drugs as well as environment chemicals may pose a potential hazard to the human population by production of gene mutations or chromosome abrasions. Either the germinal or somatic may be affected. However, from a public health point, mutation in the germinal cells is of more immediate importance because of the hazard to the further generation (Booth and McDonald, 1988).

Teratogenic effects

The term teratogen applies to a drug or chemical agent that produces a toxic effect on the embryo or fetus during a critical period of gestation. As a consequence, a congenital malformation that affects structural and functional integrity of the organism is produced (Booth and McDonald, 1988).

2. 5. 2. Environmental impact

Animals may excrete active metabolites of antimicrobials through urine and faces and reach the soil and water. The most prevalent antimicrobials found in the environment (surface waters) belong to the macrolide and the sulfonamide groups. Tetracyclines or penicillins have only been found in some cases and at low concentrations (Kolpin *et al*., 2002). Zuccato *et al*., (2000) identified some commonly used antimicrobials, such as erythromycin, sulfadimidin, and tetracycline are antimicrobials which persist in the soil and remained on surface water and soil for over a year.

Antimicrobial metabolites have also been found to be able to be transformed back to their original active substances once in the environment. Since most antimicrobials are water-soluble, up to 90% of a dose can be excreted in urine and up to 75% in animal faces. It has however been difficult to ascertain whether the residues are caused by waste management or if they are due to inputs from agriculture. It is thus generally felt that dairy animals have a low influence on the input of antimicrobials into the acquatic environment (Heberer, 2002).

2. 5. 3. Dairy industry (technological) impact

The dairy industry is an important segment in the food industry, providing both milk and meat for human consumption. Dairy farmers and all supporting groups (veterinarians, feed/supply dealer, milk processors, livestock dealers, etc) should be concerned and devoted to producing a safe as well as nutritious dairy food products .If the consumer lacks confidence in these products, the market and the livelihood of the entire dairy industry is threatened. The dairy starter cultures currently used in dairy industry for the primary acidification of the milk belong mainly to the genera Lactococcus, Streptococcus and Lactobacillus. These starter cultures are mainly lactic acid bacteria used in the production of a range of fermented milk products, including cheese, yoghurt, cultured butter and cultured milks. The primary role of starter cultures in cheese manufacture is the production of lactic acid from lactose at a consistent and controlled rate. The consequent decrease in pH affects a number of aspects of the cheese manufacturing process and ultimately cheese composition and quality (Packham *et al.*,

2001).Antimicrobial residues in milk are undesirable from a manufacturing perspective, as they can interfere with starter culture activity and hence disrupt the manufacture process. The concentrations of antimicrobials which would cause such effects is however often higher than would be found inherent as residues in milk (Katla *et al.,* 2001).

2. 6. Safety evaluation

Regulatory levels have been established for drug residues in foods in the form of maximum residue limits (MRLs) (Lee *et al*., 2000). To asses the safety of ingested antimicrobial residues national and international committee evaluated data on chemical, pharmacological, toxicological and other properties; e.g. antimicrobial, properties of drugs derived from studies of experimental animals and observations in humans (Woodward, 1998).

2. 6. 1. Acceptable daily intake (ADI)

Acceptable Daily Intake (ADI) for a given compound is the amount of a substance that can be ingested daily over a life time without appreciable health risk. Calculation of ADI is based on array of toxicological safety evaluation that takes into account acute and long term exposure to the drug and its potential impact. This defines maximum quantity which may be consumed daily by even the most sensitive group in the population without any outward effects. The ADI is determined as a consecutive estimate of safe ingestion levels by the human population based on the lowest "no effect level" (NOEL) of toxicological safety studies (EC, 2001).

2. 6. 2. Maximum residue levels (MRLs)

The maximum residue level (MRL) is the maximum concentration of residue resulting from the use of a veterinary medicinal product that may be legally permitted or recognized as acceptable in or on a food, allocated to individual food commodities. No chemical is safe under all conditions of use. It is therefore important that all are fully evaluated for safety, as the parent compound and/or as its metabolites and that the results of these evaluations determine acceptability. MRLs are fixed on the basis of relevant toxicological data (Gracey *et al*., 1999).

Substances for which no maximum residue limit can be established because residues of these substances, at whatever limit, in foodstuffs of animal origin constitute a hazard to health of the consumer (EEC, 1990). Maximum residue level of some veterinary drugs in milk is shown in Table 2.

Table 2 : Residue limits of common veterinary drugs (µg/l) set for milk

Antimicrobials	MRL (µg/l)
Ampicillin	4
Amoxacillin	4
Cloxacillin	30
Streptomycin	200
Diminazene	150
Oxefendazole	100
Isomethamidium	100
Neomycine	500
Oxytetracycline	100
Sulfadimidine	25
Erythromycin	40

Source: CAC (1997)

2. 6. 3. Withdrawal period

Use of animal medicines requires observance of the withdrawal period (Table 3). This is the time that passes between the last dose given to the animal and the time when the level of residues in the tissues (muscle, liver, kidney, skin or fat) or products (milk, eggs, honey) is lower than or equal to the MRL. Until the withdrawal period has elapsed, the animal or its products must not be used for human consumption (Jackson, 1980).

Table 3: Withdrawal times of milk for injectable drugs used

Drugs	Pre-slaughter withdrawal time(days)	Discard times for milk(hr)
Amoxicillinrihydrate	25	*
Ampicillintrihydrate	6	48
Erythromycin	14	72
Furosamide	2	48
Oxytetracyclinehydrochloride	28	60
Procaine penicillin G	10	72
Benzanthine penicillin G	30	*
Dihdrosterptomycine sulphate	30	72
Sulphadimethoxine	5	60
Sulphaethoxypyridazine	16	72

*= Not determined

Source: Booth and McDonald (1988)

As can been seen from Table 4, the prevalence of antimicrobial residues in milk had been studied in different countries. However, in Ethiopia there is no any study done about the occurrence and level of drug residues in milk and milk products.

Table 4: A comparison of reported antimicrobial residues prevalence in consumer milk

Country	Reported prevalence of antimicrobial residues	Reference
Brazil	4.3 %	Borges *et al*., 2000
	50 %	Folly and Machado, 2001
India	9 (%)	Sudershan and Bhal,1998
Poland	13-22 %	Rybinska ,*et al*., 1995
Sweden	0.08-0.26 %	Sternesjo,1998

2. 7. Penicillin in public health and veterinary practices

Antibiotic is a chemical substance produced by a microorganism that has the capacity, in dilute solutions, to kill (biocidal activity) or inhibit the growth (biostatic activity) of other microorganisms. Antibiotics that are sufficiently nontoxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases. Antibiotics interfere with the production of these bacterial characteristics, resulting in selective killing or growth inhibition of susceptible microorganisms. Different antibiotics destroy bacteria in different ways. Some short-circuit the processes by which bacteria receive energy. Others disturb the structure of the bacterial cell wall, as shown in the illustration below. Still others interfere with the production of essential proteins (Brown, 2004).

The penicillins are the oldest class of antibiotics and have a common chemical structure that they share with the cephalosporins. The two groups are classed as the beta-lactam antibiotics, and are generally bacteriocidal that is, they kill bacteria rather than inhibit growth. Penicillin refers to a group of β-lactam antibiotics used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. The name "penicillin" can also be used in reference to a specific member of the penicillin group. The term “penicillin” is often used in the generic sense to refer to one of the narrow-spectrum penicillins, in particular, benzylpenicillin Benzylpenicillin, commonly known as penicillin G, is the gold standard penicillin which contains a β-lactam nucleus in its molecular structure as can be shoen as follows (Wikipedia, 2004.

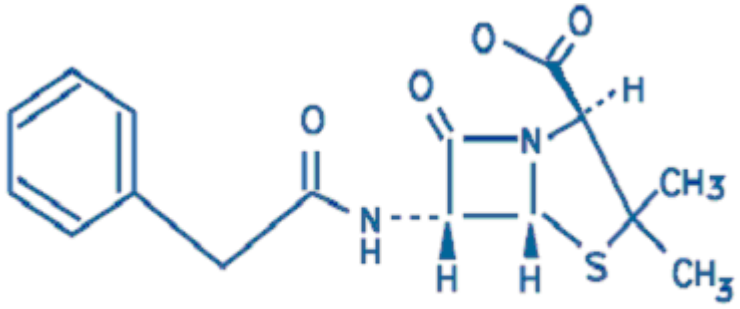


Figure 1: Chemical structure of Penicillin G

Source: Wikipedia Encyclopedia (2004)

β-lactam antibiotics are a broad class of antibiotics that include penicillin derivatives, cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors, that is, any antibiotic agent that contains β-lactam ring. They are the most widely-used group of antibiotics available.β-lactam antibiotics work by inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall. Penicillin disturbs the cell wall synthesis and more accurately the glycopeptide (or murein) formation, a substance giving rigidity or shape to bacteria (Wikipedia, 2004).

In the early there had been many descriptions of the phenomenon antibiosis, it was Alexander Fleming who, in 1928, discovered that the mould Penicillium notatum produced under certain circumstances a diffusible substance that inhibited the growth of some species of bacteria. He named it penicillin. Very little was done with this substance in the ensuing years, probably because it was found to be very unstable (Fleming, 1929).

During World War II, penicillin made a major difference in the number of deaths and amputations caused by infected wounds among Allied forces, saving an estimated 12%-15% of lives. Common adverse drug reactions associated with use of the penicillins include diarrhea, nausea, rash, urticaria, and/or superinfection (including candidiasis). Infrequent adverse effect include fever, vomiting, erythema, dermatitis, and/or pseudomembranous colitis (Antunez *et al*., 2006).

Pain and inflammation at the injection site is also common for parenterally-administered benzathine benzylpenicillin, benzylpenicillin, and, to a lesser extent, procaine benzylpenicillin. Although penicillin is still the most commonly-reported allergy, less than 20% of all patients that believe that they have a penicillin allergy are truly allergic to penicillin (Salkind *et al*., 2001), nevertheless, penicillin is still the most common cause of severe allergic drug reactions. The only adverse reaction of hypersensitivity type originating from envronment or food sources has been associated with penicillin in milk. Approximately 5 to 10% of the population is hypesensitive to penicillin or other antibiotics (Meaney, 1985) and suffer allergic reactions (skin

rashes, hives, asthma, anaphylactic shock) at concentrations as low as 0.003 IU penicillin/ml (Lindemayr, 1981).

To ensure milk safety, the maximum residue limits (MRL) in the milk were set at 0.004 mg.kg-1 for penicillin G, ampicillin, amoxicillin and at 0.030 mg.kg-1 for oxacillin, cloxacillin, and dicloxacillin (CAC, 2003). Milk supplies containing higher concentrations of penicillins are not acceptable. The presence of beta–lactam antibiotics in milk represents a potential hazard to consumers, because of their allergenic properties and inhibitory effect on culturing processes in fermented milk products. The residues may be also responsible for the development of resistant strains of bacteria.

The importance of veterinary medicines and especially of antimicrobial agents is shown, not only as beneficial compounds for animal health and animal welfare but as risks as well, being potential sources of residues in food of animal origin, when after administration of veterinary medicines to the animals the withdrawal time in relation to the maximum residue limit (MRL) is not taken into account. Health for men and animal is of utmost importance and the quality of food is considered as an important health factor (Solensky, 2003).

Mitchell, Groffiths, McEwen, McNab, and Yee (2002) reported that the beta-lactam class of antibiotics, most often penicillin G, are the most frequently used drugs in mastitis therapy. Milk may also be cocontaminated with compounds of one of the other four major antimicrobial drug classes: the sulphonamides (e.g., sulphadiazine), tetracyclines (e.g., oxytetracycline), macrolides (e.g., erythromycin) and aminoglycosides (e.g., neomycin).

2. 8. Tetracycline in public health and veterinary practices

Tetracyclines got their name because they share a chemical structure that has four rings. They are derived from a species of *Streptomyces* bacteria. Broad-spectrum bacteriostatic agents, the tetracyclines may be effective against a wide variety of microorganisms, including rickettsia and amebic parasites and are added at subtherapeutic levels to cattle feeds for prophylaxis. Tetracycline inhibits cell growth by inhibiting translation. It binds to the 16S part of the 30S

ribosomal subunit and prevents the amino-acyl tRNA from binding to the A site of the ribosome. The binding is reversible in nature. Tetracyclines may be used in the treatment of infections of the respiratory tract, sinuses, middle ear, urinary tract, intestines, and also gonorrhoea, especially in patients allergic to β-lactams and macrolides; however, their use for these indications is less popular than it once was due to widespread resistance development in the causative organisms (Wikipedia, 2004).

Side effects from tetracyclines are not always common, but of particular note is possible photosensitive allergic reaction which increases the risk of sunburn under exposure to UV light from the sun or other sources. This may be of particular importance for those intending to take on holidays long-term doxycyline as a malaria prophylaxis.They may cause stomach or bowel upsets, and rarely allergic reactions. Very rarely severe headache and vision problems may be signs of dangerous secondary intracranial hypertension also known as Pseudotumor cerebri (Deboyser *et al*., 1989).

Tetracyclines are teratogens due to the likelihood of causing teeth discolouration in the fetus as they develop in infancy. For this same reason, tetracyclines are contraindicated for use in children under 8 years of age. They are however safe to use in the first 18 weeks of pregnancy.Some patients taking tetracyclines require medical supervision because they can cause steatosis and hepatotoxicity (Amacher and Martin, 1997).

The use of antibiotics may result in drug resides if they are not used according to label directions, especialy if proper withdrawal times for treated animals heve not been used (Long *et al*.,1990). These residues may pose a health threat to consumers, depending on the type of food and the amount of residue present. For this reason, regulatory agencies have established maximum legal tolerance levels for tetracycline drug in animal derived food products (USDA, 1988).

The 36th Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting in 1990 established MRL for Oxytetraccline of 600µg/ml in muscle;100µg/ml in milk; 200µg/ml in eggs; 10µg/ml in fat for all species for which residue depletion data were provided

(EMEA,1995) Oxytetracycline was the second of the broad-spectrum tetracycline group of antibiotics to be discovered. It was first found near Pfizer laboratories in a soil sample yielding the soil actinomycete, Streptomyces rimosus by Finlay *et al*. in 1953, a celebrated Scottish American biochemist, Robert B Woodward, worked out the chemical structure of Oxytetracycline as shown below.

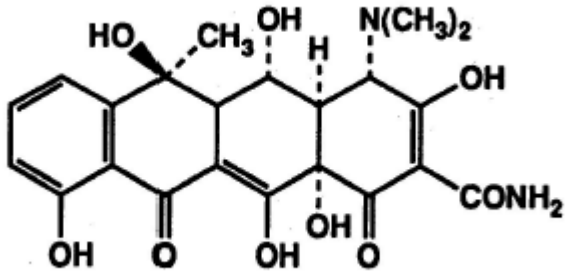


Figure 2: Chemical structural of Oxytetracycline
Source: Wikipedia Encyclopedia (2004)

Tetracycline use should be avoided in pregnant or lactating women, and in children with developing teeth because they may result in permanent staining (dark yellow-gray teeth with a darker horizontal band that goes across the top and bottom rows of teeth), and possibly affect the growth of teeth and bones. In addition, tetracyclines are poorly metabolized in animals; therefore, they can also occur in animal slurry that may pollute the environment (Sczesny *et al.*, 2003). However, a potential risk for the environment cannot be assessed yet as very little is known about the not excludible casual concentrations of antibiotics (Hirsch, *et al*., 1999).

2. 9. Antimicrobial residue detection methods

Drug residue avoidance is one of the most important issues in the dairy industry. For a drug residue-free milk supply, several methods, such as microbial growth inhibition methods, immunological methods, enzymatic assays, gas chromatography, and liquid chromatography, are used to detect drug residues in milk (Cullor, 1992). There is a requirement for a rapid, simple and cost-effective test to screen milk for the presence of the most important antimicrobial drug residues. The ideal screening method would detect most, if not all, antimicrobials at or

below their permissible limits or MRLs (Stead, Richmond, Sharman, Stark and Geijp, 2005; Stead *et al*., 2004).

2. 9. 1. Screening Tests

The most common screening methods for antimicrobial drug residues are microbiological tests, based on the growth inhibition of a test microorganism (e.g., *B. stearothermophilus*). There are on-farm screening tests devised for rapid detection of low concentrations of antimicrobial residues in milk (Tyilr *et al*., 1992). The antimicrobial residue detection assay systems that are currently available use different assay formats. The most common assay system monitors inhibition of the growth of a test organism. This type of assay system cannot identify the nature of the compound responsible for the growth inhibition. A well known assay in this category includes Charm Farm, Delvotest-P, and the regulatory standard *Bacillus stearothemphilus* disk assay. The assay systems of some of the newer residue detection tests are based on immunobinding of unique antigenic structures in antimicrobial or inherent antimicrobial receptor interactions (Calderon *et al*., 1996).

Microbiological inhibition tests

Delvotest-SP

This test is an agar diffusion test that utilizes *B. stearothermophlus*. It was introduced in 1975. It is promoted as an on-farm method of detecting antimicrobial residues. It is a sensitive, simple and comparative test. In this assay, the content of the test organism ampoule and the nutrient tablet are mixed. The milk sample then is combined with the above mixture. The mixture is incubated for 3 hrs at 64 ^{o}C the color reaction is recorded based on a pH change in the media. The assay control consists of inhibitor-free milk powder or water. A yellow color indicates a negative reaction and purple color indicates a positive reaction (Suhren and Beukers, 1998).

Disk assay

In this assay, a disk of absorbent paper impregnated with a milk sample is placed on the surface of an agar medium inoculated with *B. stearothermophlus*. A positive control disk containing 0.008 IU/ml potassium penicillin is placed on each plate. The plates are inverted and incubated at 64 ^{O}C until well-defined clear zones of inhibition surrounds the potassium penicillin control disk. The plates are then examined for clear zones. Bulk tank samples are considered positive for the presence of inhibitory substances if a zone of inhibition greater than 16 mm occurs (Bishop and White, 1984).

Brilliant black reduction test /BR-Test/

In this test, the test sample is first combined with a nutrient tablet and then incubated on an agar gel containing *B. stearothermophlus*. The microorganism multiplies during the incubation period and causes the pH indicator color to change from purple to yellow which indicates that the test is negative. If the growth inhibitor substances are present in the sample, the pH indicator color remains purple indicating that the test is positive (Bishop and White, 1984).

Charm inhibition assay

This system, in tablet form, uses *B. stearothermophlus* and a specially formulated agar medium. The antimicrobial substances in the milk sample inhibit microbial sporulation and growth, which results in reduced acid production. The pH indicator changes from blue to greenish-yellow if the milk doesn't contain an inhibitory substances; it remains blue if the milk contains a growth inhibitor (Bishop and White, 1984).

Immunoassays

This assay format is based on the ability of antibodies to bind specifically to different substances. This test measures competitive interaction between drugs (labeled or unlabeled) and sample contents. The antimicrobial, being low molecular weight compounds, generally do not

prompt any immunological response in animal. To produce such a response, the compounds have to be coupled to a large molecular such as bovine serum albumine (Haagsma and Van De Water, 1992).

Radioimmuno assay

This test uses specific antiserum, which provides the binding sites, and an antimicrobial tracer. The drug in the sample competes with the tracer for binding sites. After removal of the free (unbound) tracer, the antibody-tracer complex is counted. The more antibiotic tracer bound, the less antibiotic present in the sample (Haagsma and Van De Water, 1992).

ß -lactam lactex

This test uses a ß-lactam specific antibody. The concentration of antibiotics in the milk is determined by comparing the optical density of each sample tube with that of the test kit standard (405 nm). The results are positive for the presence of B-lactam if the color reaction (optical density) of the sample is less than of the assay standard (Haagsma and Van De Water, 1992).

Charm II receptor assay

This receptor assay is based on a binding reaction between the antimicrobial functional group and a receptor or binding site on or within added microbial cells. Antimicrobial tracers (^{14}C or ^{3}H) completes with the drugs present in the sample for binding sites. Binding is measured by a scintillation counter and is compared with standard antimicrobialfree milk. The greater the amount of antimicrobial present in the sample, the lower the counts detected by the equipment (Haagsma and Van De Water, 1992).

2. 9. 2. Chromatographic analysis

Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a mobile phase which moves in a definite

direction) through a stationary phase (immobilized on the support particles), which separates the analytes to be measured from other molecules in the mixture and allows it to be isolated (Wikipedia, 2004). These methods provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest. The tests are employed to determine presence or absence of residues in a sample found positive by routine screening test. These tests are aimed at preventing false positive results (Heitzman, 1994).

Approaches to extraction include extraction with water or buffer, direct solid phase extraction, deproteinization with tungstic acid,trichloracetic acid, water-miscible organic solvents such as methanol, acetone or acetonitrile combined with buffers or acids, ultrafiltration, partitioning into water-immiscible organic solvents, heating denature proteins, andextraction with superficial carbondioxide. With the automated sequential trace enrichment of dialysates sample pretreatment is restricted to homogenization and dilution of the samples;clean-up is by on-line dialysis and on-line solid phase extraction. Sample clean-up procedures includes column chromatography, thin layer chromatography (TLC), solid-liquid extraction, solid phase extraction and solid phase dispertion (Moats, 1997). All compounds were identified by matching the retention time and their spectral characteristics examined against those of standards (Chen, Zuo, and Deng, 2001). Quantitation was made based on the linear calibration curves between the concentration and peak area of standard compounds.

The aim of chromatography in general is the resolution or separation of different molecular species (Klassen and Edberg, 1996). The mobile phase passes over the stationary phase at a constant rate; the two phases possess different chemical properties. As the analytes in the mobile phase pass over the stationary phase, those with polarity closer to that of the stationary phase are retained selectively for a time on the column. Conversely, the analyte molecules with polarity closer to that of the mobile phase tend to remain in the mobile phase, passing through the column. Passing through the instrument monitor sequencialy, those”groups” of molecules give rise to peaks on the chromtogram (Klassen and Edberg, 1996).

HPLC methods utilize the same basic steps. Extraction of the drug with a specific solvent, separation of the drug on the solid phase detection of the effluent from the solid phase by

spectranetry and quantitation of the amount of antimicrobial present by peak height or peak area analysis, Uvabsorbance is the simplest and most widely used derivatization , either pre- or post column, is frequently used to enhance UV absorbance or to form fluorescent compounds. Compounds with little or no UV absorbance requires other approaches (Moats, 1997).

Commonly used procedures for the detection of veterinary drug residues include high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) and mass spectrometry (MS). Chemical methods usually proceed with a preliminary extraction in order to isolate drug of interest from biological matrix. The main objectives of sample treatment are removal of macromolecules and other matrix constituents that may either adversely affect the chromatographic systems or interfere with the detection and enrichment of the analytes in order to achieve the required low limits of detection. Compounds must be separated from another and food matrice. The low solubility of some antimicrobials in organic solvents has made it difficult to develop procedures to extract and concentrate their residues from biological matrices. Other antimicrobial are either insufficiently volatile or too thermally unstable or both to permit their analysis using GC or MS. Liquid chromatography has merged as the method of choice for determination of antimicrobial which are rather polar, non volatile, and some times heat stable (Shaikh and Moats, 1993).

2. 10. Prevention of antimicrobial residues

A food control system is an official institutional set up, at national and subnational level, responsible for ensuring the quality and safety of the food supply. It includes the relevant food legislation and regulation, food inspection, food analysis, food import/export inspection and certification and food control management. Bacteria are a natural and essential component of the environment. Using antimicrobials to declare “all-out war” against bacteria is a war that we cannot win. Instead, antimicrobials must be used with the precision of a surgeon’s knife, being employed strategically against target bacteria, and only as one component of an overall herd health management program. Failure to use antimicrobial with respect could lead to their eventual elimination as a tool in animal production, either through regulatory restrictions or through the loss of their effectiveness due to the emergence of resistant bacterial populations. It

is important to remember that the individuals most likely to come into first contact with antimicrobial resistant bacteria in the dairy are the dairy producers and their families (Heeschen and Suhren, 1996).

Providing on farm food safety programmes, which address the daily management of the production unit with regard to animal health and well-being, public health and environmental health must be a top priority for agriculturalists and veterinarians. Developing critical control point management (CCPM) procedures for animal and human health concerns is a viable approach to aid in alleviating public concerns about dairy products and the food supply in general. The safety and the quality of dairy products will improve as people realize that healthy animals are more profitable, which encourages them to pay attention to diagnosis and treatment of diseases, prevention costs less than cure and image of quality can increase the attractiveness of milk on the market (FAO, 1998).

The 10-point milk and dairy beef quality assurance program (Herrick, 1991) will become a valuable tool in maintaining a safe and wholesome product. These residue prevention plans are: Practice healthy herd management; establish a valid veterinarian/client/ patient relationship (VCPR); use prescription drugs with a veterinarian's guidance; maintain milk quality. Food safety and quality begins on the dairy; implement an effective mastitis management program; administer all drugs properly and identify all treated animals; maintain and use proper treatment records on all treated animals; use drug residue screening tests; implement employee/family awareness of proper drug use to avoid marketing adulterated milk and dairy beef and complete the milk and dairy beef residue prevention protocol annually.

3. MATERIALS AND METHODS

3. 1. Study area

The study was carried out in Debre Zeit and Nazareth dairy farms between October 2007 and May 2008.

3. 1. 1. Debre Zeit

The town is located at 9 ^{0} N and 40 ^{0} E. It is 47 km South East of Addis Ababa, the capital of Ethiopia with human population size of about 95,000 people. The altitude is about 1850 m above sea level. It experiences bimodal patterns of rainfall with the main rainy season extending from June to September. It accounts for 84% of the total rain. A short rainy season occurs between March and May with an average rainfall of about 800 mm. The mean annual minimum and maximum temperatures are 12.3 ^{0}C and 27.7 ^{0}C, respectively with an overall average of 18.7 ^{0}C (CSA, 2001). Highest temperatures are recorded in May. The mean relative humidity in Debre Zeit is 61.3%.

3. 1. 2. Nazareth

It is located at 39.17 ^{0} N and 8.33 ^{0} E. It is about 95 km South East of Addis Ababa at an altitude of 1622 meters above sea level. Nazareth is situated in the East African Rift Valley in Oromia Region, Central Ethiopia. The area has an annual rain fall and temperature ranges of 400-800 mm and 13.9 ^{0}C to 27.7 ^{0}C, respectively (NMSA, 2003).

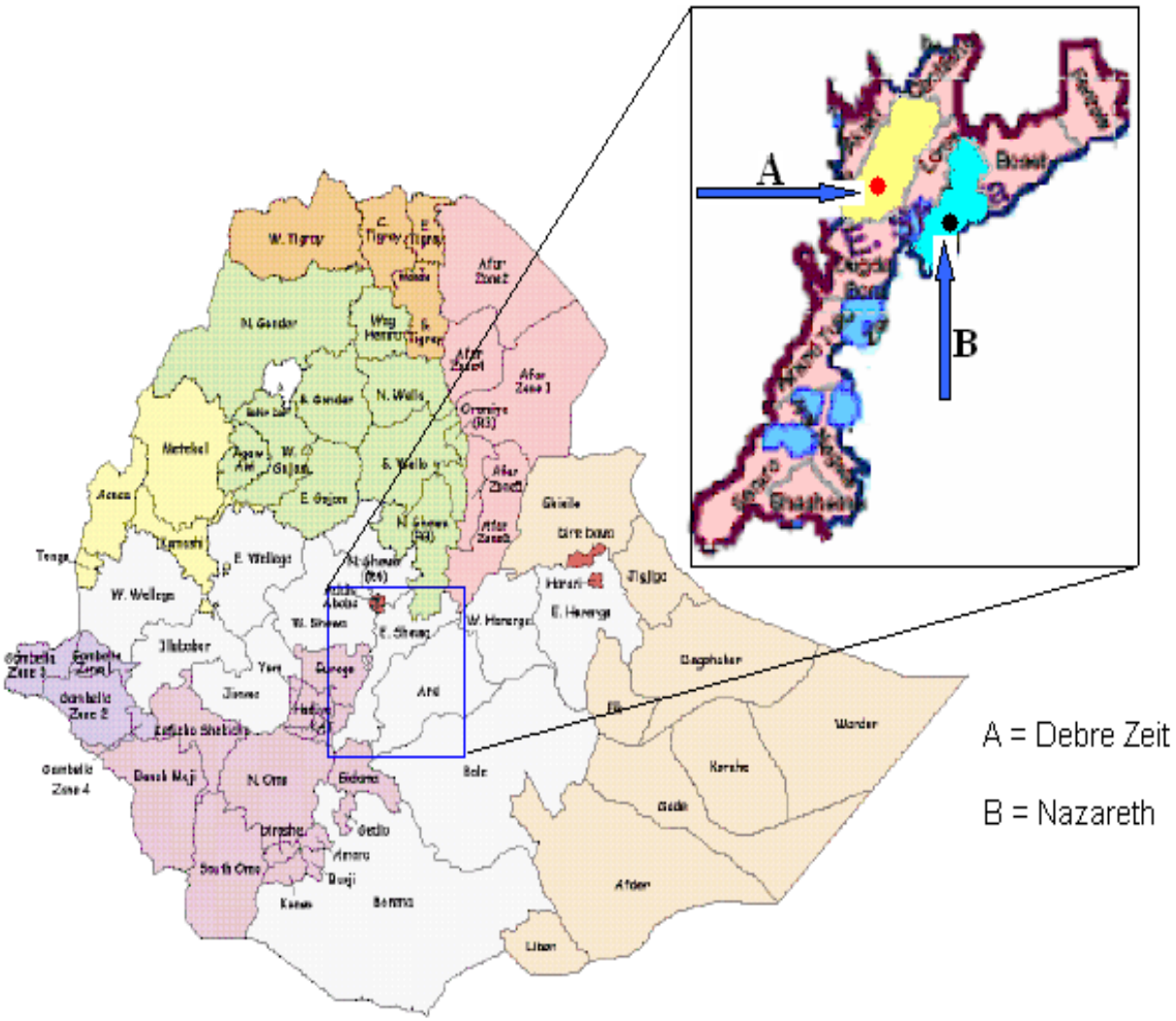


Figure 3: Map of study areas

3. 2. Study population

The study population consisted of bulk milk tanks found in Debre Zeit and Nazareth dairy farms.

3. 3. Study design

A cross-sectional study was undertaken in Debre Zeit and Nazareth dairy farms between October 2007 and May 2008. On each sampling day, about 20 ml of milk samples were randomly selected and sampled from each farm bulk tank. Ninety two dairy farms were visited after antibiotic residues were detected in samples of their bulk milk (case farms) (Debre Zeit= 34 and Nazareth=12) along with an equal number of residue free farms (control farms) (Debre Zeit= 34 and Nazareth=12). Each milk sample was aseptically collected in separate containers and transported in ice-box packed to the AAU, Faculty of Veterinary Medicine (FVM) and Drug Administration and Control Authority (DACA) laboratory.

A questionnaire survey (Annex 6) was conducted by personal interview of the dairy farm owners in case farms and equal number of control farms. It was administered to determine associations between the occurrence of antibiotic residue in milk and various risk factors like management practices, treatment factors, residue prevention methods and knowledge of the farm owners about the antibiotic residues. In management practices of the farm owners, the informations collected were the use of feed additives, use of post-milking teat dips and training on dairy management. Informations on treatment factors included sources of antibiotics, type person who administers antibiotics to cows, route of antibiotic administration, record keeping, use of dry cow therapy and number of milking cows. Regarding residue prevention methods, the information gathered were marking of treated cows, milking of treated cows using separated equipment, use of antibiotic test kit and knowledge of withdrawal periods of antibiotics.

3. 4. Sampling

3. 4. 1. Sampling procedure

Individual bulk milk tanks to be sampled were selected using random sampling technique. About 20 ml milk samples were collected in each dairy farm from their bulk milk. Each sample was labeled legibly and accompanied by necessary identification information, which included date of sampling, type of samples, breeds of cows from which the samples were obtained and identification code. All milk samples were transported under chilled conditions to the laboratory and stored at –20 ^{0}C, until analysis.

3. 4. 2. Sample size determination

The sample size required for the study were determined on the expected occurrence (prevalence) of drug residue and desired absolute precision according to Thrusfield (2005) using the following formula.

$$n = \frac{1.96^2 P\,exp\,(1-P\,exp)}{d^2}$$

Where: n=required sample size

P exp= expected prevalence

d= desired absolute precision

Using a 95% confidence level, 5% absolute precision and 50% expected occurrence of residues. The desired sample size was about 384, but sample size was raised to 400.

3. 5. Methods for oxytetracycline and penicillin G residue analysis

The type of antibiotics in milk samples was screened qualitatively by using Delvotest SP assay (microbial inhibitor tests with *Bacillus stearothermophilus* as test microorganism) and quantitatively by high performance liquid chromatography (HPLC).

3. 5. 1. Detection of oxytetracycline and penicillin G residue in milk using Delvotest SP assay

The qualitative analysis of oxytetracycline and penicillin G residues in milk was done using Delvotest SP assay as described by Suhren and Beukers (1998). This method is based on the susceptibilities of bacteria to different antibiotics. Delvotest SP ampoules were supplied by DSM (DSM Food Specialties, Delft, and the Netherlands). The method was carried out according to the instructions by the manufacturer. The chemicals, reagents, apparatus and detailed procedures are fully presented in Annex 1.

Principle of the asssay

The Delvotest SP assay is a microbial diffusion test used for the detection of antibiotic residues in milk. Milk is added to a solid agar medium containing *Bacillus stearothermophilus vr calidolactis* spores along with a nutrient agar tablet. When the test is kept cool, the spores remain viable. Upon incubation at 64 ^{0}C for three hours, the spores germinate. The germinated spores multiply with the formation of an acid. When sufficient acid is formed the colour of the indicator (bromcresol) changes from purple to yellow. If the milk sample contains antibiotic residues the antibiotic will diffuse into the agar and when present in a sufficient concentration will cause the inhibition of the multiplication process and thus acid production. The colour of the media remains purple when the concentration of the inhibitor is above the detection limits of the assay. When the concentration of the inhibitor is around the detection limit, the media exhibit partly yellow and partly purple colour.

Among the microbial inhibitor tests widely used for detection of antimicrobials in cow milk is Delvotest. It is an economical, easy-to-use screening test giving results within a relatively short

period (3.00 h). This method was recognized by the Association of Official Analytical Chemists (Katz, 1982; Kelley, 1982). The Delvotest SP method is classified visually into three categories: "negative," "doubtful," and "positive," compared with the colors of "positive" and "negative" standard sample (Suhren and Luitz, 1995).

3. 5. 2. Determination of the residue levels of oxytetracycline and penicillin G antibiotics with high performance liquid chromatography (HPLC).

The qualitatively positive samples (section 3.5.1) were further qualitatively analyzed using HPLC as described by Ghidini *et al.* (2003) for oxytetracycline and penicillin G. The chemicals, reagents, apparatus and detailed procedures for determining of these antibiotics are presented fully in Annex 3 and Annex 4.

Analysis of Penicillin G

Extraction

A total of 5ml raw milk was put into 10-ml Pyrex screw cap centrifuge tubes and mixed using vortex mixer with 400 µl of 10% acetic acid aqueous solutions. The acidified milk was then centrifuged at 3500 rpm for 10 min. The clear supernatant was taken by a syringe, avoiding taking the upper fat layer, and then filtered through a 0.50 µm nylon filter 13µm diameter (Advantec MFS, Pleasanton, CA, USA). The filtered extract was put into 2-ml autosampler vials (Chromacol Ltd, Herts, UK) and injected into calibrated HPLC system.

Analysis

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector equipped with an AS3000 autosampler. The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm) at a flow-rate of 1ml/min) with a gradient of acetonitrile and distilled deionized water. Water and acetonitrile both

acidified with 0.1% formic acid were used as mobile phase at 1ml min^{-1} constant flow, according to the elution programme. The latter operated as follows: From 100% water to 100% acetonitrile in 6 min and from 100% acetonitrile to 100% water within 6 to 12 min.

Reference standard solutions

Stock solutions of each antibiotic at 50mg l^{-1} were prepared by weighing the exact amounts of the penicillin G procaine salt as a standard and dissolving it into mixture of water and acetonitrile (75/ 25 v/v). These solutions were kept at 4 ^{0}C. They can be stored for 2 weeks.

Quantitation

Quantitation was made through external standards of penicillin G procaine salt. Calibration curves were built by injecting mixtures of stock solutions at the following concentrations: 5, 10, 200, 400 and 1000 µg l^{-1}. Quantitation was made based on the linear calibration curves between the concentration and peak area of standard compounds. A given sample was regarded as positive for penicillin G if its retention time and peak corresponded to that of the standard.

Oxytetracycline

Extraction

The extraction of oxytetracycline from milk samples was carried out by using the 500 mg SPE-C18 cartridge (J.T. Baker Inc., Phillipsburg, NJ, USA) which was activated according to the following procedure. After the addition of the sample (2 ml), the column was washed twice, first with water (2 ml) and then with a mixture containing water: methanol 70:30 v: v (2 ml). Finally, oxytetracycline was eluted with 2 ml of methanol: 0.1 M hydrochloric acid (2:1 v: v).

Analysis

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector

equipped with an AS3000 autosampler. The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm) at a flow-rate of 1ml min^{-1}. The analysis was carried out under the operational conditions given in Annex 6.

Quantitation

Based on suitable calibration curves, previously set up with solutions at decreasing concentrations of oxytetracycline hydrochloride used as a standard (range: 0.05–10 mg OTC: ml). To get the concentration of a given sample, a reference standard of a known concentration were injected into the HPLC and concentrations of the sample were extrapolated from the peaks of the curves. Curves were built by injecting mixture of working solutions at the following concentration: 0.5, 1.5, 2.25, 3.0, 6.0 mg/l of OTC eluent. A given sample was regarded as positive for oxytetracycline if its retention time and peak corresponded to that of the standard.

3. 6. Data management and analysis

The data collected through questionnaire survey, Delvotest SP and HPLC were entered in to databases using Micro-Soft computer program Excel (Version 6. 0, 2000) and analyzed using SPSS (SPSS version 11. 05, 2000) statistical computer software programs. Differences between proportions of groups with certain determinant factors were assessed by Chi-square (χ^2) test. Descriptive statistics were also used to describe the nature and the characteristics of the data. Univarable and multivariable logistic regression analysis were used to assess association between risk factors and residue occurrence. Comparisons between means were made using one way ANOVA.

4. RESULTS

A cross sectional study was conducted on bulk milk in Debre Zeit (n=279) and Nazareth (n=121) dairy farms between October 2007 and May 2008 to detect and determine penicillin G and oxytetracycline residue levels in milk. Questionnaire was also administered to selected dairy farm owners.

4. 1. Qualitative analysis of with Delvotest SP assay

Out of the total 400 milk samples analysed during this study, 46 (11.50%) had detectable levels of antibiotic residue in both dairy farms. In Debre Zeit dairy farm, out of 279 milk samples, 34 (12.19%) of them were positive for antibiotic residue. In Nazareth, out of 121 milk samples, 12 (9.92%) of them were positive for antibiotic residue (Table 5). The colour reaction of the Delvotest kit with standards (positive and negative) and samples (negative, positive and doubtful) have been shown in Figure 4 and Figure 5.

Table 5: Prevalence of antibiotic residue in Debre Zeit and Nazareth dairy farms

Diary farms	Positive bulk milk samples	Prevalence	95% CI
Debre Zeit (n=279)	34	12.19%	8.35-16.03
Nazareth (n=121)	12	9.92%	4.59-15.25

n= number of samples; CI=Confidence Interval

No significant difference between Debre Zeit and Nazareth dairy farms($p>0.05$) were observed with respect to prevalence of antibiotic residue in milk as their odds ratio was equal to 1 and 95% confidence interval included 1 (95% CI=0.394-2.536). Milk from Debre Zeit dairy farms was at similar risk of residue as milk from Nazareth dairy farms.

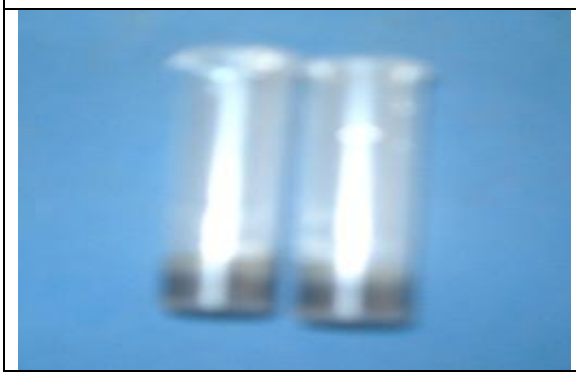
	Negative control samples
	Positive control samples

Figure 4: Colour reaction of Delvotest kit with controls (-/+)

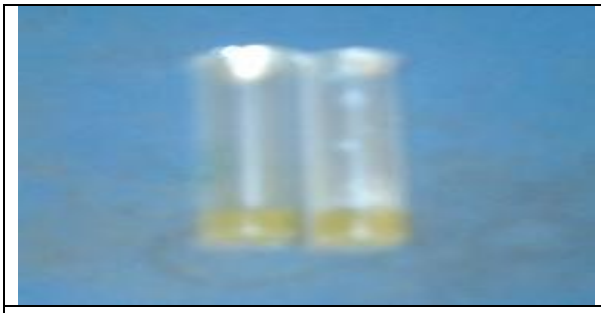
	Yellow colored (-) samples
	Purple colored (+) samples

Figure 5: Colour reaction of Delvotest kit with milk samples(-/+)

4. 2. Questionnaire survey

4. 2. 1. Response to the questionnaire survey in both dairy farms

A questionnaire survey was carried out by personal interview of the dairy farm owners in Delvotest positive (case) farms and Delvotest negative (control) farms. The study was conducted to identify various risk factors and to determine associations among the occurrence of antibiotic residue in milk and the risk factors like farm management practices, treatment factors, residue prevention methods and knowledge of the farm owners about the antibiotic residue.

When the farmers were asked whether they frequently used part-time employers for the milking of cows, 26.09% of them reported using part time helper. Use of medicated feed and post milking teat dips were not recorded in all farms. Branding of milking equipment was not used also totally in both farms. One important finding of this study was the observation that about 35.87 % of dairy farm owners had got training on dairy management. Farm management practices of the dairy owners are shown in Table 6.

Table 6: Response to questionnaire survey on farm management practices in dairy farms

Farm management practices	Frequency (n=92)	Percentage	95% CI
Part time help	24	26.09	17.12-35.06
Feed additives	0	0	0
Training	33	35.87	26.07-45.67
Post milk teat dipping	0	0	0
Branding of milking equipment	0	0	0

n= total number of samples; CI=Confidence Interval

The survey conducted in this study included questions regarding commonly observed diseases and specific antibiotics applied. Thirty-seven percent of the dairy producers admitted that mastitis was a problem in their herds. Metritis and enteritis were recorded on 17.4% and 12 % of farms respectively. Other commonly observed diseases and abnormalities recorded in the farms were abortion, dystocea, retained fetal membrane and foot problem (9.6%). Figure 3 shows the percentage of the most commonly encountered diseases in the dairy frams.

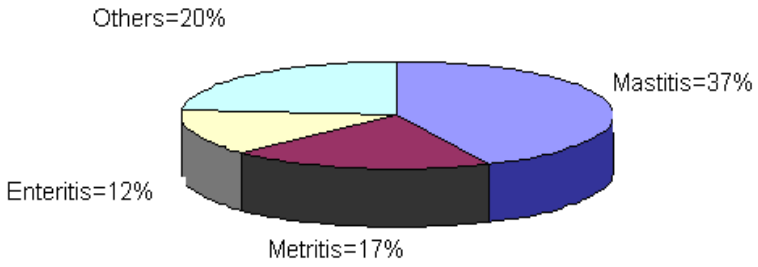


Figure 6: Percentage of the commonly encountered diseases in both dairy farms as rated by respondents

Out of the total of 92 farms interviewed, 43 (46.74%), 34(36.96%) and 17(18.48%) used oxytetracycline, pinstripe and multiject antibiotics to treat disease problems. 19(20.65%) of the farmers interviewed used also other drugs. 4(4.35 %) of the farmers were aware of dry cow therapy as a means for controlling mastitis. In 20(21.74%) of the farms, antibiotics were administered by veterinarian, while 63 (68.48 %) of the farms used assistants. Around ten percent of the dairy producers said they always completed the course of antibiotic treatment. 46(50.00 %), 27(29.35%), 14(15.22%) and 8(8.70%) of antibiotics were administered through intramuscular, intramammary, intrauterine routes and perous, respectively (Table 7).

Table 7: Response to questionnaire survey on treatment factors in both dairy farms

Treatment factors	Frequency (n=92)	Percentage	95 % CI
Drugs			
Oxytetracycline	43	46.74	58.99-77.97
Penistripe	34	36.96	27.10-46.82
Multiject	17	18.48	10.55-26.41
Other drugs	19	20.65	12.38-28.92
Route of administration			
Intramuscular	46	50.00	39.78-60.22
Intramammary	27	29.35	20.04-38.66
Intrauterine	14	15.22	7.88-22.56
Perous	8	8.70	2.94-14.46
Who administer			
Vetrinarian	20	21.74	13.31-30.17
Assistant	63	68.48	58.99-77.97
Owners	9	9.8	3.71-15.85
Dry cow therapy	4	4.35	0.18-8.52

n=total number of samples; CI=Confidence Interval

The proportions of the dairy farms using various antibiotic residue prevention methods are shown in Table 8. 39 (42.39 %) of dairy farms used separate equipment for milking treated cows. 42(45.65%) of farms reported marking of treated cows while 58(63.04%) of farms reported withholding milk from all quarters of treated cows and around twenty percent of the dairy producers said they always kept records of antibiotic treatments. There were no any farms which used the antibiotic residue test kit. Approximately 55(60%) of farmers knew that antibiotic residues were of public health significance.

Table 8: Response to questionnaire survey on prevention methods of antibiotic residues in dairy farms

Prevention methods	Frequency (n=92)	Percentage	95% CI
Marking treated cows	42	45.65	35.47-55.83
Use of separate equipment to milk treated cows	39	42.39	32.29-52.49
Keeping records of antibiotic treatment	18	19.57	11.46-27.68
Withholding milk from all quarters	58	63.04	53.18-72.90
Usage of antibiotic test kits	0	0	0
Knowledge about antibiotic residues	55	59.78	49.76-69.8

n=number of samples; CI= Confidence Interval

4. 2. 2. Response to questionnaire survey in each dairy farm

Proportions of the dairy farms which applied different management practices are summarized in Table 9. 18(26.47%) farmers in Debre Zeit and 6(25.0%) farmers in Nazareth reported using of part-time employers for the milking of cows. There was no significant ($p>0.05$) difference between the two farms in the proportion of their part-time employers. The farmers were asked if they participated in any training of dairy farm management. Around fourty-six percent of the dairy producers in Debre Zeit said as they had participated but none of the farmers participated in Nazareth. Use of medicated feed, post milking teat dips and branding of milking equipment were not recorded in both areas.

Table 9: Response to questionnaire survey on farm management practices in Debre Zeit and Nazareth Diary farms

Farm management practices	Debre Zeit (n=68)			Nazareth (n=24)		
	Frequency	%	95% CI	Frequency	%	95% CI
Part time help	18	26.47	15.98-36.96	6	25.0	7.68-42.32
Feed additives	0	0	0	0	0	0
Training	31	45.59	33.75-57.43	0	0	0
Post milk teat dipping	0	0	0	0	0	0
Branding of milking equipment	0	0	0	0	0	0

n= number of samples; CI=Confidence Interval

Commonly observed disease conditions recorded in Debre Zeit dairy farms were 38.2% mastitis, 17.6 % metritis, 10.3 % enteritis and other types of diseases were recorded in 16.2% of farms. Mastitis, metritis and enteritis were recorded in Nazareth dairy farms on 33%, 16 % and 16.7% of farms respectively (Figure 14). The other disease conditions (dystocea, retained fetal membrane, metabolic problem and foot problem) recorded in Debre Zeit and in Nazareth dairy farms were 16.2% and 20.1% of the farms, respectively.

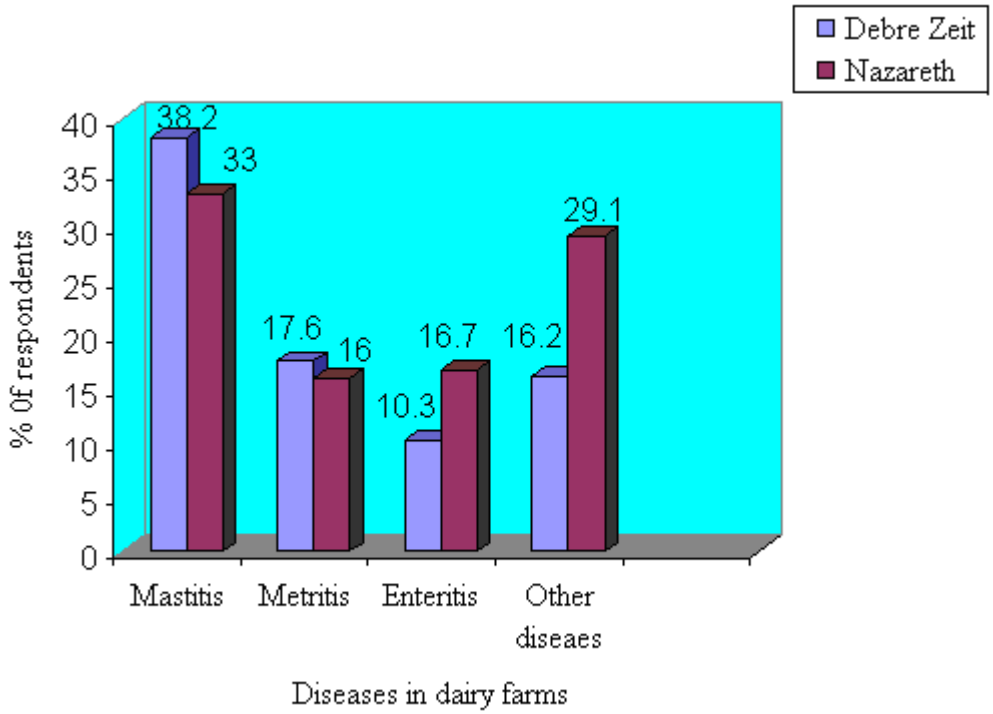


Figure 7: Percentage of the commonly encountered diseases in Debre Zeit and Nazareth dairy farms as rated by respondents

51.1%, 41.2 % and 14.7% of the farmers interviewed in Debre Zeit dairy farms utilized oxytetracycline, pinstripe and multiject antibiotics respectively. On 33.3%, 25% and 29.2% of the dairy herds surveyed in Nazareth, 0xytetracycline, penistripe and multiject were the antibiotics commonly utilized to treat disease problems. Only 5.9% of the farmers were aware of dry cow therapy for controlling mastitis in Debre Zeit dairy farms, but not in Nazareth dairy farms (Table 10). In Debre Zeit dairy farms, antibiotics were administered by veterinarian, assistants and some owners on 23.5 %, 67.7% and 8.8% of farms respectively. On 70.8%, 16.7% and 12.5% of the dairy herds surveyed in Debre Zeit, veterinarians, assistants and owners themselves were used to administer antibiotics. Administration of antibiotics was accomplished using the routes of intramuscular, intramammary, intrauterine and perous on 52.9%, 30.9%,

8.8% and 14.7% of the farms respectively in Debre Zeit and on 41.7 %, 25%, 8.3 % and 16.7 % of the farms in Nazareth respectively (Table 10).

Table 10: Percentage of the treatment factors in Debre Zeit and Nazareth dairy farms

Treatment factors	Debre Zeit (n=68)			Nazareth (n=24)		
	Frequency	Percent	95% CI	Frequency	Percent	95% CI
Drugs						
Oxytetracycline	35	51.4	39.59-63.35	8	33.3	14.47-52.19
Penistripe	28	41.2	29.48-52.88	6	25	7.68-42.32
Multiject	10	14.7	6.29-23.13	7	29.2	10.98-47.36
Other drugs	12	17.7	8.59-26.71	7	29.2	10.98-47.36
Route of administration						
Intramuscular	36	52.9	41.08-64.8	10	41.7	21.95-61.39
Intramammary	21	30.9	19.9-41.86	6	25	7.68-42.32
Intrauterine	6	8.8	2.08-15.56	2	8.3	-2.73-19.39
Perous	10	14.7	6.29-23.13	4	16.7	1.76-31.58
Who administer						
Veterinarian	16	23.5	13.45-33.61	4	16.7	1.76-31.58
Assistant	46	67.7	56.53-78.77	17	70.8	52.64-89.02
Owner	6	8.8	2.08-15.56	3	12.5	-0.73-25.73
Dry cow therapy	4	5.9	0.29-11.47	0	0	0

n=total sample number; CI=Confidence interval

The major antibiotic residues prevention methods used in Debre Zeit and Nazareth dairy farms as per questionnaire survey are presented in Figure 3. The study noted that 52% of dairy farms in Debre Zeit marked treated cows, but only 25% in Nazareth. Around 42% of farms in both areas used separate equipment for milking treated cows 64.7% and 58.3% of dairy farms in

Debre Zeit and Nazareth reported withholding milk from all quarters of treated cows respectively to prevent occurrence of antibiotic residue.

Around 20 % of farms in Debre Zeit and Nazareth dairy farms used keeping records of antibiotic treatment. None of the farms used antibiotic test kit. Nearly equal number of respondents (60%) in both areas thought that antibiotic residues were of public health significance.

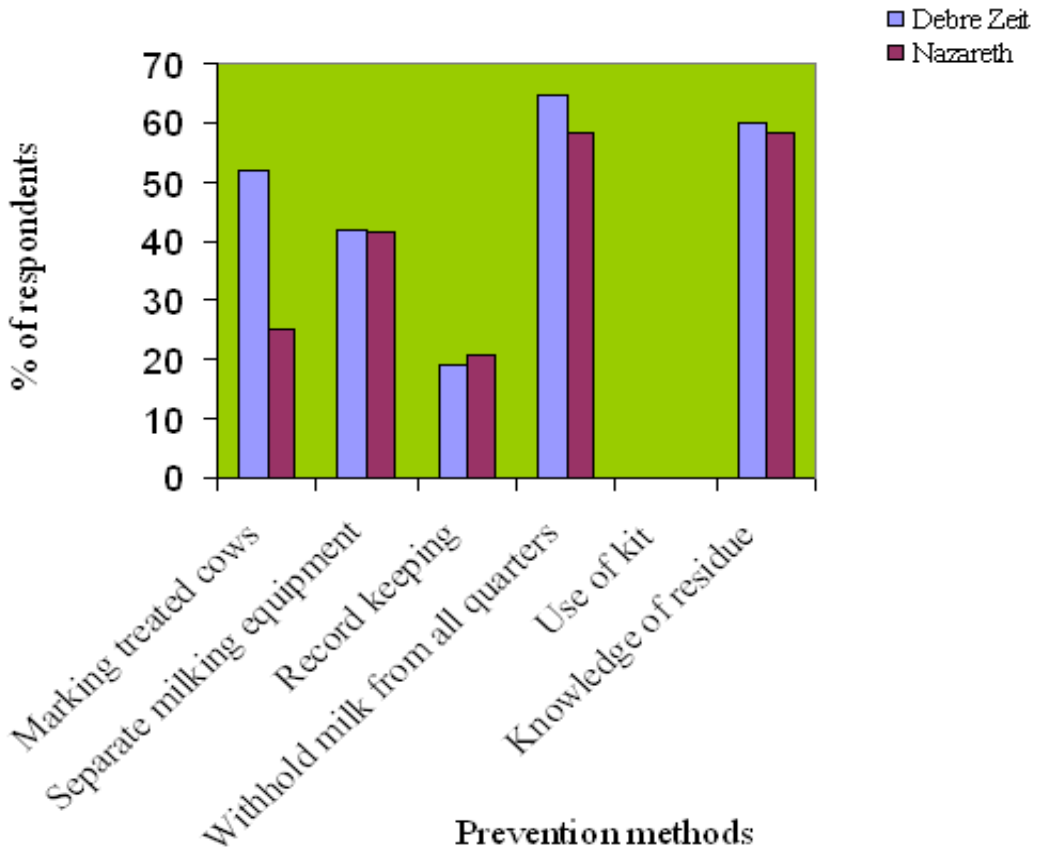


Figure 8: Percentage of responses to questionnaire survey on prevention methods in Debre Zeit and Nazareth dairy farms

Table 11 shows descriptive statitics of herd size and number of milking cows in Debre Zeit and Nazareth dairy farms. The average animals per farm in Debre Zeit were 3.94 and in Nazareth it was 2.79 animals per farm. The mean number of milking cows was 3.34 and 2.29 in Debre Zeit and Nazareth dairy farms respectively.

Table 11: Summary of descriptive statistics of herd size and number of milking cows in Debre Zeit and Nazareth dairy farms

Variables	Areas	n	Mean	Std. Deviation	95% CI for mean		Minimum	Maximum
					Lower limit	Upper limit		
Herd size	Debre Zeit	68	3.94	3.300	3.14	4.74	1	20
	Nazareth	24	2.79	1.668	2.09	3.50	1	7
	Total	92	3.64	2.997	3.02	4.26	1	20
No of milking cows	Debre Zeit	68	3.34	2.601	2.71	3.97	1	16
	Nazareth	24	2.29	1.083	1.83	2.75	1	5
	Total	92	3.07	2.343	2.58	3.55	1	16

n= number of samples; CI= Confidence interval

4. 2. 3. Association of risk factors with antibiotic residue occurrence in milk of case and control farms

A survey instrument (questionnaire) was developed for colleting information on management practices, treatment factors, residue prevention methods and knowledge of the farm owners about the antibiotic residue. The questionnaire survey was carried out in equal number of case farms (Delvotest positive=46) and control farms (Delvotest negative= 46) in both Debre Zeit

and Nazareth dairy farms to see the association between antibiotic residue occurrence and the above risk factors.

The results of farm management practices comparison in case and control farms are shown in Table 12. Case farms were significantly ($p<0.05$) more likely to make frequently use of part-time employers for milking of cows, but control farmers used part-time helpers less frequently. Significant difference ($p<0.05$) between cases and controls were observed with respect to training. Post milking teat dips were not used totally in both control and case farms. Use of medicated feed was also not recorded in both farms.

Table 12: Dairy farm management factors distribution in case and control farms for antibiotic residues

	Percentage of farm management factors				
Management factors	Case (n=46)	Control (n=46)	Chi-square	OR	P value
Frequently use part-time employee	39.96	15.22	5.637	3.266	0.018
Post milking teat dips	0	0	0	0	0
Training	23.91	47.83	3.941	0.409	0.047
Use medicated feed	0	0	0	0	0
Branding of milking equipment	0	0	0	0	0

n=number of samples; OR=Odds Ratio

Table 13 summarizes the association of mastitis between case and control dairy farms. Mastitis had odds ratios greater than 1 and the 95% confidence interval included 1 hence it was not significantly ($p>0.05$) associated with residue occurrence. Metritis, enteritis and other diseases had odds ratios less than 1 and the 95% confidence interval included 1 as a result they were not significantly ($p>0.05$) associated with residue occurrence.

Table 13: Summary of the results of chi-square analysis of association between residue occurrence and commonly encountered diseases

				95% CI	
Diseases	Chi-square	OR	P value	Lower limit	Upper limit
Mastitis	0.271	1.21	1.852	0.612	5.607
Metritis	0.103	0.748	0.813	0.230	2.878
Enteritis	0.276	0.599	1.319	0.468	3.716
Others	5.283	0.22	2.664	1.146	6.198

CI=Confidence Interval; OR=Odds Ratio

Table 14 presents farmer-estimated numbers of cows treated with antibiotics every two months through various routes of administration for each case and control farms. Significant differences ($p<0.05$) between groups of farms were observed only in the case of injectable (intramuscular or intravenous) antibiotics. Although not significant ($p>0.05$), the estimated frequencies of administration of antibiotic through intramammary routes were also higher in case farms. The estimated frequency of intrauterine antibiotics application was higher on control farms, but not significantly.

Table 14: Farmer-estimated frequency of antibiotic treatment of cows, by route of administration, in case and control farms

	Percentage of number of cows treated per two months				
Route of antibiotic administration	Case (n=46)	Control (n=46)	Chi-square	OR	P value
Intramuscular/ intravenous	63.04	36.96	6.261	2.910	0.012
Intramammary	34.78	28.26	1.311	2.910	0.252
Perous	43.48	30.43	1.680	1.758	0.810
Intrauterine	26.09	23.91	0.058	1.123	0.810

n=number of samples; OR=Odds Ratio

Farmers were asked if they treated cows with antibiotics as they thought necessary, or only under the advice of a veterinarian, or not at all. No significant differences between groups were observed in proportions of farmers that treated cows only under the advice of a veterinarian, assistant and those that treated as they felt necessary. Nearly all farmers in both groups were not used intramammary antibiotics in lactating cows as they felt, without the advice of a veterinarian.

In all farms no antibiotics were administered based on extralebel fashin and no person who has administered antibiotic treatments was always the person who milked the treated cows. In lactating cows, clinical mastitis was predominant and observed in all farms. The use of antibiotics was determined by the farm owners. In lactating cows, mastitis was predominant and was observed in 37% of farms. Other commonly observed disease condition was metritis and it was observed on 19.6% of farms. The study has not only determined the most prevalent

diseases, but also the specific antibiotics applied. Oxytetracycline was the most preferred and drug of choice for treating clinical mastitis in lactating cows in 46.7% of the farms. Penicillin G was the second preferred drug for treating clinical mastitis in 37.0% of the farms.

Proportions of case and control farms using various antibiotic residue prevention methods at the time of questionnaire administration are shown in Table 15. Significantly ($p<0.05$) more control farms used separate equipment for milking treated cows (56.52 %) than case farms (34.78%) awhile the rest tended to use the same equipment for all cows and attempted to divert the milk to tank. There was no any farm in both groups which used the antibiotic test kit.

Although not significant difference was noted ($p>0.05$), more case farms reported withholding milk from all quarters of treated cows. Similarly there were no differences between groups in the type of records kept: record book, black board and others. However, significant difference was not recorded between case and control farms ($p<0.05$) in marking treated cows. Farmers were asked to give their opinion on the importance of antibiotic residues in milk. No significant difference was observed between case and control groups regarding farmers' opinion. Totally, around 39.13 % of farmers thought that antibiotic residues were of public health significance, residues could interfere with the manufacture of cultured milk products and antibiotic residues were significant because of their effect on the public health.

Table 15: Milk antibiotic residue prevention methods employed on case and control dairy farms

	Percentage of the farms using prevention methods				
Residue prevention methods	Case (n=46)	Control (n=46)	Chi-square	OR	P value
Use separate equipment to milk treated cows	34.78	56.52	5.386	0.368	0.020
Have used antibiotic test kit	0	0	0	0	0
Keep records of antibiotic treatments	39	21.74	0.276	0.758	0.599
Mark treated cows	34.78	56.52	4.381	0.410	0.036
Withhold milk from all quarter of treated cows	69.57	56.52	1.680	1.758	0.195
Knowledge about antibiotic residues	36.96	43.48	0.045	0.914	0.832

n= number of samples; OR=Odds Ratio

Risk factors that had significant effect on the prevalence of residue using univariable logistic regression, as shown in Annex-9, were fitted in to a multivariable model (Table 16). The effects of the potential risk factors were analyzed using multivariable logistic regression and the results revealed that the risk of antibiotic residue occurrence increased significantl with the frequently use of part-time labourer ($p<0.05$) and administration of the drug through intramuscular route ($p<0.05$). The risk of antibiotic residue occurrence decreased significantly in association with the marking of treated cows ($p<0.05$) and when the farmers took training about dairy management ($p<0.05$). Risk was also reduced with the use of separate equipment for milking of treated cows, but not significantly ($p>0.05$).

Table 16: Summery of results of multivariable logistic regression analysis of association between antibiotic residue occurrence in milk and some risk factors

Variables	Coefficient	SE	P value
Part time help	1.517	0.639	0.018
Training	-1.607	0.566	0.004
IM	1.171	0.527	0.026
Marking	-1.154	0.539	0.032
Separate equipment	-0.679	0.526	0.197

IM=Intramuscular; SE=Standard Error

Comparison of the means of herd size and number of milking cows between case and control dairy farms is shown in Table 17.

Table 17: Comparison of the means of herd size and number of milking cows between case and control dairy farms using ANOVA

Variables		Sum of squares	df.	Mean square	F	P value
Herd size	Between farms	23.440	1	23.440	2.658	.107
Number of milking cows	Between farms	19.430	14.1	19.430	3.642	.060

df=degree of freedom

4. 3. Result of quantitative analysis by high performance liquid chromatography

The samples positive for Delvotest SP assay were further analyzed by HPLC for quantification. A given sample was regarded as positive for oxytetracycline or penicillin G if its retention time and peak corresponded to that of the standard. Retention time was considered a reasonably unique identifying characteristic of a given analyte. The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. To get the concentration of oxytetracycline or penicillin G, a reference standard of a known concentration had been injected in to the HPLC and concentration of the sample was extrapolated from the curve peak area. Chromatograms of reference standards; oxytetracycline HCl and penicillin G procaine salt (Figure 9 and 11) and some samples those were positive for oxytetracycline and penicillin G from Debre Zeit and Nazareth dairy farms (Figure 10 and 12) are shown as follows.

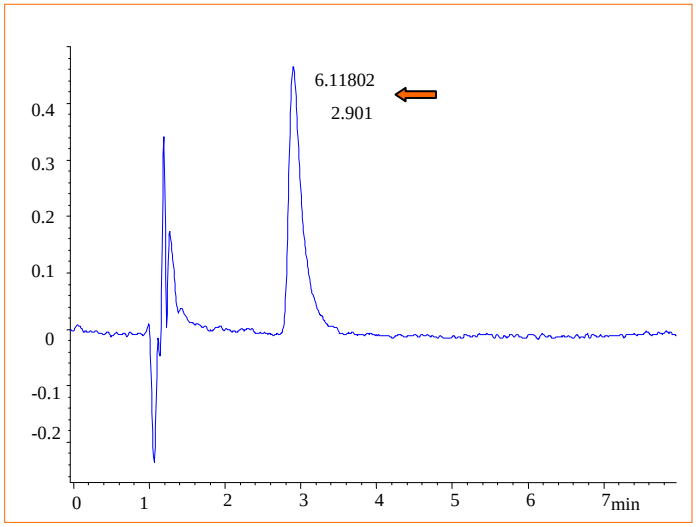
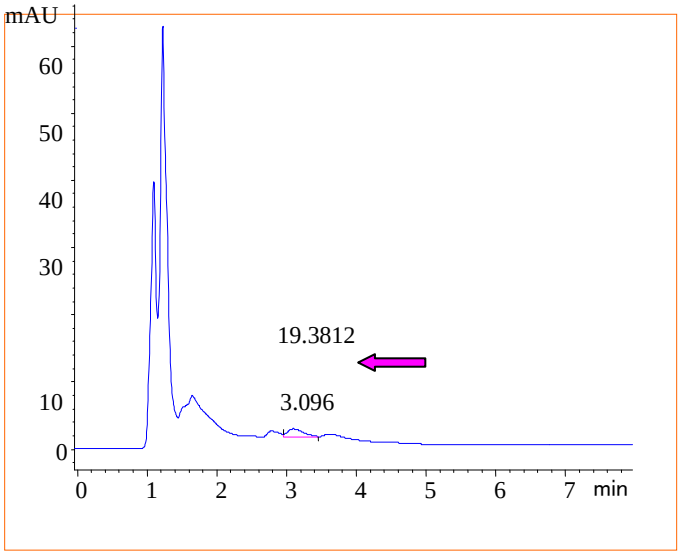


Figure 9: Chromatogrm of reference standards of oxytetracycline HCl

The arrows indicate the peak, peak area and its retention time.



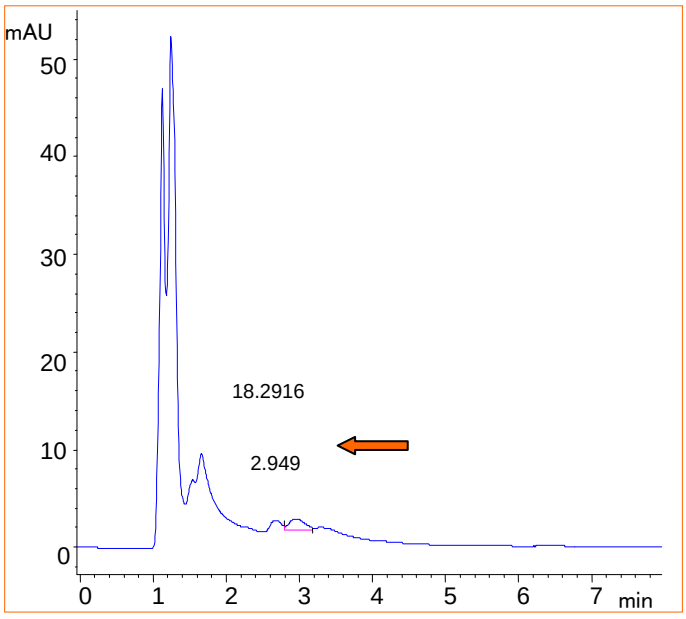


Figure 10: Chromatograms of some samples those were positive for oxytetracycline residue.

A. From Debre Zeit dairy farms B. From Nazareth dairy farms

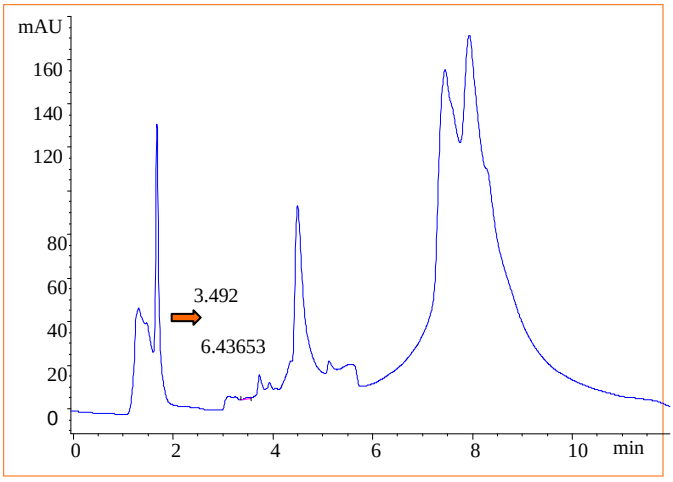
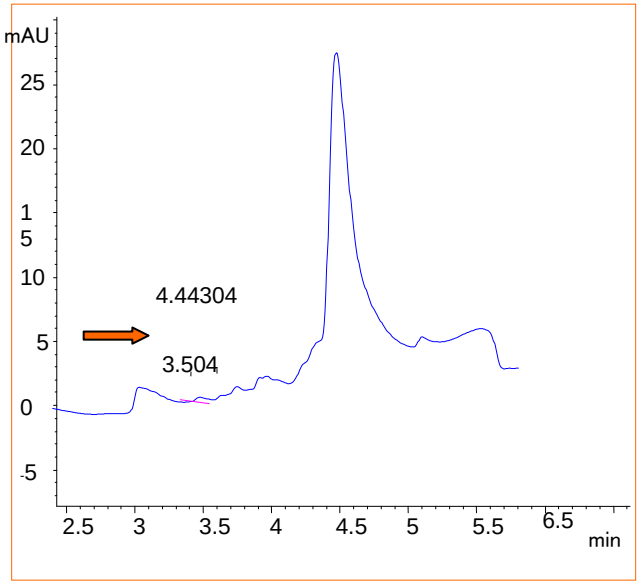


Figure 11: Chromatogrms of reference standards of penicillin G procaine salt
The arrows indicate the peak, peak area and its retention time



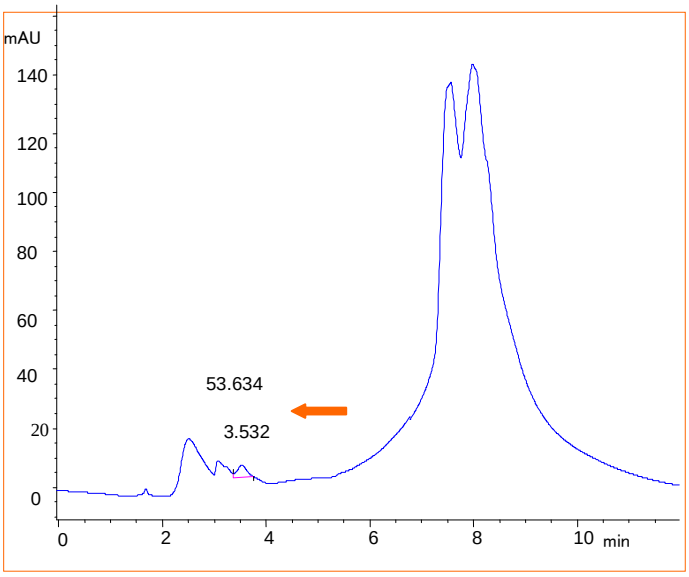


Figure 12: Chromatograms of some samples those were positive for penicillin G residue

A. From Debre Zeit dairy farms B. From Nazareth dairy farms

The range for oxytetracycline residue level from Debre Zeit dairy farms was 0 µg/l to 251µg/l and from Nazareth dairy farms; it was 0µg/l to 208µg/l. The range for penicillin G residue levels for Debre Zeit dairy farms was 0 µg/l to 47µg/l and for Nazareth dairy farms was 0 µg/l to 28µg/l. The antibiotic residue positive samples which showed residues of oxytetracycline above MRLs were 24 (70.58%) at Debre Zeit dairy farms and 10 (83.33%) for Nazareth dairy farms. The antibiotic residue positive samples which showed residues of penicillin G above MRLs were 7 (20.58%) for Debre Zeit dairy farms and 2(16.66%) for Nazareth dairy farms. Mean oxytetracycline residue levels in Debre Zeit and Nazareth dairy farms were significantly different ($p<0.05$). But penicillin G residue levels in Debre Zeit and Nazareth dairy farms were not significantly different ($p>0.05$). The Descriptive statistics such mean, range and standard deviation of oxytetracycline and penicillin G residues are shown in Table 18. Oxytetracycline was found being present in all bulk milk samples of Debre Zeit and Nazareth dairy farms in a concentration range of 27-251µg/l and 45-192 µg/l, respectively. But, no penicillin G was found in some milk samples in Debre Zeit 19(58.8%) and Nazareth 5(41.7%) dairy farms.

Table 18: Summary of desacriptive statitics of oxytetracycline and penicillin G residues concentration (µg/l) in Debre Zeit and Nazareth dairy farms

					95% CI for mean			
Antibiotics	Areas	n	Mean	Std. Deviation	Lower limit	Upper limit	Minimum	Maximum
Oxytetracycline	Debre Zeit	34	142.00	67.206	118.55	165.45	27	251
	Nazareth	12	125.25	52.091	92.15	158.35	45	192
	Total	46	137.63	63.489	118.78	156.48	27	251
Penicillin G	Debre Zeit	34	4.77	10.787	1.01	8.53	0	47
	Nazareth	12	4.52	8.097	-0.63	9.66	0	28
	Total	46	4.70	10.068	1.71	7.69	0	47

n=total number of sample; CI= Confidence Interval

The result of comparison of the mean square of oxytetracycline and penicillin G residue levels between and Debre Zeit and Nazareth dairy farms is shown Table 19.

Table 19: Comparison of the mean square of oxytetracycline and penicilline G residue between Debre Zeit and Nazareth dairy farms using ANOVA

Antibiotic analysis		Sum of squares	df.	Mean square	F	P value
Oxyteracycline	Between farms	2488.467	1	2488.467	0.612	0.438
Penicillin G	Between farms	0.572	1	0.572	0.006	0.941

df=degree of freedom

5. DISCUSSION

Out of the total 400 bulk milk samples analyzed by Delvotest SP assay during the study period, 46 (11.50%) had detectable levels of antibiotic residues. The proportion of positive samples was higher when compared to other reports elsewhere. A similar study carried out by Borges *et al.* (2000) in Brazil country reported that 4.3 % milk samples had detectable level of residue for antibiotic. The result of Rybinska *et al*. (1995) in Poland revealed that 13-22 % milk sample showed violative concentration of chemical residues. In the study undertaken by Sternesjo (1998) in Sweden, it was also indicated that 0.08-0.26% milk samples were positive for antibiotic residues which were much lower than the present study. Another study conducted by Sudershan and Bhat (1998) in India also indicated that 9% milk samples had residues of antibiotic which is comparable with the results of this study.

The prevalence of antibiotic residues in Debre Zeit bulk milk samples (n=279) was 34 (12.19%) while in Nazareth bulk milk samples (n=121) it was 12 (9.92%). The occurrence of antibiotic residues in both areas may be attributed to absence of use of post milk teat dipping, milking by contract laborers and poor record keeping systems. No significant difference was noted between Debre Zeit and Nazareth dairy farms ($p>0.05$) with respect to prevalence of antibiotic residue in milk. Therefore, milk from Debre Zeit dairy farms was not at greater risk of residue significantly than milk from Nazareth dairy farms. But, the presence of minute amount of antibiotics in milk, as a result of therapeutic use in dairy cows, can cause a wide variety of dairy manufacturing problems, including; inadequate milk curdling or sub obtimal ripening during cheese production, inadequate acidity and flavor attributes during the manufacture of butter milk, subobtimal starter culture growth; false results during quality control testing due to the presence of interfering drug metabolites (EEC, 1992).

In addition, the presence of antibiotics in raw milk poses a variety of legal concerns and health risks, including: the fact that elevated drug levels in milk are illegal; milk from drug-treated cows commonly contains live pathogenic bacteria capable of causing diseases in people; the potential for direct toxic effects of the drug on the consumer; the drugs or their metabolites may cause consumers to develop allergies or trigger life-threatening allergic reactions and the

potential for the development of antibiotic resistant strains of bacteria. For these reasons, many governments have established regulations to assure the proper collection, processing, pasteurization and sanitation of milk and milk products (IDF, 1991). But, a study that examined the use of antibiotics on conventional dairy farms in Michigan, Minnesota, New York, and Wisconsin USA reported that the use of new antibiotics such as ceftiofur was a common practice (Zwald *et al.*, 2004). The authors observed high use of dry cow therapy on these farms, and approximately half of the farms fed medicated milk replacers to calves.

Small dairy producers in Kenya were observed producing milk with beta-lactam residues exceeding the established maximum residue levels (Shitandi, 2004). The use of antibiotics in Sweden and Norway for mastitis treatment had been influenced by national policies and recommendations. In these countries, the preference for using beta-lactams (i.e., procaine and benzyl penicillin) was based on the withdrawal period. Dairy producers in Sweden use long-acting drug treatment for subclinical mastitis and dry cow therapy, whereas the same formulations are not accepted in Norway (Grave *et al.*, 1999). These examples indicate that antibiotic usage varies among and within countries and also between farms, depending on policies.

The questionnaire survey conducted during the study period included questions that were helpful to gain insights into farm management practices associated with antibiotic usage. In general, twenty six percent (26.09%) of the respondents used contract laborers or part-time employee for milking activities. Nearly equal number of respondents, (26.47 %) in Debre Zeit and (25.0 %) in Nazareth dairy farms reported use of part-time employee for milking activities. This finding was lower than the finding of Tesfaye (2007) at Nazareth who reported a proportion of (39.0%). However, in most cases, contract laborers were either not aware of giving much attention to the importance of hygienic conditions during milking, proper milking practices and the necessary precautionary measures while milking treated cows.

All the respondents indicated that they never practiced post milking teat dipping. Post milking teat dipping was carried out by 3.9% of the respondents in Addis Ababa dairy farms as reported by Mungube (2001). Those farms that practiced teat dipping used ammonium compounds

(3.9%) and lugols iodine (5.9%). Nearly all respondents reported that they never used feed additives in their dairy farms. Branding of milking equipments was not used also totally in both farms.

The overall prevalence of mastitis at herd level was 37.0%. The prevalence of mastitis at herd level was 38.2 % in Debre Zeit and 33.0% in Nazareth which is comparable with that of Workineh *et al*. (2000) who reported 25.1% in Addis Ababa. However, the present study was higher proportion than that reported by Bishi (1998) and Munguba *et al.* (2001) who reported 5.35%and 6.6%, respectively in Addis Ababa dairy farms. In addition, the prevalence was much higher than Gizat (2004) who reported 3.9% in Bahardar. Mastitis is a complex disease and the difference in results could be due to difference in management systems among farms. The high prevalence of mastitis may be attributed to improper milking hygiene, lack of use of post milking teat dipping and practices of milking by contract laborers with different skills. Mastitis has been previously reported to be the most common condition treated in lactating dairy cows (Mitchell *et al.*, 1998). Cases of metritis and enteritis in lactating cattle in both Debre Zeit (17.6%, 10.5%) and Nazareth (16 %, 16.7%) dairy farms were almost similar. Cows with other types of diseases like dystocia, retained fetal membranes and various metabolic disorders are more likely to lead to metritis (Lewis, 1997).

Based on the findings of this survey, it can be inferred that antibiotics, particularly tetracyclines and penicillin G, are extensively used for prevention and treatment of diseases in dairy farms. Oxytetracycline was the first antibiotic used in most farms (46.74 %) secondary to penicillin (36.96 %) according to the respondents. The use of antibiotics continues to be a predominant in the treatment and control of mastitis (Owens *et al.*, 1997). Dry cow therapy was only reported in Debre Zeit dairy farms 4 (4.35%) which was similar to that report by Mungube (2001) in Addis Ababa dairy farms. For dry cow therapy on farms, preferred drugs were cephapirin, penicillin G procaine and cloxacillin .These antibiotics are effective in protecting against new intramammary infections (Sol and Melenhorst, 1990; Sanchez and Watts, 1999).

Health services were given mostly by the practitioners coming to the farms or sometimes by taking the animals to veterinary clinics. Regular health programs by professionals were not

practiced, but in general, it can be hypothesized that all farms had access to health services provided by professionals when needed. This might be due to the income they get from sale of milk that allows them to pay for the veterinary services. Twenty two percent of the farmers always sought veterinarian advice before administering antibiotics. Other than the veterinarian, antibiotics were administered primarily veterinary assistants and owner or herdsmen on 69% and 10% of farms, respectively. Only 9% of the dairy producers in Debre Zeit and 12 % in Nazareth said that they always completed the course of antibiotic treatment by themselves. The tendency to rely on personal experience for antibiotic use, dosage, and withdrawal period was also observed in dairy producers surveyed by Zwald *et al*. (2004). This lapse could lead to improper antibiotic usage.

One important finding of this study was the observation that approximately equal number of respondents (60%) in both Debre Zeit and Nazareth farms thought that antibiotic residues were of public health significance. Similarly, 78.4% of the respondents had knowledge on residue (Mungube, 2001) in Addis Ababa dairy farms. Only 20% of the farms in both areas surveyed kept records of antibiotic treatment that could be verified. Kaneene and Ahl's (1987) survey of dairy producers in Michigan USA indicated that insufficient record keeping and poor knowledge about drug withdrawal periods among producers were important factors leading to drug residues in milk. All respondents in Debre Zeit and Nazareth dairy farms indicated that they never used antibiotic test kit for detection of residues.

Nearly equal number of respondents (42%) in both dairy farms said that they used separate equipment for milking treated cows. Half (52%) of the respondents in Debre Zeit reported marking of separated cows. But, it is only one-fourth (25%) of the farms in Nazareth who marked the treated cows. The average animals per farm in Debre Zeit was 3.94 which is in agreement with the existing reports by Devendra (2001) and Mekonnen *et al.* (2006) who reported average 4.56 animal per farm in Debre Zeit. In Nazareth it was 2.79 animals per farm, but this observation diverge from what was reported by Tesfaye (2007) which was 5.23 animals per farm. The mean number of milking cows was 3.34 and 2.29 in Debre Zeit and Nazareth dairy farms respectively.

The association and covariance among a number of potential risk factors for antibiotic residues in milk was analysed using Chi-square and logistic regression analysis. The frequent use of part-time help laborer in the milking of cows was associated with increased risk of residue occurrence. Similar findings were observed in a mail survey conducted by McEwen *et al*. (1991). Part-time farm workers may be less aware of the necessity of withholding milk from antibiotic treated cows or there may be a failure of communication in identifying treated cows when these people are employed. Kaneen and Ahl (1987) also found that among dairy farmers surveyed in Michigan, antibiotic residues in milk were associated with an increase in the number of people working on farms.

The number of cows milked was not significantly different on case and control farms, but other epidemiological studies of antibiotics in milk have observed this to be the case (McEwen *et al*., 1991). Although statistically insignificant, control farms had more milking cows on average than case farms in the present study. But, Kaneen and Ahl (1987) and McEwen *et al.* (1991) found significant associations between residue occurrence and herd sizes. Herd size is likely to influence the frequency of antibiotic administration to cows. This is because; large herds have more employees and hence more opportunity for errors in communication and accidental transfer of milk from treated cows.

Estimated frequency of treatment of lactating cows with intramuscular antibiotics was significantly higher in the case than control farms in simple association and also when all variables were considered together using the multiple logistic regression analysis. This type of antibiotic is especially likely to lead to milk residue problems, because it is the only route of administration commonly used by the owners and even assistants. But, McEwen *et al*. (1991) found that residue occurrence in milk was associated with an increase in the estimated frequency of treatment of lactating cows with intramammary antibiotics.

In this study, the use of medicated feeds having antibiotics and teat dips were overtly not observed. In Canada, feeds medicated with antibiotics are not licensed for use in lactating cows, although the potential exists for accidental contamination of milk cows fed with medicated feed intended for calves, swine or some other farm animals (Kaneen and Ahl, 1987). Reports from

other studies indicate prevalent use of teat dips on farms (McEwen *et al*., 1991). This practice reduces mastitis in dairy herds and perhaps in that way, use of teat dip may reduce the need to treat animals and therefore low probability of antibiotic residues in milk.

Responses from the questionnaire survey showed that farmers never used dry cow intramammary antibiotics. Only 5.9 % of farm owners in Debre Zeit used dry cow therapy. This is contrared to results from the study which found significantly more case than control farms which never used injectable or dry cow intramammary antibiotics (Kaneen and Willeberg, 1988).

With respect to residue prevention methods, other than the use of antibiotic residue detection kits, the practice of using separate equipment for milking treated cows was associated with reduced risk of milk residues. This had been observed previously by McEwen *et al*. (1991) in Pennsylvannia. And as has been suggested by others (Guard *et al*., 1985), using separate equipment is likely to be more reliable than attempting to divert milk from the tank while using the same equipment for untreated and treated cows. As expected, high proportion of all farmers knew that milk should be withheld from all quarters of treated cows because residue can be found in untreated quarters (Egan and Meaney, 1985). Further in USA, Booth (1982) reported that 8% of antibiotic residues occurred in milk from all quarters of treated cows.

Over 60% of all farmers in Debre Zeit and 58% in Nazareth thought that antibiotic residues in milk were of public importance and should be carefully guarded, but did not think that residues interfere with production of milk products. Although no significant ($p>0.05$) difference was recorded between these opinions between case and control farms, Kaneen and Ahel (1987) reported that milk residue positive farms were less likely than on negative farms to think residues are important to public health and interfered with processes of producing milk by-products.

Milk samples that were Delvotest assay positive, had detectable levels of oxytetracycline and penicillin G residues by HPLC analysis. In most countries, limits have been established for antimicrobial drug residues in food products such as milk; for instance, in the European Union (EU), the maximum residue limits (MRLs) have been set by the European Commission.

FAO/WHO Expert Committee on Food Additives established MRLs for oxytetracycline and penicillin G in milk at 100 µg/l and 4 µg/l, respectively. The accurate detection of low levels of antimicrobial drug residues in milk is not only of great importance for governmental control, laboratories and the dairy industry but also for farmers to enable them to ensure that contaminated milk from individual cows is not consigned to the bulk tank (EEC, 1990).

This study revealed that out of 46 milk samples found positive for antibiotic residues by Delvotest, 34 (73.91%) had oxytetracycline residues and 8 (17.39%) penicillin G residues above the recommended MRLs in both Debre Zeit and Nazareth dairy farms. The mean concentration of oxytetracycline residue was 142 µg/l (95% CI: 92-158 µg/l) and 125.25 µg/l (95% CI: 118-165 µg/l) in Debre Zeit and Nazareth dairy farms, respectively. For penicillin G residue, the mean concentration was equal to 4.77 µg/l (95% CI: 1-9 µg/l) in Debre Zeit and 4.52 µg/l (95% CI: -0.6-8.53) in Nazareth. However, difference between the concentrations of both antibiotics between the two areas were not statistically significant ($p>0.05$).

Oxytetracycline was found in all milk samples collected in Debre Zeit and Nazareth dairy farms with area-specific concentration range of 27-251µg/l and 45-192 µg/l, respectively. This finding was higher as compared to Zhao *et al*. (2004) who found oxytetracycline concentration within range of 13-106µg/l in USA. A study by Shitandi (2004) found out that penicillin G was the most commonly type of antibiotic residue in milk, with levels often exceeding the maximum residue limit established in the European Union (4 µg/l) . Penicillin G was not detected in 58.8 % of the milk samples from Debre Zeit and 41.7% in Nazareth dairy farms. The concentration range of penicillin G in Debre Zeit and Nazareth were 0-47 µg/l and 0-28 µg/l, respectively.

The presence of antibiotic residues in milk is strongly associated with several variables such as milk production at time of treatment, type and amount of antibiotic used, type of vehicle used in antibiotic formulations and the disease state of the animal (Mercer, *et al.,* 1970). Antimicrobials, antiinflammatories and hormones are the pharmacologically active substances most used for these purposes, but an illegal or unsuitable use increases the risk of introducing harmful residues into the human food chain. Adverse effects to consumers are connected with the intrinsic

toxicity of a drug and its metabolites. The use of antmicrobial agents in food animals has caused concern regarding the impact of them on human health.

The use of tetracyclines in the United States exceeds 5.6 million pounds annually (Mellon, Benbrook and Benbrook, 2001). The main applications of tetracyclines in animal husbandry are for prophylaxis of bacterial infections and increase in growth rates. Although the public health risks are difficult to define, it is accepted that antimicrobial drug residues may induce allergic reactions in sensitized individuals and may have negative effects on the composition of the human intestinal flora. In general, the excessive use of antimicrobials has led to the development of multi-drug resistance in animal and human pathogens (Sarmah, Meyer and Boxall, 2006). Furthermore, milk contaminated with even low concentrations of antimicrobial drug residues may also create problems in the production of fermented milk by products, because such compounds inhibit the growth of the starter cultures.

In addition, human exposure to animal products containing significant levels of antibiotic residues may affect the immunological responses in susceptible individuals and cause disorders of normal intestinal flora. Some individuals may have allergic reactions to these compounds. As undesirable side effects, tetracyclines not only discolor the primary and permanent teeth, but also cause hypoplasia in developing teeth when administered to infants, mothers during the last two trimesters of pregnancy and children under 12 years of age (Walton *et al.,* 1994). It has also been suggested that discoloration caused by tetracyclines occurs in adult dentition (Tanase *et al.*, 1998).

The main concern regarding the ingestion of antibiotic residues is the development of resistant bacterial strains. Resistant bacteria from food animals may be passed through the food chain to human resulting in resistant infections. Increasing resistance to antimicrobial agents that are important in treating human diseases, such as tetracyclines, penicilline, fluoroquinolones and the third generation cephalosporins for treatment of salmonellosis, campylobacter infections and other diseases, has significant public health implications (Alicia *et al*.,2003).

The development of resistance to salmonella was reported from Ethiopia by several authors (Alemayehu *et al.,* 2004 and Zewdu, 2004). Background Strains of salmonella that are resistant to antimicrobial agents have become a worldwide health problem. A distinct strain of Salmonella enterica serotype typhimurium, known as definitive type 104 (DT104), is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline and has become a major cause of illness in humans and animals in Europe, especially the United Kingdom (Glynn *et al.,* 1998).

The study also showed that oxytetracycline and penicillin are imprudently used in those areas which are the basic means for treatment of many diseases. As a result, these drugs will be out of use in the near future due to treatment failures by creating resistance to many species of bacteria even to those species which are isolated for the fist time in Ethiopia; for example, *S. Braenderup, S. Hall* (Becker *et al*., 1996).

Suspicious carcinogenicity of growth-promoting agents has prompted the European Union (EU) to ban the use of these compounds and to forbid the importation of meat and milk products from countries that authorize their use for fattening purposes. Therefore, drug residues remain very significant from the prospective of international trade and consumer confidence, because it results in international trade barrier (Kanneene and Miller, 1997). To increase considerable foreign currency, milk and milk products need to be exported, the requirement by World Trade Organization (WTO) and Codex Alimentarius Comission (CAC) should be adhered. One of the requirements is that antimicrobial residues in food should be below MRLs. But, the indiscriminate use of veterinary drugs can hinder the country's interest to fulfill the need to export to those WTO member countries. Therefore, attempts should be made to reduce the magnitude of the problem at various levels through the prudent use of antimicrobials such as oxytetracycline and penicillin G. Awareness need to be created at different levels including controlling authorities, concerned organizations and the consumers.

6. CONCLUSIONS AND RECOMMENDATIONS

Milk intended for human consumption must be free from antibiotic residues. Antibiotics will continue to be used extensively for treatment of mastitis and other bacterial infections in dairy cows. The actual uses of antibiotics greatly vary from established protocols. Several antibiotics are often used simultaneously for prolonged periods. Any variations in antibiotic dosage, duration of treatment, or use of multiple antibiotics lead high probabilities of the antibiotics being excreted in milk beyond recommended withdrawal times.

The survey conducted in this study included questions that were helpful to gain insight regarding farm management practices associated with antibiotic usage. Absence of antibiotic treatment records, lack of written plans for treating sick animals, failure to consult veterinarians in treating sick animals, and failure to complete antimicrobial treatment course are factors that can lead to inappropriate use of antibiotics. There was considerable variation in the management practices associated with antibiotic uses in dairy farms. The most important clinical conditions on farms for which antibiotics were used extensively included mastitis.

Delvotest SP assay and HPLC technique provided effective means to detect and determine antibiotic residues in milk. The results obtained confirmed that oxytetracycline and penicillin G were heavily and indiscriminately used in dairy farms without appropriate withdrawal times observed. Results of the present study serve as preliminary base-line information for veterinarians, drug administration and quality control authorities and other concerned organizations and professionals to take measures in controlling and preventing occurrence of drug residues in milk and more in animal-food chains.

Based on these facts, the following are recommended:

- Strong extension package that involves training on health care and feeding, record keeping, proper milking management methods, post milking teat dips, dry cow therapy and withdrawal periods of antibiotics should be introduced to dairy cow owners.

- The antibiotic screening tests should be provided to be used by dairy producers, milk processors and veterinarians to ensure the production of antibiotic residue-free milk.

- Trained man power and instruments for drug residue analysis should be put in place where these can help in reducing or eliminating residues and guaranties the supply of wholesome and safe milk to the public.

- Regulation of the dairy development enterprises, establishment of standards for antibiotic residues in milk and the use of effective enforcement of their standards are essential to fulfill the objective of consumer providing them with safe and wholesome milk and milk products.

- Since there is no information on antibiotic residue levels in milk in Ethiopia, planned and continous surveillance should be conducted to generate base line data and to monitor antibiotic residue level inorder to effectively safeguard the consumer's health.

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8. ANNEXES

Annex 1: Principle and procedure of Delvotest SP assay

1. Principle

The Delvotest SP assay is a microbial diffusion test used for the detection of antibiotics and sulphonamide residues in milk. Milk is added to a solid agar medium containing *Bacillus stearothermophilus vr calidolactis* spores along with a nutrient agar tablet. When the test is kept cool, the spores remain viable. Upon incubation at 64 ^{0}C for three hours, the spores germinate. The germinated spores multiply with the formation of acid. When sufficient acid is formed the colour of the indicator (bromcresol) changes from purple to yellow. If the milk sample contains antimicrobial residues the antimicrobial will diffuse into the agar and when present in a sufficient concentration will cause an inhibition of the multiplication process and thus acid production. The colour of the media remains purple when the concentration of the inhibitor is above the detection limit of the assay. When the concentration of the inhibitor is around the detection limit, the media will exhibit a part yellow and a part purple colour. For the most widely used antibiotics, the detection threshold of Delvotest is the MRL level.

Among the microbial inhibitor tests widely used for detection of veterinary medicines in cow milk, Delvotest SP is an economic, easy-to-use screening test giving results within a relatively short period (3.00 h). This method was recognized by the Association of Official Analytical Chemists (Katz, 1982; Kelley, 1982). The Delvotest SP method is classified visually into three categories: “negative,” “doubtful,” and “positive,” compared with the colors of “positive” and “negative” standard sample (Suhren and Luitz, 1995).

2. Equipment

2. 1. Analytical balance (5 decimal points)

2. 2. Water bath

3. Apparatus

3. 1. Test tube rack

3. 2. Disposable pipettes

3. 3. Disposable syringe

3. 4. Forceps

4. Chemicals

4. 1. Penicillin G sodium salt

4. 2. Milk known to be antimicrobial free

4. 3. Distilled water

5. Reagents provided

Each kit contains enough material for 100 tests. 100 ampoules with *Bacillus stearothermophilus var calidolactis* and nutrient in a solid agar medium. Store at +4 ^{0}C. Don't freeze.

6. Standard preparation

6.1. Positive control

Weigh 50 mg of penicillin G using an analytical balance. Transfer to a volumetric flask and make up to 50 ml using distilled water to give a solution containing 1 mg of penicillin G per ml. prepare daily and discard it.

6. 2. Negative control

Use milk known to be antimicrobial free or distilled water

7. Assay procedure

7. 1. For each sample to be assayed or control, place one ampoule in to a test tube rack.

7. 2. Open each ampoule and add a nutrient tablet

7. 3. Transfer the 100 µl sample of milk in to the ampoule. Use a clean pipette for each sample.

7. 4. Incubate the kit in water bath or dry block incubator at 64 ^{O} C for 3 hours. When incubating in the water bath the level of the sample fluid should be approximately ½ cm below the water surface. Don't let ampoules float in the water bath.

8. Interpretation of the results.

Read the assay results by judging the color of the lower two-thirds of the agar medium.

8. 1. If the sample is free from antimicrobial compounds at or below the detection limits, the color of the media in the ampoule changes to yellow.

8. 2. If the sample contains antimicrobial compounds above the detection limit, the color of the media in the ampoule remain purple.

8. 3. If the sample contains antimicrobial compounds at a concentration near the detection limit, the media in the ampoule exhibits a part yellow and part purple color.

9. Criteria for acceptability of results

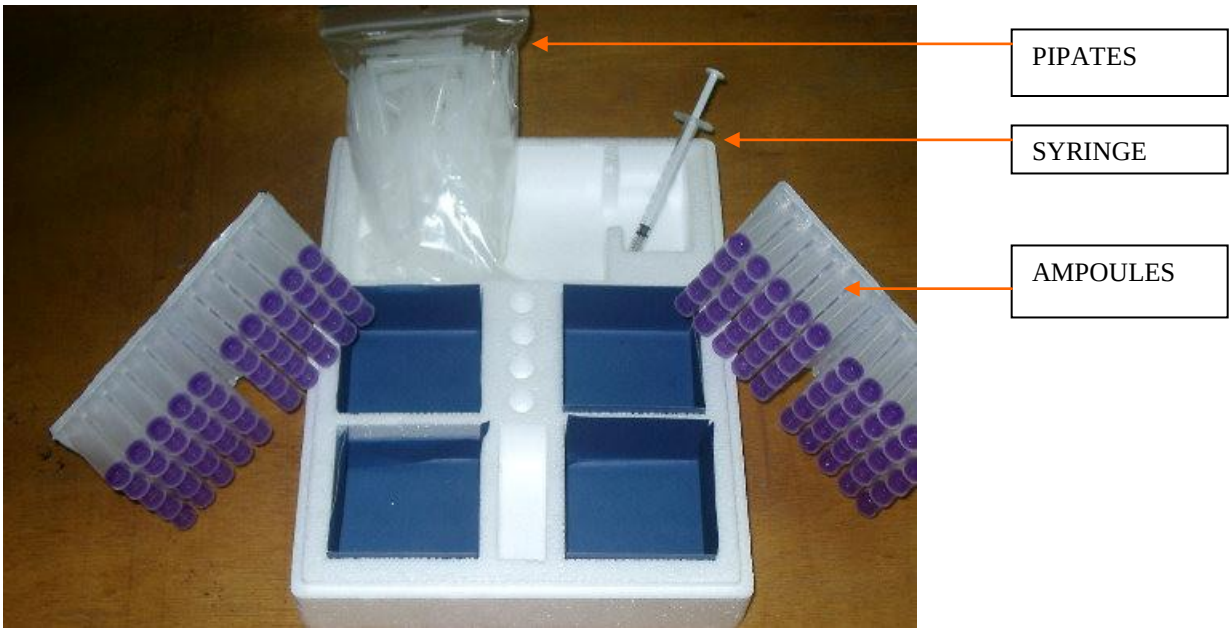
9. 1. The color of the media of the negative control sample should change to yellow.

9. 2. The color of the media of the positive control sample should remain purple.

9. 3. Samples which are presumptive positive (part yellow or part purple) should be repeated.

Annex 2: Set of Delvotest kit

A. Delvotest kit



B. Dry block incubator

Annex 3: Method for determination of penicillin G residue in milk

The residues of these antibiotics in bovine milk were determined by using High Performance Liquid Chromatography method described by Ghidini, *et al*. (2003) for penicillin G. The chemicals, reagents, apparatus and detailed procedures for determining of penicillin G in milk.

1. Principle

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector equipped with an AS3000 autosampler.The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm) at a flow-rate of 1ml min^{-1}.The analysis was carried out under the operational conditions expressed in

There are three principal stages in the sample preparation:
Extraction of the sample residues
Precipitation of proteins using trichloroacetic acid and filtration,
Cleanup on solid-phase extraction 500 mg SPE cartridge C18 and injection.

2. Penicillin G are separated on a C18 stationary phase and detected by UV absorption at 360nm. The amount of penicillin G is calculated by interpolation from a calibration curve.

3. Chemicals and reagents
Unless otherwise specified, all reagents are of analytical grade and the water is demineralized.

3. 1. Acetic acid acid aqueous solution (10%)
3. 2. Acetonitrile
3. 3. Formic aid (o.1%)
3. 4. Mobile phase: Acetonitrile and water both acidified with 0.1% formic acid, according to the following elution program: from100% water to 100% acetonitrile in 6 min and from 100% acetonitrile to 100% water from 6 to 12 min.

3. 5. Standard: Penicillin G procaine salt

3. 6. Standard solutions

The analytical standards were dissolved in methanol at a concentration of 0.1 mg/ml. The working standard solution made by dilution with water containing 0.1 % of formic acid and 25% of methanol.

The analytical standards were dissolved in water: acetonitrile (75:25 v/v) at a concentration of 50mg/l and kept in the dark at 4^{o}C.

4. Apparatus

4. 1. 1. Polypropylene centrifuge tubes, 50ml capacity, with caps.

4. 1. 2. Glass tubes, 30ml capacity

4. 1. 3. Polypropylene tubes, 5ml capacity

4. 1. 4. Amber volumetric flasks, 25ml, 50ml, 100ml, 200ml and 1000mlk.

4. 1. 5. Graduated glass pipettes, 2ml, 5ml, 20ml and 25ml.

4. 1. 6. Automatic pipettes type Gilson P1000.

4. 1. 7. Blender type moulinette (Moulines)

4. 1. 8. Analytic and precision balance model PB302 (Mettler-Toledo)

4. 1. 9. High precision analytic balance type A 120S (Sartorius).

4. 1. 10. Solvent dispensers (Brandt)

4. 1. 11. pH-meter (Tacussel)

4. 1. 12. Electric stirrer type vortex (Bioblock)

4. 1. 13. Rotary stirrer type Rheax 2 (Heidolph). Vortex mixer was used instead.

4. 1. 14. Magnetic stirrer type Nuova II (Bioblock).

4. 1. 15. Cooled centrifuge model GR 4.22 (Jouan).

4. 1. 16. Solid phase extraction cartridges Bond-Elut C18, 3cc, 200mg (Varian).

4. 1. 17. Solid phase extraction manifold (Supelco), adaptors, needles (Analytichem). Syringe was used in this study for the sample to pass-through the cartridge

4. 1. 18. Vacuum pump, 0.4 bar, 12w (Bioblock)

4. 1. 19. Whatman disposable filter funnels, 25mm diameter (Whatman, Art. 1922-1800) or 50 ml reservoirs containing these same filters.

4. 1. 20. Refrigerated ultra- speed centrifuge mode MR 1822 (Jouan)

5. Storage of samples and sampling: Sample must be stored at about -20 ^{0}C.

6. Procedure

6. 1. Extraction

6. 1. 1. Put a total of 5ml milk into 10-ml pyrex screw cap centrifuge tubes

6. 1. 2. Add 10 % acetic acid aqueous solution to the milk and mix using vortex

6. 1. 3. Centrifuge the sample at 3500 rpm for 10 minutes after placed in centrifuge tube.

6. 1. 4. Take the upper suppernatatant by syringe, avoiding taking the upper fat layer

6. 1. 5. Filter through a 0.5-µm nylon filter 13 mm daiameter (Advanctec MFS, Pleasanton, CA, USA).

6. 1. 6. Put the filtered extract in to 2ml auto sampler vials.

6. 4. Chromatographic conditions

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector equipped with an AS3000 autosampler. The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm).

6. 4. 1. The mobile phase: Distilled water and acetonitrile both acidified with 0.1 % formic acid

6. 4. 2. Flow rate: 1ml/min

6. 4. 3. UV detector wavelength: 360nm

6.4.3. Injection volume: 20 µl

6. 4. 4. Retention time: 3.5 min

6. 4. 5. Recovery: 65%

6. 4. 6. Limit of detection: 0.05µg/l

7. Calculation of results

The following calculations can be executed directly by the PHLC 2D Chemstation software.

7. 1. Derive the calibration curve from the results obtained with the working standards solutions. Quantitation was made through external standards. Calibration curves were built by injecting mixture of working solutions at the following concentration: 5, 10, 200, 400, 1000µg/l of stock solution. Peaks corresponding to the penicillin G standard have to be taken into account. Then, determine the curve equation:

Y= ax + b

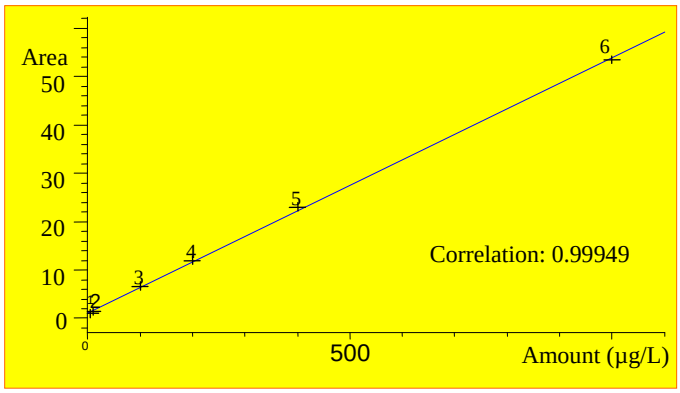
Y= peak area (TC + epimer)

X = concentration (µg/ml)

A = slope

B = intercept

7. 2. Calibration curve



Area = 0.05301397 X

R^2=0.99949

Annex 4: Method for determination of oxtetracycline residue in milk

The residues of these antibiotics in bovine milk were determined by using High Performance Liquid Chromatography method described by Ghidini, *et al*. (2003) for oxytetracycline and penicillin G. The chemicals, reagents, apparatus and detailed procedures for determining of oxytetracycline in milk are as follows.

1. Principle

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector equipped with an AS3000 autosampler.The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm) at a flow-rate of 1ml min^{-1}.The analysis was carried out under the operational conditions expressed in

There are three principal stages in the sample preparation:
Extraction of the sample residues
Precipitation of proteins using trichloroacetic acid and filtration,
Cleanup on solid-phase extraction 500 mg SPE cartridge C18 and injection.

2. Oxytracyclines are separated on a C18 stationary phase and detected by UV absorption at 360nm. The amount of oxytetracycline is calculated by interpolation from a calibration curve determined f

3. Chemicals and reagents
Unless otherwise specified, all reagents are of analytical grade and the water is demineralized.

3. 1. Acetonitrile (Merck, Art. 1.14291)
3. 2. Methanol (Merck, Art. 1.06009)
3. 3. Trichloroacetic acid (TCA) (Prolabo, Art. 20742-293): dissolve 50g in 50ml ultra pure water to obtain a 1g/ml solution.

3. 4. Potasium phosphate (0.2M) (KH_2PO_4)

3. 5. Hdrochloric acid (0.1M)

3. 6. Mobile phase: Distilled water (Ph=2.1 with H_2SO_4): Acetonitrile, 85:15 (v/v)

3. 5. Standards

3. 5. 1. Oxytetracycline hydrochloride, potency (Pfizer).

3. 6. Standard solutions

The analytical standards were dissolved in methanol at a concentration of 0.1 mg/ml. The working standard solutions were made by dilution with water containing 0.1 % of formic acid and 25% of methanol.

4. Apparatus

4. 1. 1. Polypropylene centrifuge tubes, 50ml capacity, with caps.

4. 1. 2. Glass tubes, 30ml capacity

4. 1. 3. Polypropylene tubes, 5ml capacity

4. 1. 4. Amber volumetric flasks, 25ml, 50ml, 100ml, 200ml and 1000mlk.

4. 1. 5. Graduated glass pipettes, 2ml, 5ml, 20ml and 25ml.

4. 1. 6. Automatic pipettes type Gilson P1000.

4. 1. 7. Blender type moulinette (Moulines)

4. 1. 8. Analytic and precision balance model PB302 (Mettler-Toledo)

4. 1. 9. High precision analytic balance type A 120S (Sartorius).

4.1.10. Solvent dispensers (Brandt)

4. 1. 11. pH-meter (Tacussel)

4. 1. 12. Electric stirrer type vortex (Bioblock)

4. 1. 13. Rotary stirrer type Rheax 2 (Heidolph). Vortex mixer was used instead.

4. 1. 14. Magnetic stirrer type Nuova II (Bioblock).

4. 1. 15. Cooled centrifuge model GR 4.22 (Jouan).

4. 1.1 6. Solid phase extraction cartridges Bond-Elut C18, 3cc, 200mg (Varian).

4. 1. 17. Solid phase extraction manifold (Supelco), adaptors, needles (Analytichem). Syringe was used in this study for the sample to pass-through the cartridge

4. 1. 18. Vacuum pump, 0.4 bar, 12w (Bioblock)

4. 1. 19. Whatman disposable filter funnels, 25mm diameter (Whatman, Art. 1922-1800) or 50 ml reservoirs containing these same filters.

4. 1. 20. Refrigerated ultra- speed centrifuge mode MR 1822 (Jouan)

4. 2. High performance Liquid Chromatography equipment

4. 2. 1. Series 1200 quaternary gradient pump (Hewlett Packard)

4. 2. 2. Series1200 UV-VIS detector (Hewlett packard)

4. 2. 3. Vectra 486/66VL computer (Hewlett Packard) and HPLC 2D Chemstation software.

4. 2. 4. Series 1100 auto sampler (Hewlett packed).

4. 2. 5. Analytical column Nucleosile C18, 250*4mm, 5mm

5. Storage of samples and sampling: Sample must be stored at about -20 ^{0}C.

6. Procedure

6. 1. Extraction

6. 1.1. Buffer 2 ml of milk with 2.5 ml of 0.2 M phosphate buffer (pH 5)

6. 1. 2. After 5min shaking by vortex for homogenizing,

6. 2. Proteins precipitation

6. 2. 1. Transfer the supernant in a glass tube. Place this tube in a breaker on the magnetic stirrer (4.1.14).

6. 2. 2. Add 1 ml of 20% trichloroacetic acid solution (3.3) with constant stirring. Then stir more rapidly for further 1min. remove the magnetic stirrer.

6. 2. 3. Centrifuge the sample at 3500 rpm for 10 minutes after placed in centrifuge tube

6. 3. Clean up

6. 1. 3. Activate the SPE-C18 columns by flushing with 2ml water: methanol 99:1 (v: v)

6. 1. 4. Add 2ml of milk in to the cartridge

6. 1. 5. Dry the cartridge with dry vacuum pump

6. 1.5. Wash the cartridge with a mixture containing water:methanol 70:30 v:v (2ml).

6. 1. 6. Elute oxytetracycline with 2ml of methanol: 0.1M hydrochloric acid (2:1 v/v) in to an autosampler vial.

6. 1. 7. Store the vials with the sample extracts at -20 ^{o}C until HPLC examination.

6. 4. Chromatographic conditions

Liquid chromatography was carried out using a 1200 series high performance liquid chromatography (Agilent Technologies, Germany) with an electron diode array detector equipped with an AS3000 autosampler. The separation of the analyte was performed using ZORBAX Ecllpse XDB-C_{18} anlytical column (4.6 x 150 mm, 5 µm). The chromatography was performed at 24 ^{0}C.

6 .4. 1. The mobile phase: distilled water (pH=2.1 with H_2SO_4): Acetonitrile, 85:15 (v/v)

6. 4. 2. Flow rate: 1.5 mi/min

6. 4. 3. UV detector wavelength: 360nm

6. 4. 3. Injection volume: 20 µl

6. 4. 4. Retention time: 2.9 min

6. 4. 5. Recovery: 68%

6. 4. 6. limit of detection: 0.10µg/l

7. Calculation of results

The following calculations can be executed directly by the HPLC 2D Chemstation software.

7. 1. Derive the calibration curve from the results obtained with the working standards solutions. Quantitation was made through external standards. Calibration curve s were built by injecting mixture of working solutions at the following concentration: 0.5, 1.5, 2.25, 3.0, 6.0 mg/l of OTC eluent. Peaks corresponding to the oxytetracycline standard have to be taken into account. Then, determine the curve equation:

Y= ax + b

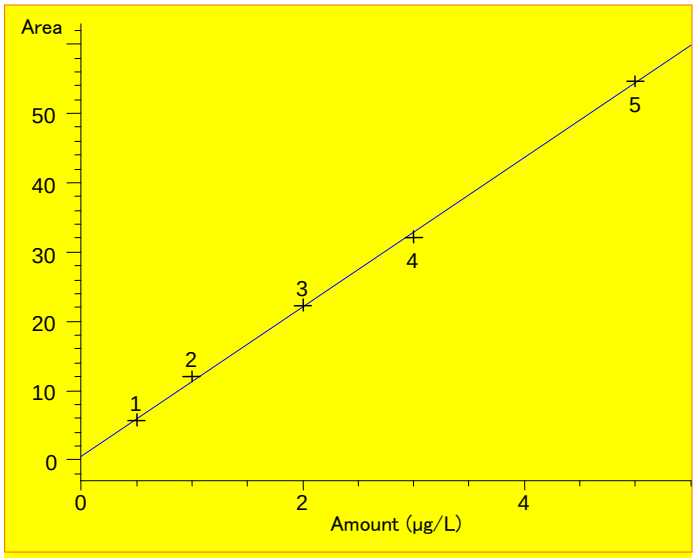
Y= peak area (TC + epimer)

X = concentration (µg/ml)

A = slope

B = intercept

7. 2. Calibration curve



$y = 10.8166724\ X$

$R^2 = 0.99963$

Annex 5: Result of univariable logistic regression analysisi of risk factors for occurrence of antibiotic residues in dairy farms

A. Management factors

Variables	Coefficient	P value	OR	(95%) CI	
				Lower limit	Upper limit
Part time help	-1.821	0.019	0.343	0.141	0.836
Teat dips	0	0	0	0	0
Training	1.800	0.021	3.266	1.198	8.903

B. Prevention methods

Variables	Coefficient	P value	OR	(95%) CI	
				Lower limit	Upper limit
Marking	-1.783	0.038	0.410	0.177	0.952
Separate equipment	-0.971	0.022	0.368	0.156	0.864
Record keeping	-0.517	0.600	0.758	0.269	2.134
Withholding milk from all quarters	0.052	0.197	1.758	0.746	4.142
Antibiotic residue test kit	0	0	0	0	0
knowledge	-0.773	0.832	0.914	0.397	2.103

C. Treatment factors

Variables	Coefficient	P value	OR	(95%) CI	
				Lower limit	Upper limit
Mastitis	0.920	0.033	2.597	1.081	6.238
Metritis	0.259	0.276	1.852	0.612	5.607
Enteritis	-1.252	0.748	0.813	0.230	2.878
Other diseases	0.504	1.319	0.468	3.716	7.453
Ooxytetracycline	1.018	0.023	2.664	1.146	6.198
Penistripe	0.631	0.197	1.758	0.746	4.142
Multiject	-0.760	1.155	0.403	3.317	8.092
Other drugs	-0.197	0.797	0.876	0.319	2.406
Intramuscular	0.778	0.086	2.132	0.898	5.065
Intramammmary	0.628	0.013	2.910	1.248	6.786
Perous	-0.187	0.197	1.758	0.746	4.142
Intrauterine	-0.830	0.810	1.123	0.437	2.888
Person to give treatment		0.065	0.536	0.276	1.039
Dry cow therapy	2.383	0.422	1.548	0.533	1.308

Annex 6: Questionnaire format

Date of sample collection _____

Dairy farm owner's name_____

Address_____

Sample code_____

Management factors

1. Use of medicated feed (feed additives) in your dairy farms. Yes ☐ No ☐
2. Participation in any training about dairy farm management. Yes ☐ No ☐
3. Do you use part-time help for milking of cows? Yes ☐ No ☐
4. Do you use post milking teat dips? Yes ☐ No ☐
5. Do use branding of milking equipment? Yes ☐ No ☐
6. Herd size of your dairy farm_____
7. Number of dairy cows in your herd_____

Treatment factors

8. Most commonly encountered diseases in your dairy farm?

☐ Mastitis
☐ Metritis
☐ Enteritis
☐ Others

9. What type of antibiotics do you use in your dairy farm? _____

_____

_____

10. Commonly used rout of drug administration

☐ Intrammamary
☐ Intramuscular
☐ Oral
☐ Intrauterine

11. What type of person administers the treatment for cows?

☐ Veterinary

☐ Assistant

☐ Dairy farmer

2. Do you use dry cow intrammary antibiotic treatment? Yes ☐ No ☐

Prevention methods

13. Do you mark the treated cows? Yes ☐ No ☐

14. Do you use separate equipment for milking of treated cows? Yes ☐ No ☐

15. Do you use treatment record keeping? Yes ☐ What type of records?

_____

_____

_____

No ☐

16. Do you use antibiotic test kit? Yes ☐ No ☐

17. Knowledge about antibiotic residue or withdrawal period of antibiotics in milk

Yes ☐ No ☐

18. The importance of antibiotic residues in milk?

-Public health significance ☐

-Interfere with the manufacture of cultured milk products ☐

-Has effect on public opinion ☐

9. CURRICULEM VITAE

Personal data:

Name	Desalegne Abebew Syit
Date of birth	December 08, 1980
Place of birth	South Gondar, Debretabour
Sex	Male
Marital status	Unmarried
Religion	Orthodox Tewahido
Nationality	Ethiopian
Language proficiency	Amharic and English

Education/ qualifications:

1987/88-1992/93	Primary Education in Hirui and Abaregai elementary School, South Gondar.
1993/94-1994/95	Secondary Education in Gafat Junior Secondary School South Gondar.
1995/96-1998/99	Secondary Education in Theodros Senior Secondary high School, South Gondar.
1999/2000	Freshman courses in Addis Ababa University, Arat killo Campus.
2000/01-2004/05	Doctorate degree in Veterinary Medicine, in Addis Ababa University, Faculty of Veterinary Medicine
2005	Diploma in Basic Computer Application Software Courses.
2006/2007-2008	Master of Science in Tropical Veterinary Public Health, Addis Ababa University, Faculty of Veterinary Medicine

Research Paper:

- The status of parasitism of donkeys found in intervention and non- intervention areas: a comparative study. DVM Thesis (2005) FVM, AAU, (Unpublished).
  Other papers

- Repeat breeder cow and its economic impact. Seminar paper (2004).

Antibacterial residue in milk and its impact on public health. Seminar paper (2007).

Work experience:

- Head of Animal and Fish development Office and Animal Doctor in Benishangule Gumuze Regional state for one year.

Additional training and certificate:

- Computer literacy: basic computer application software courses (2005), Diploma
- Participatory epidemiology in Veterinary Medicine.

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10. SIGNED DECALRATION SHEET

This thesis is my original work and has not been prepared for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Desalegne Abebew

Signature_____

Date of submission = 25/06/2008

This paper has been submitted for the examination with my approval as advisor.

Academic Advisors: Signature

Dr. Mosses Kyule (BVM, MSc, MVPM, PhD)_____

Dr. Kalay Belihu (DVM, PhD)_____

Dr. Girma Zewde (DVM, PhD) _____

Dr. Tadelle Dessie (BSc., PhD)_____