DETERMINATION OF ANTIMICROBIAL RESIDUES AND THE EFFECT OF HEAT TREATMENT ON RESIDUAL CONCENTRATION OF SOME ANTIMICROBIAL DRUGS IN FRESH COW MILK IN ZARIA, NIGERIA

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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

DECEMBER, 2015

DECLARATION

I declare that the work in this dissertation entitled "**Determination of antimicrobial residues and the effect of heat treatment on residual concentration of some antimicrobial drugs in fresh cow milk in Zaria, Nigeria**" has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, under the supervision of Prof. Junaidu Kabir and Dr. A. B. Suleiman. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____ _____

Zeenatuddeen Muhammad Date

CERTIFICATION

The project dissertationentitled**DETERMINATION OF ANTIMICROBIAL RESIDUES AND THE EFFECT OF HEAT TREATMENT ON RESIDUAL CONCENTRATION OF SOME ANTIMICROBIAL DRUGS IN FRESH COW MILK IN ZARIA, NIGERIA by ZEENATUDDEEN MUHAMMAD**meets the regulations governing the award of Master of Science (Veterinary Public Health) of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to my daughter, Naana Hauwa for her pains and sacrifice.

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ABSTRACT

Veterinary drug residues are a global health concern in many countries, with developing countries reporting high occurrence of residue contamination of edible animal products beyond FAO/WHO permissible levels.The occurrence of antimicrobial drug residues in milk in Zaria and environ, and the effect of heat treatment on residues of some antimicrobials was investigated. Three hundred fresh milk samples were collected from local Fulani milk producing herds in 11 villages and areas within Zaria and environ. Each milk sample, was screened for the presence of antimicrobial drugs using Delvotest SP-NT®. Antimicrobial residue-free milk was spiked with 0.01g/ml, 0.02g/ml, 0.25g/ml and 0.4g/ml of laboratory grade Oxytetracycline hydrochloride (OTC), Streptomycin sulphate (STREP) , Sulfamethazine (SMZ) and Sodium penicillin G (PEN G) respectively. The drug bearing milk was subjected to pasteurization (63^{o}C for 30mins) and ultra high temperature treatment (121^{o}C for 15 mins) to determine the effect of the heat treatment on antimicrobial activity of the various drug concentrations. Forty four(14.7%) of the milk samples tested were positive for residues, most of theresidues occurred in milk from Palladan and Graceland with 36.8% and 29.7% of the samples collected from these areas having residues while no residues were detected in Gabari, Hanwa and Unguwan Rimi. Residue occurrence was significantly ($P<0.05$) associated with location and recent history of antimicrobial use (within 4 weeks). Antimicrobial activity persisted up to the 10^{-7} dilution for OTC, STREP and PEN G while no activity was detected at dilution of 10^{-7} for SMZ in milk subjected to pasteurization. For the sterilised spiked milk, there was a reduction in antimicrobial residue of OTC at 10^{-6} and 10^{-7} dilutions while antimicrobial residue persisted up to 10^{-7} dilution for SMZ, STREP and PEN G. Consumers are predisposed to health hazards due to the presence of residues as a result of non observance of withdrawal period and gross misuse of antimicrobial residues. This emphasises the need for effective prevention of infectious diseases, use of

alternatives to antibiotics, strict observation of antibiotic cessation times, creation of more awareness and establishment of a national antibiotic residue monitoring program in Nigeria.

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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
CAC	Codex Alimentarius Commission
DNA	Deoxyribonucleic acid
DSM	De Nederlandse Staatmijnen translated into Dutch State Mines
EFSA	European Food Safety Authority
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FSSA	Family and Social Services Administration
IDF	International Dairy Federation
LME	Liquid Milk Equivalent
MRL	Maximum Residue Limit
NAFDAC	National Agency for Food and Drug Administration and Control
NT	Not Tested
OD	Optical Density
OIE	Office International des Epizootics
OTC	Oxytetracycline
PEN G	Penicillin G
ppb	Parts Per Billion
ppm	Parts Per Million
SCC	Somatic Cell Count
SMZ	Sulfamethazine

STREP	Streptomycin
TDL	Test Detection Limit
USA	United States of America
WHO	World Health Organisation

CHAPTER ONE

1.0INTRODUCTION

1.1 Background Information

Antimicrobial drugs have been widely used for more than 50 years to improve both human and animal health since theantibiotic golden age to date (Flynn, 2012). The discovery of the antibiotic and antibacterial agents since 1940's, revolutionizedthe treatment of infectious bacterial diseases that used to kill millions of people during the pre-antibiotic golden age worldwide (Clardy *et al.,*2009; MSU, 2011). Antibiotics are chemical substances naturally produced by various species of microorganisms such asbacteria and fungi like Actinomycetes and Streptomyces that kill or inhibit the growth of other microorganisms. Amongthe major sources of antibiotics include *Streptomyces*,*Penicilliums*, *Actinomycetes*and *Bacillus*(Godfrey and Norah 2013; MSU, 2011). It is estimated thatabout 100,000 tons of antibiotics are produced globally (Godfrey and Norah, 2013; MSU, 2011). About 80% of the antibiotic agentsproduced are used as feed additives in livestock production as growth enhancers and for prophylactic use due to thesuppressed immune system caused by the overcrowding of the animals leading to stress (EFSA, 2011; FDA, 2010). They are also used inagriculture especially horticulture and tissue cultures (Kumar *et al*., 2005), in the food industry as food preservatives (FSSA, 2013) and incommercial ethanol production (Arshad *et al*., 2011; Deutscher, 2009). In all these activities, the antibiotics eventually enter the environmentand cause deleterious effects to the microbiota (FDA, 2010).

Antibiotics belong to a group of drugs which are used in the prevention and treatment of bacterial infections in both humans and animals (Adewuyi *et al*.,2011). They are also administered to animals at higher doses for the purpose of growth promotion (Kabir *et al*., 2004).

However, widespread use could lead to the presence of persistent residues in edible parts such as muscle (meat), organs, egg, honey and milk obtained from treated animals (Bergwerff and Schloesser, 2003; Ibrahim*et al.*, 2009). These residues may include the non-altered parent residue as well as metabolites and/or conjugates (Peters *et al*., 2009). The presence of these residues is usually attributed to non-observance of withdrawal periods before the sales of edible animal food product, and also due to undesireable practices such as unregulated and indiscriminate use of drugs and lack of awareness on the proper usage of these antibiotics (Kabir *et al*., 2004; Shitandi, 2004).

Antibiotic residues in milk are of great concern to dairy farmers, milk processors, consumers and regulatory agencies. To ensure food safety for consumers, several regulatory authorities around the world including National Agency for Food and Drug Administration and Control (NAFDAC), European Food Safety Agency (EFSA), Food and Drug Administration (FDA), USA Codex Alimentarius,established tolerance (safe) levels of antibiotic residues in milk for consumer protection (Adewuyi *et al*., 2011). Antibiotic residues in milk exceeding tolerance levels not only present potential health risks to the consumer but also interfere with fermentation processes during milk processing such as cheese and yoghurt manufacturing (Adewuyi *et al*., 2011).

Milk and milk products are important sources of animal protein which contribute greatly to human growthand development (Dikko*et al.*, 2010). Milk and milk products are also important products of the Fulaniagro-pastoral and other small holder peri-urban systems of Nigeria (Oladunjoye*et al.*, 2001). In these systems, milk is sold out fresh or processed into other dairy productssuch as butter, cheese, yoghurt and ice cream and sold in the markets (Tona and Olusola, 2014).Milk, since ancient time, is known as an excellent source of protein rich in organoleptic

and nutritional supplement essential for human health (Mishra *et al*., 2011). Production of safe and quality milk for ensuring better human health is essential.

To prevent antibiotics from entering the milk supply, each tanker of milk is tested prior to acceptance at the processing plant. Milk that is contaminated with antibiotics must be discarded (FDA, 2010). In the last several years, the number of tests available to detect penicillin and other common antibiotics has multiplied. Both qualitative and quantitative tests exist; some are applicable for dairy farmer use to prevent antibiotics from entering the milk supply at the source (O'Donnell, 2011). Screening tests are needed to provide high sensitivity and specificity for testing antibiotic residues in milk on the farm, in processing plants and in regulatory laboratories.

1.2Statement of Research Problem

Antibiotics are substances either produced naturally by living organisms or syntheticallyin the laboratory, and they are able to kill or inhibit the growth of microorganisms (Godfrey and Norah, 2013).Antibiotics are also used as feed additives for the purpose of livestock health maintenance.Antibiotic residues in feedstuffs are currently a problem of some magnitude in different parts of the world(Omeiza*et al.,* 2012), particularly due to associated public health concerns that include hypersensitivity reactions, antibiotic resistance, toxicity, teratogenicity, and carcinogenicity (Pamela, 2005; Nisha, 2008;Darwish *et al*., 2013).A significant proportion of humans are atrisk. For example, about 3.4% of the human population issensitive to sulfonamides (Bigby *et al*., 1986).

In Africa, as in other parts of the world, antibiotic residues in animal-derived foods have been extensively recorded, in many African countries; these residues have exceeded the WHO maximum residue levels in many cases (Darwish *et al*., 2013).In Tanzania, antimicrobial

residues weredetected in 36% of marketed milk samples frommilk supply chains in and around Mwanza andDar es Salaam during 1999 and 2000(Kurwijila *et al*., 2006). In Ghana, a report showed that 35% ofthe raw milk marketed in two major cities,Accra and Kumasi, were contaminated withantibiotics (Aning *et al*., 2007).Tetracycline antibiotic residue was observedto be present in milk products sold out for human consumption in Ibadan, Nigeria (Adetunji, 2008). The use of antibiotics may result indrug residues being present in the milk. Residues present in milk could interfere with starter cultures forcheese, yoghurt and other dairy products. This could indicate thatthe milk may have been obtained from an animal with aserious infection (Phillips *et al*., 2004; Adetunji, 2011). There are also concernsthat the usage of antibiotics may be responsible for thepromotion of resistant strains of bacteria (Brady and Katz, 1992; Torrence, 2001; Adetunji, 2011) resulting in untreatable infectionsin both animals and humans.

The presence of drug residues in milk supplies andproducts is of public health implications and areperceived by consumers as undesirable (Adetunji, 2011; Adewuyi *et al*., 2011). Today, it is the generalconsensus that even slight traces of antibiotics in milkand food for human consumption should not betolerated (Jepsen, 1990).

The presence of residues ofthese antibiotics in some milk products at a levelexceeding the Maximum Residue Levels(MRLs) of WHO/FAO (Joint Food andAgricultural Organization) Expert Committee on FoodAdditives Standards (1999) is probably due toinjudicious use of antibiotics in the treatment ofinfections in cattle and lack of adherence to withdrawalperiod before milking(Kabir*et al*., 2004; Shitandi, 2004). Dina and Arowolo (1991) reportedthe widespread misuse of veterinary drugs in Nigeriadue to inadequate monitoring and prescription byuntrained personnel.

Antibiotic residues have also been reported in products that had undergone heat processing likecheese, evaporated milk and yoghurt. Similar reports by O'Keefe and Kennedy (2008), reported very little or no effect on antibiotic residues after processing.

1.3Justification

Residues of antimicrobial drugs in milk may causeproblems in the milk processing industry, inthe preparation of cheese, yoghurt, and other dairy products (Adewuyi *et al*., 2011). Moreover, the presence of residues in milk may have serious consequences for public health; antibioticresidues in milk may provoke antibiotic resistance or allergies in consumers (Pamela, 2005;Fagbamila*et al.,* 2010; Adetunji, 2011; Omeiza*et al.,* 2012).

In Nigeria, specific legislation regarding drug use and veterinary drug residue control is lacking; basic facilities for determination of residues are also unavailable at the level of the abattoirs, farms and markets (Kabir *et al*., 2004). Similarly, there are no specific residue monitoring programs (Fagbamila*et al.,* 2010), however, there are scanty reports on veterinary drug residues and on the extent of the residue problem in Nigeria (Yaqub *et al*., 2009; Kabir *et al*., 2004; Fagbamila *et al*., 2010; Ibrahim *et al*., 2009).

Nowadays, quality controls are essential in milk to establish the price that the producerreceives, as well as to assure the quality of the product. In control laboratories, it is a common practice to carryout treatments by heating the sample for its homogenisation,inactivation of pathogenic organisms or for inactivation of the natural antimicrobials inthe case of analysis of inhibitors (Berruga *et al*., 2006).On the other hand, milk arrives at the consumer after undergoing different heat treatments in the industry, suchas pasteurisation and sterilisation. The studies referring to

the influence of these treatments on antimicrobialresidues in the dairy industry are very few, and centred on a very limited number of antibiotics (Berruga *et al*., 2006).

1.4Aim and Objectives

1.4.1 Aim:

To determine the occurrence of antimicrobial residues in milk and the effect of heat treatment on residual concentration of some antimicrobial drugs in fresh cow milk in Zaria.

1.4.2 Objectives:

The specific objectives of the study are to:

1. determine the occurrence of antimicrobial residues in fresh cow milk in Zaria and environ usingthe Delvotest SP-NT test.
2. determinethe effect of age, parity, stage of lactation, and antimicrobial use on the occurrence of antimicrobial residues in milk in Zaria.
3. determine the effect of pasteurization and high temperature treatment on residual concentrationof some commonly used antimicrobial drugs in fresh cow milk in Zaria.

1.4.3 Research Hypothesis

1. Fresh cow milk in Zaria and environ are not contaminated with antimicrobial residues
2. Antimicrobial residues in Zaria and environ are not affected by age, parity, stage of lactation and antimicrobial use
3. Antimicrobial residues in fresh cow milk in Zaria and environ are not affected by pasteurisation and high temperature treatment

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Antimicrobial drugs

The term antimicrobial agent has been defined as ‘anaturally occurring, semi-synthetic or synthetic substancethat exhibits antimicrobial activity (kills or inhibits the growth of microorganisms) at concentrations attainable *invivo*’ (OIE, 2011), and includes agents active against bacteria,protozoa, viruses and fungi. Antimicrobial drugs have been widely used for more than 50 years to improve both human and animal health since theantibiotic golden age up to date (Flynn, 2012). The discovery of the antibiotic and antibacterial agents since 1940’s, revolutionizedthe treatment of infectious bacterial diseases that used to kill millions of people during the pre-antibiotic golden ageworldwide (Clardy *et al*., 2009). Antibiotics are chemical substances naturally produced by various species of microorganisms such asbacteria and fungi like actinomycetes and streptomyces that kill or inhibit the growth of other microorganisms. Amongthe major sources of antibiotics include *Streptomyces*, *Penicilliums*, *Actinomycetes* and *Bacilli* (Godfrey and Norah, 2013). It is estimated thatabout 100,000 tons of antibiotics are produced globally (Clardy *et al*., 2009; Godfrey and Norah, 2013).The use of antibiotics in animals shortlyfollowed their use in humans for the purpose ofdisease prevention and treatment (Gustafson, 1993). Today,antimicrobial drugs are used to control, prevent,and treat infection and to enhance animal growthand feed efficiency (Tollefson and Miller, 2000). Currently, approximately80% of all food-producing animals receivemedication for part or most of their lives.

The most commonly usedcategory of antimicrobial agent and the one currently ofgreatest public health interest is the antibacterial class and this overview will focus exclusively on the antibacterialsubset of antimicrobial agents and will use the termantimicrobial in this narrower context.

2.1.1Veterinary antimicrobials

Antimicrobials are the main group of veterinary medicinal productsused since the 1950s to treat bacterial infectious diseases in both foodproducingand companion animals. The substances used belong to thesame families as those used in human medicine (Sanders *et al*., 2011)). These medicinal products are administered to prevent and treat infectious diseases thatcould cause significant morbidity and possible mortality. The mostcommonly treated disorders are digestive and respiratory (Mensah *et al*., 2014). Forseveral types of integrated farm systems where animals (poultry, pigs,calves and fish) are raised in groups indoors, production conditionsprompt veterinarians to prescribe treatments for both preventiveand curative purposes. For other production systems, treatments areindividual and mostly curative (Mensah *et al*., 2014).

The three types of veterinary treatment (Sanders *et al*., 2011) are: preventive treatment (prophylaxis), administered at a time in the animal's life when the riskof bacterial infection is considered to be very high; curative treatmentadministered to sick animals; and control treatment (metaphylaxis)prescribed for groups of animals in contact with sick animals (Labro, 2012; Mensah, 2014).

Authorised veterinary antimicrobials: In the European Union (EU), the antimicrobials approved for use as veterinary drugsfigure in the list of substances in Annex 1 of Commission

Regulation(EU) 37/2010 (EC, 2010). The medicinal products containing theseantimicrobials authorised for veterinary use are those that have passed the marketing authorisation process of the competent national orEuropean authority. After an evaluation of the scientific data provingthe efficacy of the product and its safety for humans, animals and theenvironment, the Competent Authority authorises its importation, distribution and use (Mensah, 2014)).The statutory marketing authorisation mechanism is virtually identicalacross most African countries. No medicinal product may be marketedunless it has first been authorised by the Competent Authority (Mensah, 2014).

However, there are huge shortcomings in the implementation becausethe technical evaluation of a marketing application is limited to an administrative procedure alone. These countries have no effectivescientific control tools to ensure the validity of the data provided bythe applicant. In addition, the procedures do not cover all veterinaryspecialities marketed in these countries (Mensah, 2014)). In the West African Economic and Monetary Union(WAEMU) region,the accreditation system for veterinary medicinal products wasintroduced in 2006 but only came into operation in 2010 (Mensah, 2014).

Prohibited antimicrobials are substances for which it is not possible to determine the Maximum Residue Limit (MRL). These are listed in Annex 2 of EuropeanCommission Regulation 37/2010 (EC, 2010; Mensah, 2014). It should also be borne in mindthat antimicrobial substances not listed in Annex 1 of this regulationmust not be used in food-producing animals.Chloramphenicol is a broad-spectrum antimicrobial against Gram-positiveand Gram-negative bacteria. While it is an effectivetherapeutic for a wide range of animal diseases, historicepidemiological data have

shown that its use in humans may beassociated with haematological disorders; in particular, aplasticanaemia. During its assessment, it was not possible to determine anMRL based on the available data. The inability to set a threshold valueand shortcomings in the marketing authorisation application led tochloramphenicol being classified in 1994 as a prohibited substance foruse in food-producing animals in the European Community (EC, 2010; Mensah, 2014).

Nitrofurans have been banned from use as a veterinary medicinalproduct and as additives in the EU since 1998 (EC, 2010; Mensah, 2014).Dapsone, which is used to treat leprosy in humans, is not authorisedfor use in food-producing animals in Europe because of insufficienttoxicology data, making it impossible to determine the acceptabledaily intake (ADI)(EC, 2010; Mensah, 2014).

2.2 Uses of Antimicrobial Drugs

2.2.1 Antibiotics/antibacterial drugs use in humans

In humans, antibiotics/antibacterial drugs are commonly prescribed, sold and used to treat bacterial diseases worldwide in irrational manner (Abimbola, 2013). The problem has overwhelmingly increased due to financial and budgetary constraints, marketinefficiencies, distortion and behavior of health systems, health workers and pharmaceutical companies (WHO, 2011). Thecomplementary medicine specialists or traditional herbalist and quacks also use allopathic drugs like antibiotics to treatall types of infections, with lack of expertise and knowledge on their use (WHO, 2011). It is reported that about 1/3 to 2/3 ofprimary health care patients receive twice the antibiotics than clinically needed contributing to irrational antibiotic

use (WHO, 2006). Complex socioeconomic and behavioral factors have been associated with antibiotic misuse among health careprofessionals, unskilled practitioners and public (Thriemer *et al*., 2013). Poor drug quality and inadequate surveillance by the regulatoryauthorities especially in poor developing countries greatly contributes to antibiotic misuse (Amin, 2011).

Access to affordable health care is limited in many low andmiddle income countries hence many people rely mainly on self-medication and purchasing of antibiotics directly frompharmacies, street vendors or markets (Godfrey and Norah, 2013). Self-medication involves the ‘‘use of medicinal products by the consumer totreat self recognized disorders or symptoms or the intermittent or continued use of the medication prescribed by thephysicians for a chronic or recurring diseases or symptoms’’ (WHO, 2000). Self medication with antibiotics or the use ofnon-prescribed antibiotics including leftover antibiotics is common in both developed and developing countries, inwhich the point prevalence ranges from 3% to 75% thus increasing irrational antibiotic use globally (Godfrey and Norah, 2013).

2.2.2. Antibiotic/antibacterial drugs use in treatment of food producing animals

Antibiotics/antibacterial drugs have been widely used globally in animals for more than 50 years, with tremendousbenefits in animal production and economic development (Flynn, 2012). Several antibiotic/antibacterial drugs are used in thetreatment of many bacterial diseases of animals’ especially in food-producing animals globally. Many of these agentsare at the same time used in bacterial infections in humans(FDA, 2010). However, many of these drugs are abusedby veterinarians as healthcare professionals and the general public where many farmers treat their sick animals withantibiotics/antibacterial drugs without seeking professional consultation. The

problem is worse in developing poorcountries that have privatized veterinary services making the cost of treatments to be very expensive for the farmers (Godfrey and Norah, 2013).

Many farmers access these agents and treat their animals even in cases where use of antibiotic/antibacterial agentswould be unnecessary. As a result of this, massive quantities of antibiotics/antibacterial drugs used, are released in theenvironment thus increasing selection of the antibiotic resistant bacterial organisms that can spread from the animals tohumans especially the bacterial zoonoses, increasing the cost of treatments in both animals and humans (Martinez, 2009). Theproblem is likely to increase globally leading to severe future consequences similar to the pre-antibiotic golden agesituation (Godfrey and Norah, 2013).

2.2.3 Antibiotics/antibacterial drugs use in the animal feed industry

Feed industries and farmers have been adding antibiotics to livestock feed since 1946, after it was realized that use ofthese antibiotics caused animals to grow faster and put on weight in a short time (Chee-Sanford*et al.,* 2001). It is reported that more than80% of all antibiotics produced globally are used in animals and some are used in aquaculture, prophylactically tocontrol bacterial diseases of fish and other aquatic animals (WHO, 2011). The antibiotics/antibacterial drugs cause a changein physiological, nutritional and metabolic processes of the animals.

Many bacterial organisms from humans, animals and the environment commonly contaminate foods and food productsin food industries and other perishable foods especially during processing and transportation to the markets in un-sterile conditions (Godfrey and Norah, 2013). The bacteria attack and utilize nutrients needed by humans in the foods and also cause spoilage of foodsleading to economic and financial loss. The bacterial contamination also increases the

cost of de-contamination of industrial facilities (Bischoff *et al*., 2009; Lushia and Heist, 2005). As a result, many antibiotic/antibacterial drugs are used irrationally in most cases to controlbacterial contaminants in food industries.

2.2.4 Antibiotic drugs use in ethanol production

During ethanol production especially in commercial brewery plants, yeast is used to convert starch to ethanol (Bischoff *et al*., 2009; Lushia and Heist, 2005; Skinner and Leathers, 2004).However ethanol fermentation tanks occasionally become contaminated with bacteria especially "lactic acidbacteria"(Bischoff *et al*., 2009; Lushia and Heist, 2005; Skinner and Leathers, 2004). Among the lactic acid bacteria species that have been reported to contaminate fuel ethanolfermentations, include both Gram-positive and Gram-negative bacteria species such as *Lactobacillus, Pediococcus,Enterococcus Acetobacter*, *Gluconobacter*, *Leuconostoc*, *Weissella* and *Clostridium*, with the *Lactobacillus* species themost predominant contaminant (Bischoff *et al*., 2009; Lushia and Heist, 2005; Skinner and Leathers, 2004). The *Lactobacillus* species have the capacity to grow rapidly, tolerate ethanoldisinfection and the low pH allows these bacterial organisms to effectively compete favorably with the yeast (Bischoff *et al*., 2009; Skinner and Leathers, 2004).

The increased and prolonged bacterial contamination drains the available sugars that can be converted to ethanol. Thebacterial organisms convert the sugars to lactic or acetic acid that lowers the quality and quantity of the ethanolproduction (Bischoff *et al*., 2009; Skinner and Leathers, 2004). Also the bacteria scavenge essential micronutrients required for optimal yeast growth andefficiency in ethanol production. The most commonly used antibiotics/antibacterial

agents to selectively kill bacterialcontaminants and spare yeast in ethanol production include penicillin, virginiamycin, erythromycin, tylosin and tetracycline (Biscoff *et al*., 2009). The massive use of the antibiotics/antibacterial agents in commercial ethanol productioncontributes to destruction of the useful bacteria (normal flora) in the hosts and in the environment as well as increasedselection of antibiotic resistant pathogenic bacteria that can spread globally in the environment to human and animals aswell as lowering the effectiveness of the antibiotics (Godfrey and Norah, 2013).

2.2.5 Antibiotics as food preservatives

Various antibiotics/antibacterial drugs have long since 1940's been used in the preservation of perishable foodstuffsand food products. Among the commonly used antibiotics include chlortetracycline, oxytetracycline, chloramphenicol,penicillic acid, penicillin and streptomycin. Also these agents are used in preservation of fish (Davidson and Harrison, 2002). The tetracyclines arecommonly used in food industries to prevent growth of harmful bacteria in poultry product, fish, canned foods, cheese,meat, sausages and other non-sterile animal products (Mattia *et al*., 2013). Natamycin (pimaricin) is a polyene macrolide antibioticproduced by submerged aerobic fermentation of *Streptomyces natalensis* and related species (Mattia *et al*., 2013). It is used in the foodindustry as a "natural" preservative for dairy product like cheese and meat, wine, soft drinks like juice, convenient food and baking food (Davidson and Harrison, 2002; Mattia *et al*., 2013).

In developing and poor countries, some local milk coolants commonly useantibiotics to prevent milk spoilage by bacteria especially *Lactobacillus* organisms thus predisposing consumers to

subtherapeuticlevels of these drugs that causes selection of antibiotic resistant bacterial pathogens in humans, animals and environment (Alexander *et al*., 2008).

2.2.6 Antibiotics/antibacterial drugs use in agriculture (horticulture)

Several antibiotics/antibacterial agents are commonly used in horticulture and agronomy to control bacterial diseases.Oxytetracycline and streptomycin have long been used in horticulture since 1950s. They are used in the control ofbacterial diseases in high-value fruits, vegetables, trees producing timber and ornamental plants (Bryden *et al*., 1994). In 1997, it wasreported that more than 30,800 pounds of streptomycin and 26,700 pounds of oxytetracycline were used to controlbacterial infections in fruit trees in the USA (Godfrey and Norah, 2013). Antibiotics are widely used prophylactically in plant tissue culture andbiological research to prevent bacterial contaminants that would attack the media rich in sugars (Bryden *et al*., 1994).

However, the useof these drugs conflicts with the normal principles of prophylactic use, ‘‘in that the ‘pathogen’ is unknown and is ofuncertain susceptibility and the period of use of these antibiotics is prolonged’’ (Falkiner, 1998). Oxytetracycline, is used intreatment of bacterial spot in peach pecan trees and sometimes it is combined with gentamicin . Streptomycin was thefirst antibiotics used in the treatment of plant diseases since 1955 and sometimes combined with oxytetracycline andoxolinic acid in the control of fire blight bacterial diseases in rosaceous plants like pears and apples (Bryden *et al*., 1994).Chloramphenicol is used to control bacterial leaf blight in rice (Bryden *et al*., 1994); polyoxins are used to control rice sheath blight andblack spot(Bryden *et al*., 1994); the polyene macrolides act as systemic fungicides(Bryden *et al*., 1994); cellocidin is used to control rice blight spot (Bryden *et al*., 1994); griseofulvin used in

apple blossom blight and *Fusarium wilt* of melon; cycloheximide is used in control ofdowny mildew on onions (Bryden *et al*., 1994) and blasticidin-S inhibits the growth of many Gram \]positive and Gram negativebacteria and has antiviral and antifungal activity (Bryden *et al*., 1994). However the use of many of these agents in agronomy andhorticulture globally has become a public health importance since these drugs are also used in humans and animals totreat bacterial diseases (Peeples, 2012). Their massive use promotes the selection and emergence of resistant bacterial strains inboth humans and animals that may be difficult to control.

2.3. Antimicrobial use in Dairy Animals

Drugs are administered to dairy cows for treatment of mastitis through intramammary or intravenous infusions and for disease therapy by intramuscular or intravenous injections, oral administration, feed supplementation, or reproductive infusions (Blood *et al.,* 1989). FDA surveys indicate that improper use of drugs in the control of mastitis is the major source of residues found in the milk supply.

The prevalence of antibiotic residue in milk product had been observed due toindiscriminate use of various antibiotics in the treatment of mastitis. Sundlof *et al*. (1995) observedthat pencillins, tetracyclines, sulphonamides and aminoglycosides were most frequently used in lactating animals, which led to occurrence of their residues in milk. Several workers havereported the presence of antibiotic in the milk after the treatment of mastitis cases (Sundlof *et al.,* 1995). Although the use ofthese drugs has significantly improved thehealth and production efficiency of food-producinganimals (Alomirah *et al*., 2007), incorrectuse, improper administration or mistakesby farmers and veterinarians in observing thewithdrawal time for treated animals can

resultin antimicrobial residues in milk and dairyproducts. The beef industry claims that a great percentage of the drug residues found in beef carcasses are in those of culled dairy cows (Jones, 2009).

The population of cattle in Nigeria was estimated to be about 13.9 million (Bourn *et al*., 1994) and believed largely to be owned by nomadic rearers (Schafer *et al*., 1997). The present situation where both trained and untrained persons are involved in offering animal health services and coupled with an increased uncontrolled variety of veterinary drugs marketed freely to herdsmen requires that the levels of these substances be monitored (Yaqub *et al.,* 2009). These drugs given to animals may be found in tissues and other edible products, particularly when the animals are slaughtered or milked without observing the withdrawal periods (Kabir *et al.,* 2004). Long acting agents such as Oxytetracycline, Streptomycin and Penicillin are particularly favoured for use (Yaqub *et al.,* 2009). Under field conditions, compliance with drug use guidelines, including observance of withdrawal periods is difficult (Kabir*et al.,* 2004).

The importance of monitoring antimicrobialresidues in milk is emphasized by the role ofmilk and dairy products in human nutrition(Ortelli *et al*., 2009). The European Union(EU) has established safe maximum residuelimits (MRLs) for pharmacologically activesubstances in foodstuffs of animal origin (EC, 2010)..

In dairy plants, it may be difficult to implement a control program to avoid residues of veterinary antimicrobials in milk due to the large number of different compounds and formulations commercially available (Serraino *et al*., 2013).

2.4 Antimicrobial Drug Residues

Residues are defined as all active ingredients or metabolites of thoseingredients that remain in meat or other foodstuffs from the animal towhich the medicinal product in question has been administered (EC, 1981).Regulation No. 470/2009 of the European Parliament and of theCouncil defines residues as all pharmacologically active substances,whether active ingredients, excipients or degradation products, andtheir metabolites, which remain in animal-derived food.

The concept of drug residues in food was developed over the secondhalf of the 20th Century, resulting in the definition of a 'no observedeffect' level, an acceptable daily intake (ADI) and an Maximum residue limit (MRL) in food (CAC, 2011).

The use of antibiotics in food-producinganimals may leave residues in foodstuffs ofanimal origin like meat, milk, and eggs. Theoccurrence of these residues may be due to among other situations, anyone of the following: a failure to observe thewithdrawal periods of each drug, extra-labeldosages for animals, contamination of animalfeed with the excreta of treated animals or medicated feed, bulk milk contamination or theuse of unlicensed antibiotics (Paige, 1994).

Antibiotic residues in foods of animalorigin may be the cause of numerous healthconcerns in humans. These problems includetoxic effects, transfer of antibiotic resistant bacteria to humans, immunopathologicaleffects, carcinogenicity (e.g., sulphamethazine,oxytetracycline, and furazolidone), mutagenicity,nephropathy (e.g., gentamicin), hepatotoxicity,reproductive disorders, bone marrow toxicity (e.g.,chloramphenicol), and allergy (e.g., penicillin) (Nisha, 2008).

2.4.1Occurrence of antibiotic residues in foods

Colins*et al.* (1988) reported that about 28% of commercial milk supply in the US indicated thepresence of antibiotic residues. Approximately 4.2 million milk samples were tested forantibiotic residues and 30.9 million kg of milk was dumped because of positive assay result. Lee*et al.* (1996) examined antibiotic residues in milk after intramammary injections, I/U injections and oral administration in dairy cows. Amoxicillin residues were detected in milk after intramuscularinjection. They concluded that type of antibiotic, dosage and route of administration influencesthe duration over which antibiotic residues could be detected in milk. They further concludedthat milk yield on the sampling day and lactation stage had no influence on the duration overwhich antibiotic residues could be detected in milk. Podhorniak *et al*. (1998) observed that no loss ofcloxacillin residues in milk was observed after 48 hrs storage at 4^0C or 24 hrs at 25^0C. Slightloss was noted after 72 hrs storage at 4^0C and 48 hrs storage at 25^0C.Lack of awareness of withdrawal times or deliberate abuse may lead to elevated levels ofdrug residues in the milk. The most common causes of occurrence of drug residues in milk areinsufficient identification of treated cows, insufficient knowledge about withdrawal periods andfailures due to hired staff.

Singh (2010) reported that raw milk samples collected from dairy farms atJabalpur, India were free from residue concentration of amoxicillin and cloxacillin. He also observed that animals treated with amoxicillin and cloxacillin showed significant level of residuesconcentration of amoxicillin and cloxacillin on day one (post-treatment).

In manyAfrican countries, antibiotics maybe used indiscriminately for the treatment ofbacterial diseases or they may be used as feedadditives for domestic animals and birds. The ongoing threat of antibiotic contamination is oneof the biggest challenges to public health that isfaced not only by the African people, but alsoby the human population worldwide (Cars *et al*., 2008). Suchresidues are spreading rapidly, irrespective ofgeographical, economical, or legal differencesbetween countries (Harbarth and Samore, 2005).

In a study carried out in Kenya, a high prevalenceof antimicrobial residues was also reported. Forexample, 11% of raw bulk milk samples sold inNakuru was found to have penicillin-G residues(Shitandi and Sternesjo, 2001)and 9-16% of the marketed milk in rural andurban households in the Dagoritti division,Nairobi, contained higher levels of antibiotics (Ekuttan *et al*., 2007; Kan'gette *et al*., 2005).Additionally, there was a steady increase in the consumption of quinolones, which was first seen in 1998 (Mitema *et al*., 2001).In Ghana, a report showed that 35% ofthe raw milk marketed in two major cities,Accra and Kumasi, were contaminated withantibiotics (Aning *et al*., 2007). Additionally, 3.1% of the raw milk samples contained antibiotic levels above theEuropean Union maximum residue limit. Theantimicrobials detected included β-lactams,sulphonamides, aminoglycosides, tetracyclines, and macrolides (Addo *et al*., 2011).

In South Africa, a survey was done in1977/78 to investigate the incidence of antibioticcontamination in milk marketed in the marketsof Pretoria. In milk from 1081 cattle herds, 60

tankers, and 112 pasteurized batches, antibioticswere found in 2.13% of the herd samples, 11.7%of the tanker samples, and 2.1% of thepasteurized samples (Bester and Lombard, 1979). Erythromycin was rapidly passed from blood tomilk in experimentally treated lactating ewes (Goudah *et al*., 2007).

In Nigeria, a study was designed to determine the prevalence of antibiotics in eggs from poultry farms and retail outlets in Enugu State. Eggs from 25 selected commercial farms and ten retail outlets were screened for the occurrence of antibiotic residues. All 25 farms surveyed used oxytetracycline. Eggs from nine of the surveyed farms and tested positive for antimicrobial residues (Ezenduka *et al*., 2011). In a survey of chloramphenicol use in poultry farms in Kaduna State, the presence of antimicrobials was confirmed in eight out of 144 pooled egg samples (10 eggs per sample). The only positive chloramphenicol sample was recorded on a farm that used a human chloramphenicol preparation (Omeiza *et al*., 2012). Adetunji (2008) reported tetracycline residue levels range between 0.008 and 0.1μg/ml in milk products.

Meat and other edible tissue from cattleslaughtered in the Akure metropolitan abattoirwere analyzed from January to June 2008 foroxytetracycline residues. Out of a total of 180beef samples analyzed in this study, 98 (54.44%)had detectable levels of oxytetracycline of which62 (34.44%) were contaminated to levels consideredviolative by the WHO/FAO (Olufemi and Ehinmowo, 2009; WHO, 1999). In another report, a microbiological screening wasperformed for 50 cattle slaughtered in the Sokotometropolitan abattoir, Nigeria, in order to detectantibiotic residues in meat. Penicillin wasthe drug with the highest rate of occurrence (14%) followed by tetracycline (8%) andstreptomycin (4%) in the samples in question (Ibrahim *et al*., 2010).

In the literature discussed above, it is clearthat antibiotic residues in animal-derived foodsare frequently recorded in several African countries. These residues exceeded the WHO limits in many cases (Darwish *et al*., 2013). Tetracyclinesare highly predominant antibiotics, and represent41 % of all antibiotic contaminants, followed byβ-lactams at 18% (Darwish *et al*., 2013). Great care should betaken to observe the antibiotic cessation of useperiods before the production of animal-derivedfoods intended for human consumption.

2.4.2 Factors associated with residue occurrence

All licensed antibiotics intended for animal use have clear cessation of use periods,pharmacokinetics and pharmacodynamics.Failure to observe the instructions for antibiotic

use can lead to antibiotic residues enteringanimal-derived foods. Improper maintenance of treatment records or a failure to identify treatedanimals adequately may lead to the omission of these animals (Sundlof, 1989). Residues may also transmitvertically to calves consuming milk from cowsreceiving antimicrobials(Guest and Paige, 1991). Fecal recycling, wherethe drug excreted in the feces of treated animalscontaminates the feed of untreated animals, canbe the cause of traces of certain antimicrobial substances being passed on (McCaughey*et al*., 1990). Contamination ofanimal feedstuffs with a variety of compoundsmay also occur. The significance of thiscontamination depends on the pharmacodynamicsof the compound and the species affected(McEvoy, 2002; Myllyniemi*et al*., 2000).

2.4.3 Factors that influence the occurrence of antimicrobial drug residues in milk

Milking frequency: Limited and contradicting information is available on the influence of milking frequency onexcretion of antibiotic residues in milk. Investigations were mainly focused on more frequentmilking after treatment of clinical mastitis in order to determine the effect on therapeutic concentrationsof antibiotics.Schluep and Heim (1980) found slightly different concentrations of cefacetrile or penicillinresp. in milk of healthy cows in dependence of interval between treatment and milking, butthis did not influence the duration of excretion in milk. When quarters were milked out at 4additional times within the first 10 hours after treatment no difference in excretion of cefacetrilein milk was found compared to quarters of the same cow milked only at regular milkingtimes twice per day (Schluep and Heim, 1980). In contrast, European Union (2004) reported lower concentrationsof cefoperazone in milk of cows milked in intervals of 4 hours after intramammary treatmentcompared to cows milked after 12 hours.According to European Union (2004) the excretion time of procain-penicillin G or oxytetracycline in milk was significantly shorter when cows were stripped 5 times at 2 hour intervals after treatment compared to cows milked at regular milking times. From the concentrations in milk it was concluded that already 2 hours after intramammary application the major part of the antibiotic is distributed within the udder and is not milked out with the first stripping (Schluep and Heim, 1980) .

Udder health status: It has to be regarded that in studies on excretion of residues the udder health status is not alwayswell defined. If no further explanation is given cows usually are referred to as healthywhen no clinical symptoms of mastitis were detected.After intramammary treatment Edwards (1964) found irregular distribution of penicillin inudder quarters during later stages of lactation. This was explained by an increase in connective tissue and shrinkage of ducts and

alveoli. Similar findings occurred in cows with mastitisdue to inflammatory changes going along with leukocytic invasion and partial occlusion ofducts. Higher concentrations and longer retention of penicillin were found in milk of quarterswith chronic mastitis compared to healthy quarters. This was caused by increased fibrous tissueleading to poor distribution and absorption of penicillin.Anderson *et al*. (1998) investigated the excretion of amoxicillin and penicillin G in cowswith clinical mastitis. Antibiotics were not detected in milk after the end of the withholdingperiod, but one sample with somatic cell count (SCC) exceeding 22 000 x 103/ml reacted positive in a microbialinhibitor test. No antibiotics were detected in this sample by liquid chromatography althoughthe detection limit was lower than that of the screening test.

Schluep *et al*. (1979) observed that the elimination of cefacetrile was slightly retarded in experimentallyinfected quarters when a slow release formulation was applied for intramammary treatment, but no differences were detected between infected and healthy quarters when a quick release formulation was used.

After intravenous application of high dosages of ceftiofur in cows with induced *Escherichia* (*E.) coli* mastitis significant longer excretion of ceftiofur in milk was observed compared to healthy cows (147 hours versus 1.3 h) (Erskine *et al*., 1995). No significant differences were found betweenconcentrations in milk from healthy quarters and quarters with subclinical mastitis afterudder treatment with cefoperazone or cephradine. Concentrations of cefoperazone above 0.5mg/ml were only found at 8 hours after application and no significant differences were observedbetween quarters with subclinical and clinical mastitis and healthy quarters.

For the macrolide antibiotics erythromycin and spiramycin lower concentrations were found in mastitic milk with pH>7.2 than in milk with pH<7.2 after intramammary or intravenous

application (Nouws and Ziv, 1979). The excretion period in milk was shorter with more severe abnormalities of milk. These findings were consistent with those from European Union (2004) who reported lower concentrations in mastitic milk than in milk of healthy cows after intramammary application of spiramycin.Malvisi *et al*. (1991) determined higher concentrations of oxytetracycline, neomycin andoleandomycin in milk of clinically diseased cows than in healthy cows, but the amount excretedvia milk in percent of the total amount applied was lower in mastitic than in healthycows for neomycin and oleandomycin.

Polymyxin B was not resorbed after intramammary treatment of healthy cows or cows withchronic mastitis. More than 90 % of the applied amount of drug was excreted via milk (Zivand Schultze, 1982). Concentrations in healthy quarters were significantly higher than in acutely diseased quarters. In cows with experimentally induced *E. coli* mastitis only 55 %were excreted via milk within 24 hours. In these cases polymyxin B was also detected in serumand urine and in milk from untreated quarters. The excretion period was independentfrom the total amount of drug applied, but the excretion time was shorter in mastitic quartersthan in healthy quarters.

Excretion from untreated quarters: The transfer from treated quarters to non-treated quarters has been described for a number ofantibiotics. Transfer of penicillin to untreated quarters was already shown in 1964 by Edwardsand was confirmed by later investigations in cows with or without clinical symptoms of mastitis(Rollins *et al*., 1970, Aureli *et al*., 1990). Rollins *et al*. (1970) also described the excretionof dihydrostreptomycin from untreated quarters. Freke and Bates (1981) detected low concentrationsof penicillin and cloxacillin in milk from untreated

quarters in clinically healthy cows. European Union (2004) showed excretion from untreated quarters after treatment of healthy cowswith drug combinations of penicillin+neomycin, penicillin+neomycin+oleandomycin andneomycin+tetracycline. In contrast, transfer of polymyxin B was only observed when cowswith acute clinical mastitis were treated (Ziv and Schultze, 1982). Excretion of spiramycinfrom untreated quarters was dependent on treatment interval and dosage (Schällibaum *et al*.,1981).For oleandomycin a higher transfer rate to untreated quarters was found compared to neomycinand oxytetracycline (Malvisi *et al*., 1991). Cefotaxime and ceftazidine were determinedin milk of non-treated quarters already one hour after application (Rule *et al*., 1998).

Extra label use of antibiotics: Studies in extra label use of antibiotics are mainly related to dosages deviating from recommendationsof the drug manufacturer.Administering doses exceeding label directions were reported as one reason for inhibitor positivemilk samples after the end of the withholding period in cows treated with different antibioticsfor various reasons (Seymour *et al*., 1988).A prolonged excretion period was observed in cows treated with a dosage of spiramycin twice as high as recommended by the manufacturer (Schällibaum *et al*., 1981).Koppinen *et al*. (2001) determined factors influencing the excretion time of penicillin in milkin a field trial including 467 cows. In addition to the product used and the number of days inlactation also the total dosage applied had a significant influence on excretion time.No exceeding of the withholding time for milk was observed after intramuscular applicationof ampicillin or amoxicillin at higher dosages than recommended by the manufacturer(Anderson *et al*., 1996).

For certain antibiotics a combined intramammary and intramuscular therapy may increase the excretion time although Poelarends *et al*. (2001) did not find significant differences compared to only intramammary treatment in cows with clinical mastitis.After intramammary treatment of cows with *Staphylococcus (S.) aureus* mastitis higher concentrationsof ceftiofur in udder tissue were achieved by repeated intramammary infusionscompared to single application (Owens *et al*., 1990). Highest concentrations of benzylpenicillinin untreated quarters were observed after repeated treatment in 24 hour intervals (Bjorland*et al*., 1998).

Other factors influencing the excretion of residues in milk: The drug formula has a significant influence on the distribution within the udder and the excretiontime in milk (Edwards, 1964, Moretain and Boisseau, 1989, Andrade *et al*., 1993,Koppinen *et al*., 2001)In an investigation by Seymour *et al*. (1988) the antibiotic drug applied significantly influencedthe excretion period in milk. Other factors like route of administration, case number,number of days treated, body weight, lactation number and milk yield had no significant influenceon excretion time. The total dosage applied, the product used and the number of daysin lactation had a significant influence on excretion time according to Koppinen *et al*. (2001).Moretain and Boisseau (1989) found the elimination of residues to be dependent on the factors such asquantity of antibiotic applied, nature of the vehicle and volume of the ointment infusedfor intramammary treatment.Also after parenteral administration of antibiotic drugs residues are detected in milk. Cannon*et al*. (1962) found variation in penicillin excretion in milk between individual cows after intramuscularor intravenous administration, but no correlation could be established with milkproduction, fat content of milk or body weight.

2.5 Regulatory Control of Residue in Milk Supply

The maximun residue level (MRL) or tolerance is the target concentration in a residue-depletion study. It should be established purely on the basis of safety to the person consuming the product and has no pharmacodynamic reality in the animal to which the drug has been administered (Hisham, 2013). Tissue tolerances are normally established in fat, milk, muscle, liver, kidney, skin, or sometime meat by-products. The first step in calculating the tolerance is to determine the safe concentration of drug that could be consumed by individuals eating the animal products:

Safe concentration = (ADI) (Body weight)/ Food consumption factor

In this equation, ADI refers to acceptable daily intake which is the maximum amount of chemical (mg/kg) that may be consumed daily over a lifetime without producing an adverse effect. Body weight is the average weight of humans consuming the product (usually assumed to be 60 Kg). The food consumption factor is the amount of edible product estimated to be consumed daily by an individual. The food consumption factor is based upon the average individual's daily intake of different types of foods. The Food and Drug Administration (FDA) and other regulatory agencies have tabulated food-specific consumption factors. Examples (Kg consumed per day) are 0.3 for muscle, 0.1 for liver, 0.05 for kidney, 0.05 for fat, and 1.5 for milk in USA (Riviere, 1999). The milk consumption in children is especially high since the total diet for an infant may entirely be the milk. Other countries use similar food consumption factors but distribute the ADI based on independent organ consumption data.

Regulatory levels have been established for drug residues in foods in the form of maximum residue limits (MRLs) (Lee *et al.,* 2000). MRLs for veterinary drugs refer to the maximum

concentration of a residue (resulting from the use of a veterinary drug) that is acceptable in food (CAC, 1997). Sampling and testing protocols are based on standards set by Codex Alimentarius Commission (CAC) and Table 2.1 gives some examples of those, which have been set for milk from veterinary cows

Table 2.1:CAC Residue Limits of common veterinarydrugs

Antimicrobialdrug	CAC MRL (µg/kg)
Benzylpenicillin/Procaine benzylpenicillin	4
Ceftiofur	100
Dihydrostreptomycin/Streptomycin	200
Diminazene	150
Febantel/Fenbendazole/Oxfendazole	100
Isometamidium	100
Neomycin	500
Oxytetracycline	100
Spectinomycin	200
Spiramycin	200
Sulfadimidine	25
Thiabendazole (used also as pesticide)	100

Source: CAC, (1997)

The MRL is based on the Acceptable Daily Intake (ADI) for a given compound, which is the amount of a substance that can be ingested daily over a life time without appreciable health risk. MRLs are fixed on the basis of relevant toxicological data including information on absorption, distribution, metabolism and excretion (Anadon and Martinez, 1999). The European Union (EU), through a regulation No. 508/1990 (EU, 2002), has also set MRLs for antibiotics of which for the ß-lactams group includes, penicillin G 4 µg/kg, ampicillin 4 µg/kg, oxacillin 30 µg/kg, amoxicillin 4 µg/kg, dicloxacillin 30 µg/kg, cephalexin 100 µg/kg and cephapirin 60 µg/kg. Maximum residue limits are today assessed and established by the respective expert groups of different regions. In most low-income countries, regulatory bodies do not present MRLs and only specify a zero tolerance. The specification is where no detectable residues are permissible in animal foodstuffs. This standard is not practiced internationally as they are no analytical techniques with the sensitivity to achieve it (Girma *et al*., 2014).

2.6Screening/ Surveillance for Residues in Milk

The first test for establishing antimicrobial agentresidues in milk (microbial inhibitor test) was developedas early as 1952 (Mitchell *et al*., 1998).It was then known, too, that the presence of theseagents could cause the inhibition of the startercultures used in dairy industry, and for this reasonthe development of such methods was initiated soas to establish the inhibitor agent levels in milk (Navratilova, 2008).It was important that the methods be relativelycheap, simple to carry out, and capable of detectinga wide variety of antimicrobial agents. Of themethods used, microbial inhibitor methods suitedbest these requirements. A drawback which limitstheir use is a long incubation period. Therefore,rapid assays for antibiotic agent detection in milkhave been developed which enable obtaining theresults in the course of several minutes; they are simple,

sensitive, and specific. One of a plethoraof rapid assays is the Penzyme test which wasdeveloped at the start of the 1980's. Later on, in1988, Charm II. test for detecting 7 types of antimicrobialagents was introduced to the market,followed by other rapid assays, e.g. the LacTec test (1991), SNAP test (1994), Beta Star test, andCharm Safe Level test (Mitchell *et al.*,1998).

International Dairy Federation (IDF) deals inits publications (bulletin) with the issues of establishingantimicrobial agents in raw milk. In1987, the IDF issues a bulletin in which methodsare described which can be used for the detectionand confirmation of inhibitory agents in milkand milk products. In 1991, another bulletin wasissued dealing with screening and confirmationmethods for establishing antimicrobial agentsand methods for specific analysis of these agents.IDF has set a conception and a control strategyconcerning antibiotic and sulfonamide residuesin milk and milk products – an integrated system.In a nutshell, the strategy of antibiotic andsulfonamide residues control is to be guided bythe following rules (Heeschen, 1993): microbiologicalscreening inhibitory assays are to be usedespecially in primary food production.

In orderto establish agents which cannot be detected by commonly used microbiological screening tests,other methods are to be used, e.g. microbiologicalreceptor tests, immunological methods. Thetesting for antibiotics and sulfa drugs in the silomilk and in the heat-treated milk should use testswith the sensitivity required under toxicologicalaspects (microbial penicillin screening tests andin addition receptor and antibody tests).

In general, antimicrobial agent control systemcan be divided into two steps. The first of them is

general monitoring of these agents during whichtests are used making it possible to establish rapidlythe presence of inhibitory agents. The second step isa specific analysis which makes use of the methodswhich enable the identification and quantificationof inhibitory agents. For the general monitoringof antimicrobial agents in milk, common use isestablished of microbial inhibitor methods andrapid specific tests (Navratilova, 2008).

2.6.1Microbial growth inhibition methods

Microbial growth inhibition methods make useof a standard culture of the tested microorganismin a liquid or solid medium (Heeschen, 1993).*Geobacillus stearothermophilus* var. *calidolactis*,*Bacillus subtilis, Bacillus megaterium, Sarcinalutea, Escherichia coli, Bacillus cereus* var. *mycoides*or *Streptococcus thermophilus*. The analysed milksample is applied on the agar surface either directlyor with a paper disc (disc assay plate methods). Inthe course ofincubation, the diffusion of the sampleinto the medium takes place (the agar diffusionprinciple), and if the sample contains inhibitoragents, reduction or total inhibition occurs of the tested microorganism growth. Depending on themethod used, the presence of inhibitor agents inthe tested sample is indicated by the formation of aclear zone of inhibition around the disc (disc assayplate methods) or a change in the medium colour(Hui, 1993; Mitchell *et al*., 1998; Botsoglou and Fletouris, 2001).

Microbial growth inhibition methods (widespectral rapid tests) vary in the type of the testingorganism, indicator, incubation period andtemperature, spectrum and detection levels of the

agents analysed. A series of these methods use asthe testing microorganism *Geobacillus (Bacillus)stearothermophilus* var. *calidolactis*: BR-test/AS/Blue Star/6/7 (Enterotox Lab., Germany), CharmBlue Yellow Test (Charm Sciences Inc., USA), Delvo test SP-NT (Gist brocades BV, The Netherlands),CMT – Copan milk test (Copan Italia,Italy), Eclipse 50 (Zeu-Inmunotec S.L., Spain).*Geobacillus stearothermophilus* is an outstandingtesting microorganism for its properties fromwhich the most important, according to Katzand Siewierski (1995), are: the ability of rapidgrowth at higher temperatures (64°C) and a highsensitivity to the β-lactam antibiotics.

Commercially available microbial inhibitortests play an important role in the integrated detectionsystem. At present, many commerciallyproduced microbial inhibitor tests are applied simultaneously with selective rapid tests formilk screening in primary production, in dairyindustry, and in accredited laboratories (Suhren, 1995; Honkanen-Buzalski and Reybroeck, 1997; Honkanen-Buzalski and Suhren, 1999; Botsoglou and Fletouris, 2001). The advantage of these methods is that they have a wide detectionspectrum, they are simple to carry out, and theyare not costly and can be used for the screening ofa large number of samples (Mitchell *et al*., 1998).These methods have their drawbacks, however, thatlimit their use: they do not enable specific antibioticidentification, they have limited detectionlevels for a series of antibiotics, and they are onlyqualitative and require a long incubation period(2.5–3.5 h). They are highly sensitive to β-lactamantibiotics, mostly penicillin, but evidently lesssensitive to other antimicrobial agents such asmacrolides, sulfonamides, tetracyclines, or chloramfenicol (Botsoglou and Fletouris, 2001). Manystudies proved that natural antimicrobial agents, if present

in milk in higher concentrations, canbring about false – positive results (Carlsson *etal*., 1989; Andrew, 2001; Kang & Kondo, 2001;Kang *et al.*,2005).

Commercially produced microbial inhibitor testsare delivered in the form of ampoules (monotests)or in the form of microplates with a high numberof testing cells. Apart from water bath or incubator,they do not require special laboratory equipment. To avoid subjective differences in the visual interpretationand to take the readings in an automated and more objective manner, some authors performingphotometric measurements use the appropriatewavelength (590 nm) and another wavelength asreference (650 nm) in ELISA reader (Althaus *et al*., 2003). When performing microbial inhibitor tests,it is necessary to meet the standards of good laboratorypractice (protection against the contaminationof the test), checking the pH value of the sample,observing carefully the correct temperature andthe incubation period as specified by the producer'sinstructions and testing a positive as well as anegative control alongside with the sample. Out ofmicrobial inhibitor screening methods, in frequent use are, for example: Eclipse test, Charm Cowsidetest, Charm AIM-96, Charm Farm test, VALIO T101, Copan Milk test, and others.

2.6.2Receptor binding and enzymaticcolorimetric assays

An alternative method for establishing the β-lactamgroup antibiotics (active forms of the β-lactam structure)is the use of the receptor proteins. β-Lactamspecific receptor proteins or penicillin-bindingproteins (PBP) were successfully used in somemethods and commercially produced tests (Biacoreanalysis, Penzym test, Beta Star test, SNAP test,Charm Safe Level test and DELVO-X-Press testand others) for establishing the β-lactam antibioticsresidues (Mitchell *et*

al., 1998; Gustavsson, 2003).PBP is found frequently in bacterial cell walls. Penicillin sensitive bacteria have various penicillinbinding proteins which, judging on their molecularweights, can be divided into two groups: proteinswith low or high molecular weights (Massova and Mobashery, 1998). These proteins are further dividedin subgroups by the aminoacid sequence (Ghuysen, 1991). Various PBPs have different functions. Theyinclude transpeptidase, transglycosylase, and carboxypeptidase activities (Massova and Mobashery, 1998).The low molecular weight PBPs probably control theextent of cross-linking of the peptidoglycan in thecell wall by acting on d-alanyl-d-alanine-terminatedpeptides, but the high molecular weight PBPs lackthis DD-peptidase activity (Granier *et al*., 1994).

The greatest deal of attention was paid to solubleDD-carboxypeptidases *Streptomyces* R61 and *Actinomadura*R39 (Ghuysen *et al.*,1973; Frere *et al*., 1976). Bacteria excrete intracellular enzymes while ingrowth (e.g. R 61 and R 39), which are believed to bethe soluble forms of membrane – bound transpeptidasesparticipating in the bacteria cell wall synthesis.The enzymes can act as carboxypeptidases andtranspeptidases (Leyh-Bouille *et al*., 1970). Naturalsubstrates for these enzymes are peptides endedwith d-alanyl-d-alanin (d-Ala-d-Ala). Penicillin isa structural analogue of the dipeptide d-Ala-d-Alaand therefore the enzymes react with the β-lactamstructure. β-Lactam antibiotics create a covalent bondwith carboxypeptidase giving rise to a very stablecomplex. Due to the formation of this complex, theenzyme activity is inhibited. The reaction is reversibleif the enzyme is released from the complex – it hasan identical affinity to the β-lactam antibiotic as thatbefore the reaction. β-Lactam antibiotics are, however, degraded to phenylacetylglycine and N-formyl-d-penicillamine (Ghuysen, 1977; Massova and Mobashery, 1998).

Enzymatic colorimetric assays:

Penzym test (UCB Bioproducts Belgium). Penzymtest is a qualitative enzymatic colorimetricmethod for a rapid determination of β-lactamantibiotics in milk. The test principle is basedon establishing the level of inactivation of theDD-carboxypeptidase enzyme by β-lactam antibiotics.These residues bind specifically with theenzyme and inactivate it, thus interfering withthe bacterial cell wall formation. In the course ofthe Penzym test, lyophilised enzyme *Streptomyces*DD-carboxypeptidase is enclosed in an ampouleinto which a sample of milk is introduced (50 µl).Then the incubation is observed for 5 min at 47°C.β-Lactam antibiotics present in the sample createa stable complex with DD-carboxypeptidasein the sample. The degree of inactivation of theenzyme depends on the amount of antibioticspresent in the sample. After a reagent tabletcontaining synthetic d-alanin oligopeptid andd-amino acid-oxidase is added, another incubationprocess follows for 8 min at 47°C. If the sampledoes not contain β-lactam antibiotics residues,after the specific substrate is added, the enzymeDD-carboxypeptidase hydrolyses the tripeptide(Ac-l-Lys-d-Ala-d-Ala) onto the dipeptide

(Ac-l-Lys-d-Ala) with a simultaneous release ofd-Alanin (d-Ala). The amount of the d-Ala releaseddepends on the amount of the active DD-carboxypeptidaseenzyme. Free d-Ala is further oxidisedby d-amino-acid-oxidase onto pyruvic acid givingrise to hydrogen peroxide. The end products of thesubstrate and enzyme reaction (pyruvic acid andhydrogen peroxide) are measured using a redoxcolour indicator and the comparison of the finalcolour with the colour chart provided with the kit.Hydrogen peroxide is used to oxidise the organicredox indicator that will change into a pink-orangecolour compound, indicating a negative result. If the colour is peach-coloured, the sample is closeto the detection limit. If a yellow or yellow-orange colour is observed (test-positive outcome), thesample is suspected of containing an antibioticresidue

(Cullor, 1993; Mitchell *et al*., 1998; Botsoglou and Fletouris, 2001; Gustavsson and Sternesjo, 2004).

Receptor binding assays:

Delvo-X-PRESS βL test (Gist-brocades BV,The Netherlands). The Delvo-X PRESS βL test isa qualitative, competitive, receptor-enzyme assay.Although analogous in principle to immunochemicalassays, the Delvo-X-PRESS test does notuse antibodies to bind specifically β-lactams andtherefore cannot be classified as an immunochemicaltest. The test involves a reagent substance (atracer) containing a conjugate – a specific receptorprotein isolated from the bacteria of *Bacillusstearothermophilus* species conjugated with anenzyme (horseradish peroxidase). The positivity of the sample is assessed photometrically by comparingoptical densities (OD) of the sample and thestandard. The samples yielding OD higher than0 are classified as positive, while those yieldingnegative OD are assessed as negative (Mitchell*et al*., 1998; Angelidis *et al*., 1999; Botsoglou and Fletouris, 2001).

IDEXX SNAP test (Idexx Laboratories Inc., Westbrook,ME, USA). SNAP test is an enzyme-linked,receptor binding assay in which β-lactams arecaptured by a binding protein on a solid supportadsorbent matrix housed in a moulded plastic unit.SNAP residues test consists of three components:SNAP device, pipette, sample tube. Using this test,penicillin can be detected in the amount of 4 ppb,ampicillin or amoxicillin in the amounts of 10 ppb,cephapirin 8 ppb, and ceftiofur 50 ppb.The SNAP test utilises a β-lactam receptor proteinconjugated to an enzyme. The samples are declared positiveor negative on the basis of the comparison of theintensity of the colour development between thesample and control spots on the SNAP test. If thecolour of the

test spot is weaker than that of thecontrol spot, the result is interpreted as positive(Bell *et al*., 1995; Neaves, 1995; Mitchell *et al*., 1998; Gustavsson, 2003).

Beta-Star test (UCB Bioproducts, Belgium).

Thetest involves a specific β-lactam receptor linkedto gold particles. It is a dipstick test that detectspenicillins and cephalosporins. The milk sample(0.2 ml) is added to a vial containing the test reagents(receptor protein linked to gold particles),mixed and incubated at 47.5°C in the incubatorfor 3 minutes. With milk samples free ofβ-lactam residues, the receptor protein will becaptured by a biomolecule immobilised at the test field of the chromatography paper. Since thereceptor protein is linked to gold particles, thecaptured protein-gold complex will appear as apink-coloured band. The colour intensity of the test band is visuallycompared with that of the reference band: if thecolour intensity of the test band is weaker thanthat of the reference band, the sample is classifiedas positive (Gustavsson, 2003; Gustavsson and Sternesjo, 2004).

2.6.3Microbial receptor assays

CHARM I and II tests (Charm Sciences, Inc.,USA). CHARM I and II tests are qualitative microbialreceptor assays. The CHARM I test developedfor β-lactams in milk was the first rapid test recognised by the AOAC (Association of OfficialAnalytical Chemists) with a test time of 15 minutes. In 1984–1985, the CHARM I testwas further developed to a test for antibioticsincluding, apart from β-lactams, tetracyclines,sulfonamides, aminoglycosides, chloramphenicol,

novobiocin, and macrolides. The extended version ofthe CHARM I test has been referred to as CHARM II test (Botsoglou and Fletouris, 2001).

CHARM II test is based on the irreversible bindingreaction between the functional groups of antibacterials and receptor sites on or within thecells of the added microorganisms. Two types of bacterial cells (*Bacillus stearothermophilus*) areused to provide the binding sites for seven drug families. In tetracycline and chloramphenicoltest kits, an antibody coating is used. The testemploys 14C- or 3H-radiolabelled antibacterials(tracer reagent) to compete for the binding sites.

This competition for the receptor sites preventsthe radiolabelled antibacterial from binding. Thus,the more radiolabelled compound binds, the lessanalyte is in the sample.The rate of binding is measured with a scintillation counter and compared to the positive andnegative controls. The higher the amount of theantibiotic present in the sample, the lower thecounts detected by the equipment (Cullor, 1993;Hui, 1993; Mitchell *et al*., 1998; Botsoglou and Fletouris, 2001).

2.6.4Immunoassays

Immunochemical methods are based on the reactionof an antigen with an antibody – a reaction ofantigenic determinants with the antibody linkingsite. Chemically, antigens are polymers proteins,polypeptides, polysaccharides, or nucleoproteins.They have two fundamental properties – theyincite a specific immune response, and they reactspecifically with the products of this response (withantibodies and immunocomplementary cells). Bothproperties can be found in the complete antigenanimmunogen which is made of a macromolecularcarrier and antigenic determinants-epitopes.A low molecular substance which itself cannotincite the antibody

production but which reactsspecifically with the products of the immune responseis termed a hapten. Antibodies are proteins,specifically immunoglobulins, formed by the hostanimal in response to the invasion by antigens. Animmunoglobulin molecule contains two identicallight (L) and two identical heavy (H) polypeptidicchains mutually linked with disulphide bonds. Oneof the Ig molecules always contains one light andone heavy chain type. The light chain determine the immunoglobulin molecule type. The heavy chainsare decisive in classifying into the immunoglobulinclass. C-ending sections of the chain make up theirconstant area, N-endings of chain are denoted as the variable sections and they represent the sectionof the molecule binding to the antigen – aparatope. Immunochemical methods make use ofmonoclonal or polyclonal antibodies. Modern immunoanalyticalmethods accomplish an increasein the sensitivity by labeling one of the reagents– the antigen or the antibody. The label can be aradio-isotope, enzyme, fluorescence or chemicalscintillation agent (Mitchell *et al*., 1998; Roeder and Roeder, 2000; Stepaniak *et al*., 2003)

Nonisotopic immunoassays such as ELISA(enzyme linked immunosorbent assay), FPIA (fluorescence polarisation immunoassay), PCIA(particle-concentration immunoassay), PCFIA (particle-concentration fluorescence immunoassay),and monoclonal-based immunoassays will, in alllikelihood, play an increasingly important role inantibiotics screening immunoassay determinations (Roeder and Roeder, 2000). The most frequentlyused immunochemical method for rapiddiagnostics of veterinary drug residues is enzymeimmunoanalysis (EIA). As an enzyme label, horse radish peroxidase, alkaline phosphatase, glucose oxidase, pyruvate dehydrogenase and recombinantβ-galactosidase are used. These enzymes catalysethe reactions that cause the

substrates degradationand form coloured products that can be readspectrophotometrically or visually (Botsoglou and Fletouris, 2001).

LacTec test (β-lactam) is based on the immunobindingprinciples of the enzyme-linked immunosorbentassay system (Cullor *et al*., 1992;Cullor, 1993; Botsoglou and Fletouris, 2001). Among other methods is used e.g. the FluorophosBeta Screen E.U. test – qualitative enzyme immunoassay based on fluorescence detectionfor the determination of six β-lactam antibiotics – amoxicillin, ampicillin, cloxacillin, dicloxacillin,oxacillin, penicillin G in raw milk (Sternesjo and Johnsson, 1998). Parallux (IDEXX LaboratoriesInc., USA) can be used to detect different antimicrobialagents. The method is based on competitivefluorescent immunoassays in glass capillary tubes (Gustavsson and Sternesjo, 2004).Most of immunoassays are normally performedin a laboratory and therefore it takes quite a longtime before the outcome of the test is known. Forthis reason, there is a growing need for on-sitescreening at the beginning of the food chain usingfast and easy to perform test methods. A number of immunochemical tests are commercially availablein a kit format for many drugs (card format, one step strip test).

A promising approach in therapid antibiotics detection is the use of a dipstickformat (lateral flow devices).The sample is appliedto the sample filter, housed inside the devicecasing. The sample runs through the sample filterand conjugate pad. This conjugate pad contains a labelled antibody, specific for the analysis under test. Antibiotics, if present, will form a complex with the conjugate and migrate further slowly to themembrane in the test zone. This zone contains animmobilised antibody, specific for the analysis,but preferably not competing with the conjugatedantibody for the same or adjacent epitopes. Thetest line will thus capture the migrating analysed conjugatecomplex. The intensity of the test linecorrelates well with the

amount of analyte in thesample (Van Herwijnen, 2006). Immunoassaycan be sensitive, class specific, accurate, andcanprovide a means for a rapid screening of samples for antibiotics.

2.7 Public Health Implications of Antimicrobial Drug Residues in Milk

Antibiotics used as therapeutic agents or as feedsupplement in milking animal lead to secretion of their residue in milk (Stolker and Brinkman, 2005). These residues not onlycreate problems in dairy industry but also have immense public health significance.

The antibiotic residues in milk are of concern in two respects (Swantara *et al*., 2014). First they may curtailproper lactic acid fermentation in cultured products resulting in spoilage and the possibility thatstaphylococci may proliferate before curd formation. Secondly, the ingestion of antibioticcontaminated milk may cause a reaction in human already sensitized to the contaminants. Thepotential hazards of ingesting antibiotic residues in contaminated milk include "allergicreactions, interference in the intestinal flora and resistance population of bacteria in the general population (Althaus *et al*., 2003). Allergies to antibiotic occur when the body's immune system attacks theantibiotic, which is often a haptenic metabolite of the antibiotic and some carrier tissues (Swantara *et al*., 2014). Smalllevels of antibiotics can be very hazardous to susceptible humans causing acute to severereactions.Penicillin raises most apprehensive in this respect because it strongly inhibits lactic acidbacteria and because it may trigger anaphylactic shock in unusually sensitive patients to whom it has been administered previously (Swantara *et al*., 2014). Other antibiotics may cause similar reactions but evidencesindicate that penicillin is of great concern. It has been suggested that the present recommendedlimit of 0.05 IU of penicillin/ml of milk is too high and offers no guarantee of safety (Swantara *et al*., 2014).

2.7.1Toxicity of Antibiotic residues

Possible adverse effects of antibiotics residues have been suggested. These includesallergic/anaphylactic reactions to residues, chronic toxic effects occurring with prolongedexposure to low levels of antibiotics, development of antibiotic resistance bacteria in treatedanimals and disruption of normal human flora in the intestine. There are few reports whichindicate that sensitive individuals may experience allergic reactions to antibiotic residues,particularly of penicillin residue. Anaphylactic reactions have been reported to result from theconsumption of meat containing penicillin (Hamann *et al*., 1979). Estimates of the prevalence of drug sensitivityvary but are estimated to be around 7% in the general population.Symptoms of chronic exposure to Oxytetracycline (OTC) include blood changes such as leucocytosis,atypical lymphocytes, lung congestion, toxic granulation of granulocytes and thrombocytopeniapurpura. Liver injury and delayed blood coagulation may also occur (Swantara *et al*., 2014). It can damage calcium richorgans like teeth and bones and sometimes erosion of nasal cavities. Children under 7 years ofage may develop a brown discolouration of the teeth. Infants born from the mothers treated withOTC during pregnancy may develop discolouration of the teeth (Swantara *et al*., 2014). Estimate shows that 10 IU (0.6 ug) of penicillincould cause an allergic reaction in a sensitive individual; 0.01 IU/ml of milk in a very sensitiveindividual. Enrofloxacine a fluroquinolone antibiotic acts by inhibition of bacterial DNA gyrase.Embryo lethality and teratogenecity of fluroquinolone in rats and rabbits has been suggested (Guzman *et al*., 2003).

The Food and Drug Administration center for Veterinary Medicine has proposed to withdrawapproval for use of fluoroquinolone antibicrobial, enrofloxacine in poultry. Their decision is not

based on drugs direct toxicity but on potential for increasing human pathogen resistance (Swantara *et al*., 2014). Lemus *et al*. (2008) suggested that antibiotic residues that may be present in carcasses ofmedicated livestock could pass to and greatly reduce scavenger wild life populations. They foundhigh concentrations of antibiotics in the plasma of many nesting cinereous (57%) and Egyptain(40%) vulture. Enrofloxacine and ciprofloxacine were also found in liver samples of all deadcinereous vultures.

2.7.2Antibiotic resistance threat to human health

Antibiotic resistance is a global public health concern today. The U.S. Centers forDisease Control and Prevention has described resistance as one of the world's most pressinghealth problems, because the number of bacteria resistance to antibiotics has increased in the lastdecade and many bacterial infections are becoming resistance to the most commonly prescribed

treatment. The WHO has identified antibiotic resistance as one of the three greatest threats tohuman health.

Microorganisms resistance to antibiotic drugs emerged and spread soon after theintroduction of those drugs and in parallel with their use. Many well known antibiotics are nolonger effective to treat common infections such as otitis, pneumonia, gonorrhoea andtuberculosis. Thus, microbial resistance is the main cause of newly emerging and reemerginginfectious diseases.

In 1993, WHO declared tuberculosis a global emergency, it was stated that it is a leadinginfectious killer of adults and will have killed at least 30 million people within the next 10 years

if current trends continue. A cost effective and proven drug treatment exits but carelesstuberculosis treatment practices are triggering bacilli that are resistance to once effective drugs.

Multidrug resistance tuberculosis develops when doctors or other health workers prescribed the wrong drugs or wrong combinations of drugs（Samvardhan,1997）. Antimicrobial resistance results in higher costsdue to the use of more expensive combinations of antibiotics to increased rate of hospitalizationfor infections once easily treated and an outpatient basis.

Drug use in livestock is implicated in antimicrobial resistance in humans because manyantibacterial drugs used for livestock are same as or similar to drugs used for humans. Somepathogens can pass from livestock to humans, either directly through contact (Feinman, 1979) or through foodproducts that are improperly processed, handled or prepared. Some food borne illness in humanscaused by resistant pathogens have been traced in livestock products and have been linked to liveanimals on farms (Swantara *et al*., 2014).

Tragically *Salmonella* bacteria are only one of many diseases producing organism thatare becoming increasingly resistant to antibiotics due to the continuous feeding of theseantibiotics to livestock. For example in 1990s less than 10% *Staphylococci* bacteria wereresistant to penicillin but in 2000 over 90% of the *Staphylococci* became resistant topenicillin. The chronic use of antibiotics in factory forms has created the likelihood of anepidemic of untreatable *Salmonella*

food poisoning (Molotsky, 1987). A recent example is a clone of *S.typhimurium* DT104, resistance to tetracyclines, ampicillin, streptomycine, chloramphenicol andsulphonamides which have become prevalent in many countries. An outbreak of human nalidixicacid resistant *S. typhimurium* DT 104 infection in Denmark was traced in a pig farm. Anotheroutbreak of the same infection, reported in the United Kingdome was traced in a dairy farmwhere fluroquinolones had been used in the cattle a month before the outbreak. Following theintroduction of fluroquinolones for use in food producing animals, the emergence of *Salmonella*

serotypes with reduced susceptibility to fluroquinolones in humans has become a cause for particular concern (Molbak *et al*., 1999). Following the introduction of fluroquinolones for use in poultry there hasbeen a dramatic rise in the prevalence of fluroquinolone-resistant *Campylobacter jejuni* isolatedin live poultry, poultry meat and from infected humans (WHO, 2009).

The use of avoparcine as a growth promoting feed additives in animal husbandry hascontributed to the reservoir of transferable resistance genes to glycopeptides includingvancomycine, in the commensally enterococci of animals. Glycopeptides-resistant enterococcicause serious infections in hospitalized immune impaired patients. In this setting they contributeto increased morbidity and mortality, in part because of limited therapeutic options (Swantara *et al*., 2014).

Multiresistant *Escherichia coli* have been evolved by the use of broad spectrumantimicrobials in both livestock and humans. The development of antimicrobial resistance in *E.coli* creates problem due to their propensity to disseminate antimicrobial resistance genes.Resistance genes

have been traced from *E. coli* in animals to *E. coli* in humans Certain *E. coli*are food borne pathogens and most of these strains are currently susceptible to antimicrobials.

The WHO has recommended that antibiotics which are also licensed in humanmedicine should not be used any more as growth promoters in livestock.Not only is there a risk to human health from direct toxicity and from allergic reactions inpersons sensitized to the antibiotics involved but antibiotic residues may also interfere with anymicrobial examinations which may be necessary in assessing the fitness of the carcass. The agerestrictions have been imposed for feeding of antibiotics in animals. It is given in Table 2. If useof antibiotics is necessary as in treatment of animals a withholding period must be allowed tillresidues can no longer be detected.

2.8 Heat Treatment of Milk

In control laboratories, it is common practice to carryout treatments by heating the sample to destroy pathogens, its homogenisation, or for inactivation of the natural antimicrobials inthe case of analysis of inhibitors. To date, there have been no studies investigating how these treatments affectthe presence of antibiotic residues in milk (Berruga *et al.*, 2006).On the other hand, milk arrives at the consumer after undergoing different heat treatments in the industry, suchas pasteurisation, sterilisation, etc (Berruga *et al.*, 2006).

2.9Effect of Heat on Antimicrobial Drug Residues

Nowadays, quality controls are essential in milk to establish the price that the producerreceives, as well as to assure the quality of the product. In control laboratories, it is common practice to carryout treatments by heating the sample for its homogenisation, or for inactivation of the

natural antimicrobials inthe case of analysis of inhibitors. For the past 50 years, many researchers have been interested in evaluating whether antibiotic residues can be destroyed by cooking procedures, pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose *et al*., 1995; Isidori *et al*., 2005; Hassani *et al*., 2008). Traditionally, heat stabilities of antibiotics have been studied based on either the evaluation of the decrease in antimicrobial activity or by specific chromatographic analysis of change in concentration after heat treatments. Relatively few studies have been carried out using both microbiological and chemical analyses in evaluating the heat stability of veterinary drug residues (Franje *et al*., 2010). Moreover, whether or not the heating of these compounds and any structural changes generated result in altered genotoxicity that could contribute to the mutagenicity of bacteria remains unclear (Hsieh *et al*., 2011).

Previous studies have suggested that sulfamethazine (SMZ) (Rose *et al*., 1995; Papapanagiotou *et al*., 2005) oxacillin (OXA), chloramphenicol, aminoglycosides, quinolones, clindamycin, novobiocin, trimethoprim, vancomycin, and azlocillin are heat-stable (Traub and Leonhard, 1995) while oxytetracycline (OTC) (Hassani *et al*., 2008) and erythromycin were shown to be heat-labile. On the other hand, several β-lactams such as penicillin (PCN) G, ampicillin (AMP) and amoxicillin (AMX) appear partially heat-labile (Traub and Leonhard, 1995). Antibiotics of the same class were also reported to have different heat stabilities depending on different types of matrices and heating treatments involved (Kitts *et al*., 1992; Rose *et al*., 1996; Franje *et al*., 2010). Most of the findings and the conclusions drawn were summarized by Moats (1999).

On the other hand, most heat stability studies evaluated the degradation of parent drugs with few studies carried out on the possible production of toxic breakdown products (Gratacos-Cubarsi *et al*., 2007; Franje *et al*., 2010).

Although reports on antibiotics residues in milk and milkproducts did not eliminate the antibiotics especially withproducts that had undergone heat processing likecheese, evaporated milk and yoghurt. These findingsagree with an earlier study by O'Keefe and Kennedy(2008), who reported very little or no effect on antibiotic residues after processing.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

The materials used include; Delvotest SP-NT test kit, incubator, foil paper, weighing balance, water bath, autoclave, serocapped glass test tubes, cotton wool, test tube rack, pipettes, handgloves, hand sanitizers, sterile universal bottles.

3.1.1Study Area

Zaria is a major city in Kaduna State, located on the plateau of 2200 feet above sea level within latitudes $11^0 7'$, $11^0 12'$ N and longitude $7^0 41'$ E (Mamman *et al*., 2000). It is a medium sized city with an estimated population of 547,000 and a growth rate of 3.5% per annum. Zaria metropolis is divided administratively into Zaria and Sabon gari Local Government Areas (Ministry of Economic Development, 1996). It is characterised by a tropical climate, humid wet season, a rainy season of approximately 210 days (May to October). The mean monthly temperature shows a maximum of 39.7^0C in June and 13.8^0C in December (Agbogu *et al*., 2006). Hausa-Fulani are the main ethnic groups and their major occupation is Agriculture: Approximately 40-70% of the population's livelihood is from agriculture (Agbogu*et al*., 2006).

Fresh milk and fermented milk are mainly produced and marketted by the nomadic fulani in Zaria. Fermented milk, also known as 'nono' forms a major part of the staple food in Zaria, as well as other parts of Northern Nigeria (Yabaya *et al.*, 2012).

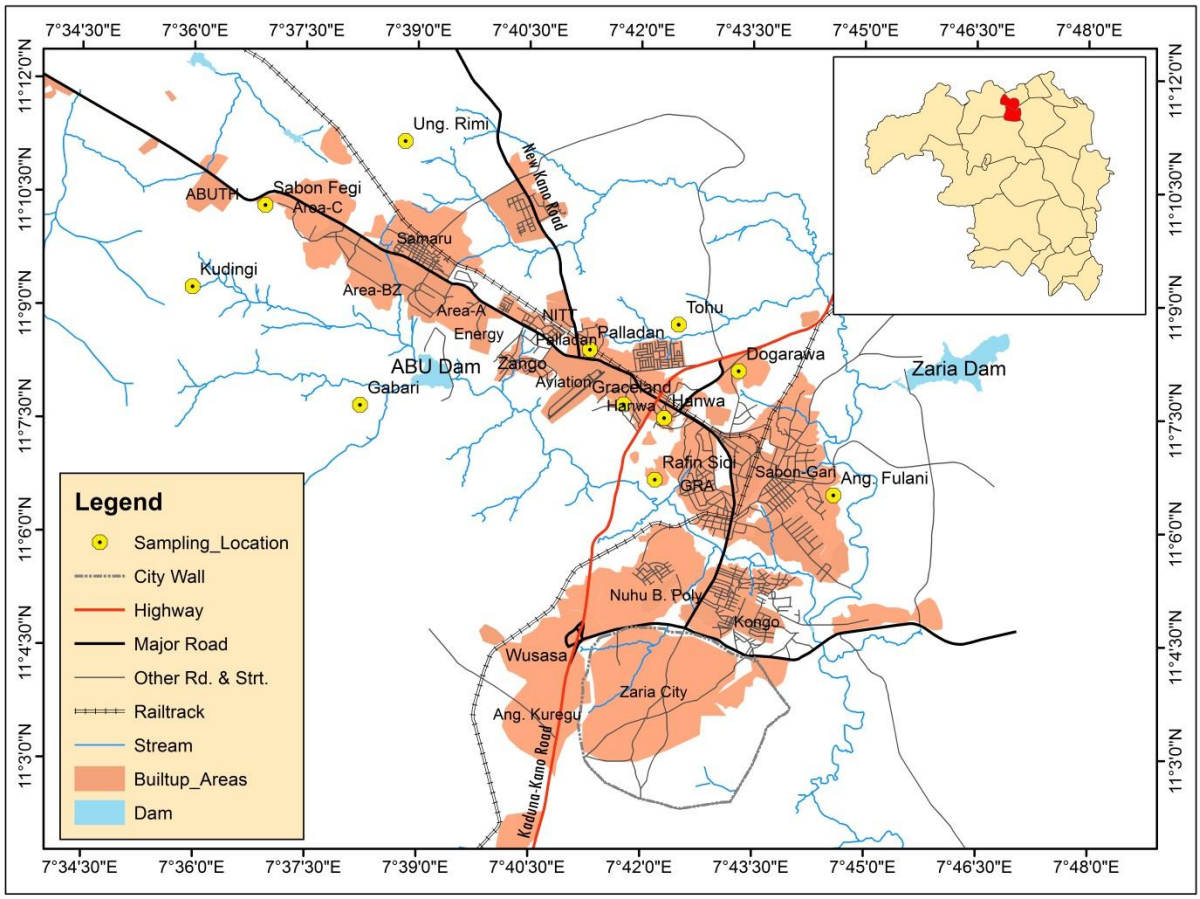


Figure 3.1: Zaria Urban Area showing the Sampling Locations

Source: Adopted and modified from Satellite Imagery, 2013.

3.2 Methodology

3.2.1Study Design

A cross sectional study was carriedout to determine the occurrence of antimicrobial residues. An experimental study was also conducted to determine the effect of heat treatment on residual concentration of some antimicrobial drugs in fresh cow milk in Zaria.

3.2.2 Sampling Technique

A convenience sampling was carried out in which fresh milk samples were collected from localfulani milk producing herds based on availability and willingness of the cattle owners to allow their animals to be sampled.

3.2.3 Sample Collection and Transportation

5ml of fresh milk samples were collected from each lactating animal sampled based on availability from local fulani milk producing herds in eleven (11) villages within Zaria and environ; Anguwan Fulani, Dogarawa, Gabari, Graceland, Hanwa, Kudingi, Palladan, Rafin Sidi, Sabon Fegi, Tohu and Unguwan Rimi. Fifty (50%) of the herds in each village visited were sampled, a minimum of two (2) and a maximum of ten (10) samples were collected from each

herd sampled. Information on the age, breed, parity, presence of mastitis, stage of lactation and antibacterial administration within the last four (4) weeks were recorded on the sample collection form for each animal sampled (Appendix I). The samples were collected using sterile universal bottles and transported on ice to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria for laboratory analysis.

3.2.4Screening for antimicrobial residues using Delvotest SP-NT Test kit:

Screening for antimicrobial residues was carriedout using the procedure described by Delvotest SP-NT manufacturer (DSM, Netherlands). The procedure is outlined below:

The aluminium foil was carefully removed from the ampule(s).A fresh diposable pipette tip was placed onto the syringe for each milk sample to be tested.0.1ml of the milk sample was added carefully onto the agar using the dosage syringe provided.The ampules were placed in a pre-heated water bath or dry incubator specifically designed for Delvotest at 64^0C after which the ampules were incubated for 3 hours.

The negative control changes from purple to yellow while the positive control retained its purple colouration after 3 hours.If the test sample changes from purple to yellow, then it was negative for antimicrobial residues. (Plate 3.1).

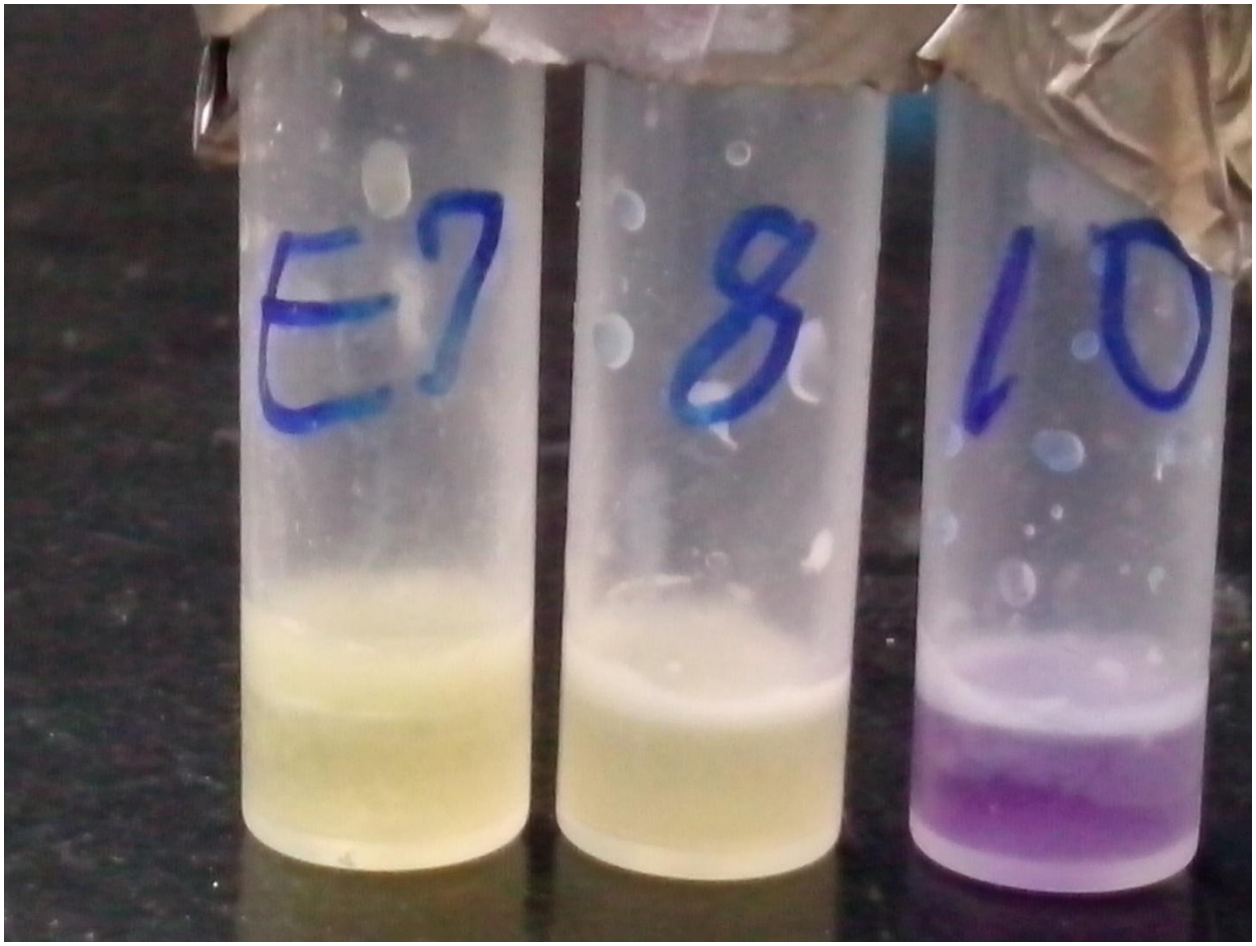


Plate3.1: Delvotest SP-NT, the negative is yellow and the positive is purple

3.2.5Effect of heat treatment on antibiotics:

Milk sample froman apparently healthy cow that is not producing colostrum and have not been treated with antibiotics for at least a month was screened for antimicrobial residues using Delvotest SP-NT. The pure antibiotic powders for tetracycline hydrochloride (95%), penicillin G sodium salt (98%), sulfamethazine (99%) and streptomycin sulphate salt were purchased from Sigma-Aldrich (St. louis, MO, USA).Stock solutions at a concentration of 0.01g/ml, 0.02g/ml, 0.025g/ml and 0.04g/ml were prepared for Oxytetracycline (OTC), Streptomycin (STREP), Sulfamethazine (SMZ) and Penicillin G (Pen G) respectively by dissolving each antibiotic in fresh milk. The working solutions were prepared by serial dilution of the spiked milk samples (10 fold dilution). All working solutions in 10ml glass tubes with "sero-tab" caps were heated in a thermostatic water bath (at 63^0C for 30 minutes) at pasteurisation temperature. Another set of prepared working solutions were autoclaved (at 121^0C for 15 minutes) at ultrahigh temperature (Berruga *et al*., 2006; Hsieh *et al*., 2011). The heat treated working solutions, both atpasteurised and ultra high temperature, were screened for antimicrobial drug residue activity using the delvotest SP-NT. Dilutions of 10^{-4} to $10^{-7}$for OTC, STREP,SMZ andPEN G were tested using five (5) ampules of the test kit for each dilution.

3.2.6Data Analysis

The data generated from the screening test and sample collection forms were collated and subjected to descriptive statistical analysis using SPSS 20.0.Chi-square (χ^2) and Fisher's exact tests were used to analyze categorical variables. P value less than 0.05 ($P<0.05$) was considered statistically significant (Appendix IIA-IIF).

CHAPTER FOUR

4.0 RESULTS

4.1 Distribution of Antimicrobial Residues in Fresh Cow Milk Sampled in Zaria using Delvotest SP-NT Test

A total of 300 samples were collected from 11 villages within Zaria and environ and screened for antimicrobial residues. The screening test produced 44 (14.7%) positive results (Plate I). These positive results were obtained from 7 villages.

All the samples were collected from White Fulani cows. Thirty (10%) of the samples were collected from Tohu, thirty eight (12.7%) from Palladan, twenty one (7%) from Anguwan Fulani, thirty seven (12.3%) from Graceland, thirty three (11%) from Kudingi, seventeen (5.7%) from Unguwan Rimi, twenty seven (9%) from Rafin Sidi, thirty (10%) from Gabari, nine (3%) from Sabon Fegi, thirty three (11%) from Dogarawa and twenty five (8.3%) from Hanwa (Table 4.1)**.** Most of the samples were collected from apparently healthy cows, 292 (97.3%). On the ages of the cows, 53 (17.7%) were less than 5years, 227 (75.7%) were between 5-10years and 20 (6.7%) were more than 10years (Table 4.2). Also, 168 (56.0%) of the cows were not upto 3rd parity, 111 (37.0%) range from 3-5th parity and 21 (7%) were morethan 5th parity (Table 4.3).

36 (12%) of the cows were lessthan one (1) month into lactation, 114 (38%) were between 1-6months into lactation and 150 (50%) were morethan 6months into lactation (Table 4.4). On the administration of antimicrobial drugs, the results showed that 144 (48%) of the cows were given antibiotics while 156 (52%) were not given antibiotics within four (4) weeks before sample collection (Table 4.5).

Table 4.1: Distribution of antimicrobial residues based on areas sampled in Zaria

Areas sampled	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Anguwan fulani	3 (1)	18 (6)	21 (7)	14.3
Dogarawa	4 (1.3)	29 (9.7)	33 (11)	12.1
Gabari	0 (0)	30 (10)	30 (10)	0
Grace land	11 (3.7)	26 (8.7)	37 (12.3)	29.7
Hanwa	0 (0)	25 (8.3)	25 (8.3)	0
Kudingi	5 (1.7)	28 (9.3)	33 (11)	15.1
Palladan	14 (4.7)	24 (8)	38 (12.7)	36.8
Rafin sidi	2 (0.7)	25 (8.3)	27 (9)	7.4
Sabon fegi	0 (0)	9 (3)	9 (3)	0
Tohu	5 (1.7)	25 (8.3)	30 (10)	16.7
Unguwan rimi	0 (0)	17 (5.7)	17 (5.7)	0
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Fisher's exact test $P<0.05$)

Table 4.2: Distribution of antimicrobial residues based on ages of cows in Zaria

Ages of cows (years)	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Less than 5	11 (3.7)	42 (14)	53 (17.7)	20.8
5-10	32 (10.7)	195 (65)	227 (75.7)	14.1
Over 10	1 (0.3)	19 (6.3)	20 (6.7)	5
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Fisher's exact test P>0.05)

Table 4.3: Distribution of antimicrobial residues based on parity of cows sampled in Zaria

Parity	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Less than 3	25 (8.3)	143 (47.7)	168 (56)	14.9
3-5	17 (5.7)	94 (31.3)	111 (37)	15.3
More than 5	2 (0.7)	19 (6.3)	21 (7)	9.5
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Fisher's exact test P>0.05)

Table 4.4: Distribution of antimicrobial residues based on stage of lactation of cows sampled in Zaria

Stage of lactation (months)	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Less than 1	4 (1.3)	32 (10.7)	36 (12)	11.1
1-6	12 (4)	102 (34)	114 (38)	10.5
Over 6	28 (9.3)	122 (40.7)	150 (50)	18.7
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Chi square P>0.05)

Table 4.5: Distribution of antimicrobial residues based on mastitis status (active clinical mastitis) of cows sampled in Zaria

Mastitis	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Present	3 (1)	5 (1.7)	8 (2.7)	37.5
Absent	41 (13.7)	251 (83.7)	292 (97.3)	14.0
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Fisher's exact test P>0.05)

Table 4.6: Distribution of antimicrobial residues based on drug administration in cows within the last 4 weeks in Zaria

Drug administration	**Positive (%)**	**Negative (%)**	**Total (%)**	**Prevalence (%)**
Yes	37 (12.3)	107 (35.7)	144 (48)	25.7
No	7 (2.3)	149 (49.7)	156 (52)	4.5
Total	**44 (14.7)**	**256 (85.3)**	**300 (100)**	

(Chi square $P<0.05$)

4.2 Effect of Heat Treatment on Milk Sample Spiked with Antibiotics in Zaria

Fresh milk sample from an apparently healthy cow that is not producing colostrum was collected from National Animal Production Research Institute (NAPRI), Zaria and screened for antimicrobial residues. The screening test showed that the milk was free from antimicrobial drug residues. The milk was spiked with four antimicrobial agents (powders); Tetracycline hydrochloride, Sulfamethazine, Streptomycin sulphate and Penicillin G sodium salt. The effects of pasteurisation and sterilisation on antimicrobial activity were observed.

For the pasteurised milk, Oxytetracycline hydrochloride (OTC), Streptomycin Sulphate and Penicillin G Sodium Salt tested positive for all the dilutions and ampules tested while Sulfamethazine (SMZ) tested positive for all the dilutions tested except 10^{-7} in which all the five (5) ampules tested negative (Table 4.7).

For the sterilised samples, Streptomycin Sulphate, Sulfamethazine and Penicillin G Sodium Salt tested positive for all dilutions and ampules tested. For Oxytetracycline hydrochloride, dilutions of 10^{-4}and10^{-5} tested positive for all the ampules tested while at $10^{-6}$and 10^{-7}dilutions, only one (1) ampule tested slightly positive for both dilutions (Table 4.8).All the positive results obtained at 10^{-7} dilutions were very faint, indicating that the concentration of the drug is very low.

Table 4.7: Effect of pasteurisation on antimicrobial residues in milk treated at 63^0C for 30 minutes in Zaria

Antibiotics	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	TDL (ppb)
Oxytetetracycline hydrochloride (1×10^8 ppb)	5	5	5	5	NT	NT	NT	100
Sulfamethazine (2.5×10^7 ppb)	5	5	5	0	NT	NT	NT	25-50
Streptomycin Sulphate (2×10^8 ppb)	5	5	5	5	NT	NT	NT	300-500
Penicillin G Sodium Salt (4×10^7 ppb)	5	5	5	5	NT	NT	NT	1 - 2

Key

NT: Not tested

TDL: Test detection limit

Shaded cell:Maximum residue level (MRL) as permitted by FAO/WHO JECFA

ppb: parts per billion

Source of test detection limit: *www.dsm-foodspecialties.com*

Table 4.8: Effect of sterilisation on antimicrobial activity of residues in milk treated at 121^0C for 15 minutes in Zaria

Antibiotics	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**	**TDL (ppb)**
Oxytetetracycline hydrochloride (1×10^8 ppb)	5	5	1	1	NT	NT	NT	100
Sulfamethazine (2.5×10^7 ppb)	5	5	5	5	NT	NT	NT	25-50
Streptomycin Sulphate (2×10^8 ppb)	5	5	5	5	NT	NT	NT	300-500
Penicillin G Sodium Salt (4×10^7 ppb)	5	5	5	5	NT	NT	NT	1 - 2

Key

NT: Not tested

TDL: Test detection limit

Shaded cell:Maximum residue level (MRL) as permitted by FAO/WHO JECFA

ppb: parts per billion

Source of test detection limit: *www.dsm-foodspecialties.com*

CHAPTER FIVE

5.0 DISCUSSION

The result showed that the overall prevalence of antimicrobial residues in milk in Zaria was 14.7% which is high for raw cow milk in Zaria. Similar studies in Kenya (Kang'ethe *et al*., 2005; Shitandi, 2004 and Shitandi and Sternesjö, 2004) have shown a range of prevalence of 9 – 16 % which is compareable to the result obtained in the present study. In Tanzania, antimicrobial residues weredetected in 36% of marketed milk samples frommilk supply chains in and around Mwanza andDar es Salaam during 1999 and 2000(Kurwijila *et al.,* 2006). In Ghana, a report showed that 35% ofthe raw milk marketed in two major cities,Accra and Kumasi, were contaminated withantibiotics (Aning *et al.,* 2007). Additionally, 3.1% of the raw milksamples contained antibiotic levels above theEuropean Union maximum residue limit.In South Africa, a survey was done in1977/78 to investigate the incidence of antibioticcontamination in milk marketed in the marketsof Pretoria. In milk from 1081 cattle herds, 60tankers, and 112 pasteurized batches, antibioticswere found in 2.13% of the herd samples, 11.7%of the tanker samples, and 2.1% of thepasteurized samples (Bester and Lombard, 1979).

The degree of contamination of milk and dairy products with antmicrobial residues differs, depending on the level of legislation and effectiveness of methods in different countries (Ergin Kaya and Filazi, 2010). The health risk associated with antimicrobial residues in milk will continue to exist in Zaria and Nigeria as a whole, until an effective residue monitoring program is legislated (Kabir *et al*., 2004).

The overall prevalence of 14.7 % recorded in the present study also indicates that consumers of locally produced milk are exposed to milk samples with unacceptable levels of antimicrobial residues. At such a high prevalence estimate, thisshows that the probability of surpassing the

acceptable daily intake (ADI) for the various drugs is high, depending on the frequency of consumption, quantities consumed and concentration of the residues in milk - which this study did not quantify. The highest risk of ingesting residues above the ADI levels prescribed by the Codex Alimentarius Commission was deemed to be for penicillin G-type or beta-lactam antibiotics due to their lowADI of only 30 μg/day and the high frequency of usage of these antibiotics locally, especially in the treatment of mastitis (Aning et al., 2007).

This study has exposed a potentially serious public health problem for consumers of locally produced milk in Zaria. It is conceivable that repeated exposure over long periods to such levels of contamination of milk may lead to mass antimicrobial resistance within communities (Oslon and Sanders, 1975), thus leading to the need for the populace to resort to purchase of more expensive drugs than they can afford.

Nowadays, rapid test kits (microbiological or immunoreceptor-based) for the common antimicrobial drug residues in milk are increasingly used and the risk of milk contamination not detected by the available screening test is very limited. (Serraino et al., 2013). The commercial test kit provides a quick and efficient screening test for drug residues and can be used in samples containing natural inhibitors (e.g. lysosomes).

There is a significant association ($P < 0.05$) between occurrence of antimicrobial residues and drug use. This is in line with a study carried out by Fagbamila *et al*. (2010) who, concluded that 33.3% of farms were not adhering tothe recommendations on veterinary drug use.This may be because there has not been any residue monitoring program put in place by the governmentneither has there been a deliberate effort to sensitize thepopulace on the dangers associated with residues inanimal products (Kabir *et al*., 2004).

There is a significant association ($P<0.05$) between the level of drug use in the villages sampled and occurrence of antimicrobial residues in Zaria. This could indicate a variation in non prudent use of antimicrobial drugs by cattle owners in these villages. The ages of cows, parity, stage of lactatation and clinical condition (active clinical mastitis) did not significantly ($P>0.05$) influence the prevalence levels of antimicrobial residues in the study area. The reason for this may be because, even though the 300 animals sampled within Zaria, are located at and serve various zones, the consumers and commercial raw milk dealers can purchase raw milk obtained from any animal irrespective of the age, breed, parity, stage of lactation and clinical condition. This indicates that non observance of withdrawal requirements following drug therapy- the main factor considered to underlie the presence of the residues - may be unrelated to variations in these factors (Fagbamila *et al*., 2010, Omeiza *et al*., 2012). The high prevalence proportions that were observed call for an urgent need for a greater level of awareness among farmers regarding withdrawal requirements following antimicrobial therapy.

The result of the experimental study showed that for Penicillin G Sodium salt, no loss in activity was detected at all the concentrations tested for both the pasteurised and sterilised spiked milk. A similar result was observed for Oxytetracycline where no loss of activity was noticed for the pasteurised milk while a low inactivation was noticed for the sterilised milk at dilutions of 10^{-6} and 10^{-7}. This is in agreement with a study carriedout by Berruga *et al.,(* 2006) with a slight variation where they observed that Penicillin and tetracycline are averagely stable in low pasteurisation and ulrta high temperature (UHT), and unstable in conventionalsterilisation.

Moats (1999) indicated that milk samples with Penicillinreach low percentages of inactivation (8%) for a treatment at 62ºC for 30 minutes. Theinactivation percentages of Penicillin were also

higher in other works, between 20-60%, when the milk sampleswere heated to temperatures over 100ºC (Pilet *et al*., 1969; Konecny, 1978).

As for the Oxytetracycline, the result obtained is comparable to a study carried out by Moats (1999) where similar reductions in theactivity of oxytetracycline (23.6%) was obtained with pasteurisation treatments (62ºC for 30 minutes). Likewise, in other studies, it is indicated that high temperatures over 100ºCproduce inactivation percentages of between 75-100% for Oxytetracycline (Sanz et al., 2002) and tetracycline (Pilet *et al*., 1969).

Sulfamethazine (SMZ) was observed to be stable after both pasteurisation and sterilisation with a very slight reduction in activity after pasteurisation (10^{-7}). This is supported by Hsieh *et al*. (2011) who observed that the sulfonamides are thermotolerant. It is also in agreement with Papapanagiotou *et al*. (2005) and Rose *et al*. (1995) who use similar method to analyse SMZ.

Streptomycin sulphate was also found to be stable after both pasteurisation and sterilisation for all the dilutions tested. Due to lack of data on the effect of heat treatment on streptomycin, the result was compared using the general class (Aminoglycosides). The result from this studies is in agreement with previous studies by Traub and Leonhard (1995) who suggested that chloramphenicol, aminoglycosides, quinolones, clindamycin, novobiocin, trimethoprim, vancomycin, and azlocillin are heat-stable.

It is important to note that unlike health risks fromzoonotic pathogens that may contaminate milk, antimicrobialagents in milk cannot be eliminated by heat treatment. (Aning et al., 2007).

Antibiotics of the same class were also reported to have different heat stabilities depending on different types of matrices and heat treatments involved (Kitts *et al*., 1992; Rose *et al*., 1996; Franje *et al*., 2010). Most of the findings and the conclusions drawn were summarized by Moats (1999).

CHAPTER SIX

6.0CONCLUSION AND RECOMMENDATION

6.1 Conclusions

There is high level of contamination of milk produced by cattle in Zaria and environ with antimicrobial residues. The residues were more likely to occur in herds or animals that have been treated with antimicrobial drugs. When antibiotic residues occur in milk, their activity remain intact with partial reduction during pasteurisation or in the production of UHT milk. Failure to observe antibiotic withdrawal periods by cattle rearers in Zaria and environ will likely expose consumers to products containing residues above tolerable limits.

6.2 Recommendations

1. There is need for the creation of awareness among farmers, consumers and policy makers on antimicrobial residue contamination of foods of animal origin and of the health hazards.
2. A national monitoring program of foods of animal origin is required in order to establish safe and tolerable limits.
3. Farmers and animal health workers should observe drug withdrawal periods at all times.
4. Socio-economic analysis of non-compliance would be required to understand the potential incentives that would lead to behavioural change among farmers.
5. Further research on the effect of temperature on antimicrobial residues should be carried out.

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APPENDICES

APPENDIX I

SAMPLE COLLECTION FORM

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA

DETERMINATION OF ANTIMICROBIAL RESIDUES AND THE EFFECT OF HEAT TREATMENT ON EXPERIMENTAL ANTIMICROBIAL RESIDUES IN FRESH COW MILK IN ZARIA, NIGERIA

SAMPLE COLLECTION FORM

Sample Number: _____Date: _____

Location:_____ Village_____ Ward_____

Type of Sample: **Raw Milk**

Source: **Milking Cow.**

Age: _____ Breed: _____

Parity _____ weeks into lactation _____

Clinical condition:

_____
_____

History of antibiotic administration within the last four weeks:

_____
_____

Any Other Information:

_____
_____

Sign: _____