"ANTIMICROBIAL RESIDUES DETECTED IN MARKETED MILK IN URBAN AND RURAL AREAS IN KENYA"

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This thesis is dedicated to my parents Mr. and Mrs. Aboge.

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ABSTRACT

Anti-microbial agents are widely used in Kenya for the treatment of diseases in both man and animals. Of major public health concern is the possible presence of anti-microbial residues in milk that is not withheld from human consumption or due to addition by market agents as preservative. This is aggravated by inadequate national anti-microbial residue surveillance and control programme in Kenya. A study was therefore conducted to establish the extent of contamination of marketed milk by anti-microbial residues in urban and rural areas in Kenya.

Respondents were randomly selected and questionnaires completed in consumer households and market agents from Nairobi, Kiambu, Nakuru and Narok districts. A total of 916 raw and processed milk samples were collected seasonally between January 1999 and January 2000 from the four districts and analyzed for anti-microbial residues using CharmAIM96 test. Of the 916 milk samples analyzed, 348 samples were collected from consumer households while 458 samples were from informal market agents of various cadres. The remaining 110 samples screened were processed milk from Nairobi and Nakuru districts.

Positive milk samples on Charm AIM96 were screened for tetracyclines and beta lactam antibiotics using Charm ROSA tests. Simple tests of association were first used to assess the relationship between the presence of anti-microbial residues in rural and urban households milk samples. A logistic regression model was thereafter fitted to evaluate risk factors associated with anti-microbial residue presence in marketed milk. Test agreement between the CharmAIM96 and Charm SL tests, for analysis of anti-microbial residues in milk was assessed. In addition, the Charm ROSA test was experimentally validated.

The Charm AIM96 test showed that 41(11.8%) and 25(5.4%) of milk samples from consumer households and market agents had anti-microbial residues above the FAO/WHO Codex MRL respectively. Nine out of 110(8.2%) pasteurized milk samples had resides above the FAO/WHO Codex-MRL. None of the consumer and market level milk samples was positive on the Charm ROSA tests. The proportions of rural and urban consumer household samples with anti-microbial residue above the FAO/WHO Codex-MRL were 18.7% and 5.1% respectively. This was a significant difference at 95% confidence level in proportions of rural and urban consumer household samples with detectable residues level (X^2=15.5: P value=0.000).

The proportions with the residues above the FAO/WHO Codex-MRL decreased with increasing levels of milk bulking with small mobile traders, milk bars and milk shop kiosks having 8.5%, 6.4% and 4.2% respectively. Season, farming systems, sales volume, and market access and channels were not associated with anti-microbial residue presence in marketed milk (P >0.05). Charm AIM96 and Charm ROSA tests detected penicillin G and oxy-tetracycline residue in all the eight milk samples collected up to 48 hours after treatment at level above 5ppb and 125ppb respectively. Charm ROSA tests detected oxy-tetracycline, penicillin G and amoxycillin at levels above 125ppb, 5ppb and 3.1ppb respectively.

In conclusion, anti-microbial residues were more likely to have originated at farm level. The sensitivities of Charm SL tests obtained from this study were in agreement with what was indicated by Charm Sciences the manufacturers of Charm ROSA kit. Although Charm AIM96 and Charm ROSA tests detected penicillin G or oxy-tetracycline residues in post-treatment milk samples up to 72 hours, the

detection agreement between the two tests was low for samples collected beyond the third day after treatment, and inconclusive for field milk samples tested.

CHAPTER ONE:

1.0 GENERAL INTRODUCTION

1.1 Background information

Anti-microbial drugs are a group of chemical substances that inhibit the growth of microorganisms, and include antibiotics and sulphonamides. They have been used worldwide in the treatment and prevention of diseases in both man and animals. Anti-microbial families that have been used include amino-glycosides, beta-lactams, sulfa drugs, tetracyclines, macrolides, floroquinolones, lincosamides and amphenicols. Others are tiamulin, polymixins and nitrofurans. In Kenya, the most commonly used veterinary anti-microbial are tetracyclines, sulfa drugs, beta-lactams amino-glycosides and nitrofurans.

Over the years, there has been much concern about the possible hazards to human health associated with contamination of milk with anti-microbial residues after misuse. Some of the health problems reported in man are hypersensitivity, as in the case of penicillin, and drug resistance (Oslon and Sanders, 1975). Hypersensitivity reaction (mainly dermatitis) after consumption of penicillin G contaminated milk by man has been reported (Dewdney and Edwards. 1984). Other toxicological effects on human health include bone marrow depression and the "grey syndrome" in newborn infants (Prescott and Baggot.1988b). The residues in milk are also of special importance to industrial milk fermentation process used in cheese and yogurt marking.

In this regard, FAO/WHO Codex Alimentarius have recommended the Maximum Residue Limit (MRL) for various anti-microbials in animal food products including milk. They have recommended that the MRL for oxy-tetracycline and

chlortetracycline in milk be 100ppb and that of penicillin G and sulphamethazine in milk be 4ppb and 100ppb respectively (FAO/WHO, 1996). Anti-microbial residues in milk above these MRL are considered as unsafe for human consumption.

Anti-microbial residues can result in milk following treatment of mastitis (Suliman *et al.*, 1990; Anderson *et al.*, 1998), intravenous injection of lactating cows (Roudant and Moreitain, 1990) and feeding of dairy cows with feeds contaminated with the drugs (McEvoy *et al.*, 2000). After administration, the drugs are absorbed into the blood stream, and are distributed and metabolized in the body, then excreted through milk resulting in residues in milk. Contamination of milk can also occur by deliberate addition of anti-microbial to milk by market agents to prolong shelf life

Since the liberalization of milk marketing in Kenya in 1992, the proportion of raw milk sold in urban centres has markedly increased, thereby raising public health concerns (Omore *et al.*, 1999). Besides residues that may result from lack of adherence to withdrawal times following therapy, there have been concerns that some anti-microbial agents may be added to informally marketed milk to extend its shelf life. This study was therefore conducted to test for anti-microbial agents in milk marketed by various market agents in Kenya using Charm AIM96 and Charm ROSA (Charm sciences Inc. USA) kits. Charm AIM96 was used to detect a wide range of anti-microbial agents while Charm ROSA was used to test beta-lactams and tetracyclines specifically at levels above the EU or FAO/WHO Codex Alimentarious Maximum Residue Limits.

1.2. General objectives

The general objective of the study was to assess the presence of anti-microbial residues present in marketed milk in Kenya.

1.3 Specific objectives

The specific objectives of the study were,

(i) To detect the presence of a range of anti-microbial residues, and tetracycline & beta-latam antibiotics specifically, in marketed milk in Nairobi, Kiambu, Nakuru, and Narok districts

(ii) To evaluate risk factors associated with anti-microbial residues in marketed milk in the target districts and make recommendations for reducing health risks from such residues.

1.4. Importance of the study

Anti-microbial residues in animal food products including milk are undesirable because of the ill health effects that they cause. These include hypersensitivity, resistance, carcinogenesis and bone marrow depression. Therefore, this study was important in generating information on the state of milk contamination by anti-microbial residues in the Kenyan milk market. Such information can be a basis for establishing national anti-microbial residue surveillance and control policies in Kenya with an aim of protecting consumers from these health hazards.

CHAPTER TWO:

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

Anti-microbial drugs are a group of chemical substances that inhibit the growth of microbial agents. They include various antibiotics and sulphonamides used in the treatment of both animal and human diseases. There are seven major families of anti-microbial drugs including sulphonamides, beta-lactams, amino-glycosides, tetracyclines, amphenicols, macrolides/ lincosamides and flouroquinolones. Other anti-microbials include novobiocin and spectinomycin.

The widespread use of anti-microbial drugs in human and veterinary medicine started in mid-40s after the discovery of benzyl-penicillin in 1929 by Alexander Fleming (Bywater, 1991). Streptomycin, an amino-glycoside was first introduced in 1944 while chloramphenicol and chlortetracycline were introduced in 1947 and 1948 respectively. Later on, semi-synthetic penicillins and cephalosporins were introduced in 1958 and 1960s respectively. The most recent anti-microbial, the flouroquinolones were developed in 1980s(Bywater, 1991).

The main sources of anti-microbial agents include; mould and fungal metabolites such as *Penicillium* spp and *Streptomyces* spp, synthetic chemistry, bacteria (*Bacillus* spp) and semi-synthetic derivatives of natural products such as amoxycillin derived from benzyl-penicillin nucleus (Bywater, 1991). Chlortetracycline was isolated from the fungus *Streptomyces aureofaciens* by Duggar in 1948 (Dornbush and Abbey, 1972). Other antibiotics isolated from fungi are streptomycin and benzyl-penicillin.

The undesirable health effects of anti-microbial residue in man include hypersensitivity, resistance problem, carcinogenesis and bone marrow depression as in

the in case of chloramphenicol (Prescott and Baggot.1988b: Prescott and Baggot.1988c).

2.2. PHYSICAL AND CHEMICAL PROPERTIES OF ANTIMICROBIAL DRUGS

2.2.1. Beta-lactams

Beta-lactam antibiotic, such as benzyl-penicillin, exist both as amorphous powder and as white crystalline substance, which is the sodium or potassium salts of the acid. Penicillin–G is soluble in water, but insoluble in fixed oils and paraffin. It has a salty taste and a faint mould like odour. Other beta-lactams like ampicillin and amoxycillin are sparingly soluble in water and are stable in acid solution (Bywater, 1991).

2.2.2. Aminoglycosides

Streptomycin, an amino-glycoside, is a white crystalline substance as benzyl-penicillin but has a slight odour and saline taste. It is very soluble in water but only slightly in alcohol, ether and chloroform, and is insoluble in fixed oils, and paraffin (Bywater, 1991). The compounds are stable over ranges of pH from 2.0 to 14. Other aminoglycosides like gentamicin and tobramycin are heat stable and can withstand boiling temperatures and even brief autoclaving (Gee, 1974).

2.2.3 Tetracyclines

Tetracyclines are amphoteric compounds that form salts with acids or bases. The bases are yellow, crystalline compounds that are odourless and slightly bitter. Aqueous solutions of oxy-tetracycline show appreciable loss of activity at elevated pH within 24 - 48 hours (Thompson, 1976).

2.2.4. Macrolides

Erythromycin, a macrolide, is a white or yellowish white crystalline powder that is oduorless and has a bitter taste. Unlike, penicillin and streptomycin, it is sparingly soluble in water but is freely soluble in alcohol and ether (Bywater, 1991). Erythromycin is a weak base with a bitter taste existing as hygroscopic crystals in solid state and is acid labile in solution (Wood and Wilson, 1974).

2.2.5. Amphenicols

Chloramphinecol, which belongs to the group known as amphenicols group has a bitter taste and is only slightly soluble in water but soluble in most organic solvents (Bywater, 1991).

2.3. CHEMICAL STRUCTURES OF ANTIMICROBIAL DRUGS.

2.3.1. Beta-lactams

Penicillins and cephalosporins belong to the beta-lactam drugs because both shares a four member beta-lactam ring in their structures, whereas penicillin structure is based on 6- amino-penicillanic nucleus and cephalosporins structure has 7- amino-cephalosporanic acid nucleus. Penicillin nucleus has a five-member thiazolidine ring that is relatively stable. However, the beta-lactam ring is unstable and is prone to cleavage by chromosomal class enzymes (beta-lactamase) leading to development of resistance (Bywater, 1991).

The chemical structure of benzyl-penicillin that belongs to beta-lactam group is shown in Figure1.

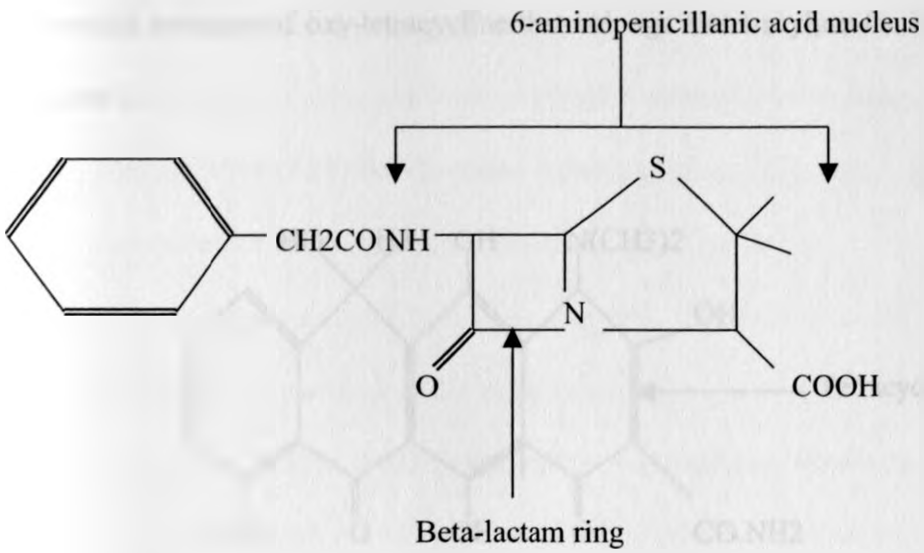


Figure 1: The structure of benzyl-penicillin with 6-aminopenicillanic acid nucleus and beta-lactam ring.

2.3.2. Aminoglycosides

Streptomycin and dihydrostreptomycin, both amino-glycosides, have a glycoside and cyclitol ring with amino groups in their structures. Different parts of the aminoglycoside molecule can be attacked by enzymes produced by bacteria such as aminoglycoside acetyltransferase and aminoglycoside phosphotransferase thus destroying the antibiotic resulting in resistance (Bywater, 1991).

2.3.3. Tetracyclines

The chemical structure of the tetracycline group is based on the tetracycline nucleus. The differences in structures among individual members in the group result

only from substitution of atoms attached to the nucleus with others. For example, the hydrogen atom attached to the carbon atom-7 in the nucleus in oxy-tetracycline is replaced by chlorine atom in the case of chlortetracycline (Bywater, 1991). The chemical structure of oxy-tetracycline that belongs to tetracyline family is shown in Figure 2.

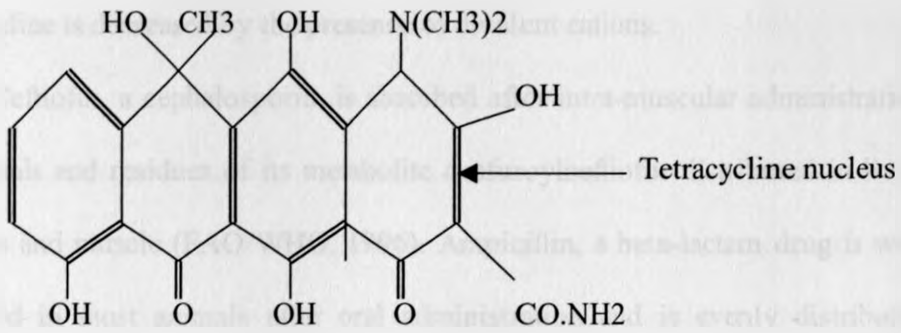


Figure 2: The structure of oxy-tetracycline with tetracycline nucleus that forms the basis of tetracyclines.

2.3.4. Sulphonamides

The sulphonamide group has a para-aminobenzoic acid nucleus in their structure that is essential for antibacterial activity (Bywater, 1991).

2.3.5. Amphenicols

For amphenicol group, the compounds are derived from nitrobenzene and dichloroacetic acid. Among the four isomers of chloramphenicol only the D-threo form has anti-microbial activity (Bywater, 1991).

2.4. PHARMACOKINETICS OF ANTI-MICROBIAL DRUGS.

2.4.1. Absorption and distribution

After oral and parenteral administration, anti-microbial drugs are absorbed and distributed in the body. Chlortetracycline and tetracycline are both rapidly absorbed and quickly cleared from edible tissues of meat animals following oral administration (FAO/WHO, 1996). Like chlortetracycline, the oral absorption of tetracycline is decreased by the presence of divalent cations.

Ceftiofur, a cephalosporin, is absorbed after intra-muscular administration in animals and residues of its metabolite desfuroylceftiofur distributed in liver, kidneys and muscle (FAO/WHO, 1996). Ampicillin, a beta-lactam drug is well absorbed in most animals after oral administration and is evenly distributed throughout the body tissues and then concentrated in the liver and kidneys. For amino-glycosides, such as neomycin, absorption occurs after intra-muscular injection and then penetrates blood-brain barriers. It is only slightly absorbed from the intestine and not at all from the skin (Bywater, 1991).

The parent sulfonamides are well absorbed into the bloodstream after oral or topical administration, and diffuse widely into the tissues, penetrating into all fluids including the central nervous system (CNS), urine, bile and milk (Bywater, 1991).

2.4.2. Metabolism and Excretion.

FAO/WHO (1996) reported that ceftiofur, a beta-lactam drug, was rapidly metabolized to desfuroyl-ceftiofur and excreted in urine and faeces following intra-muscular administration in cattle. The other metabolite of ceftiofur is furoic

acid. The residues of the metabolites have also been demonstrated in milk of a lactating cow.

In another study, FAO/WHO (1996) reported that chlortetracycline and tetracycline undergo minimal metabolism. They are eliminated in both the urine and faeces, either unchanged or in microbiologically inactive form after administration in animals. Chlortetracycline residues were also detected in milk of lactating dairy cow following intra-mammary and intrauterine administration. Penicillin G is excreted in milk of cows treated with the drug subcutaneously at an extra-label dose (Krainock, 1991).

The metabolism of sulphonamides takes place with acetylation, oxidation and glucuronidation occurring to varying degrees. Acetylation is the most important route. The unbound sulphonamide is excreted by the glomerulus whereas the ionized molecules of sulphonamides are excreted actively in the proximal tubules. Other excretion routes of anti-microbial drugs include sweat and biliary excretion (Bywater, 1991).

2.5 MODE OF ACTION OF ANTIMICROBIAL DRUGS.

Anti-microbial drugs inhibit the synthesis of nucleic acid and protein in microbes. Others interfere with the formation of bacterial cell wall and cell membrane. Sulphonamides indirectly prevent replication of the nucleic acids of the bacterial cell by inhibiting bacterial dihydrofolate reductase enzymes.

Aminocyclitols, tetracyclines, chloramphenicol and macrolides all act by interfering with the synthesis of the protein combination necessary for bacterial growth. Tetracyclines inhibit bacterial cellular metabolism by blocking the attachment of amino-acyl transfer RNA to ribosomes. This interferes with protein

synthesis and destroys the cell membrane (Schnappinger and Hillen, 1996). Penicillins and cephalosporin affect cell wall synthesis resulting in abnormal cell growth leading to filamentous forms of bacteria or spheroplast and cell death. Some anti-microbial drugs such as colistin interfere with cell membrane formation in bacterial cell (Bywater, 1991).

2.6 ASSAY OF ANTIMICROBIAL RESIDUES IN MILK.

A number of laboratory techniques have been used to detect anti-microbial drug residues in bovine milk. The techniques are mainly grouped into microbiological and chemical methods.

2.6.1. Microbiological Assay Method.

Schiemann. (1976) assessed bovine milk for inhibitory substances by using microbiological assay method. The technique was based on disc assay method utilizing *Bacillus subtilis* (ATCC6633) as test organism. The test had a detection level of 0.0125 unit of penicillin per milliliter of milk. Other methods have been based on the use of *Staphylococcus aureus* Oxford strain (NCTC 6571) (Ombui, 1994) and *Bacillus cereus* ATCC11778 *mycoides* (Nouws *et al.*, 1998) as the test organisms. The detection limits for *B. cereus* test plate was 30ng /ml of milk for oxy-tetracycline and tetracycline residues and 10ng /ml of milk for chlortetracycline and doxycline residues (Nouws *et al.*, 1998).

Recently, Charm Sciences Inc. (USA) introduced microbial inhibition tests for the assay of anti-microbial residues in bovine milk. The different types of tests include; Charm AIM 96, Charm Farm test, Charm Rapid Inhibition Test (RITe) and *Bacillus stearothermophilus* Tablet Disc Assay Test. Charm AIM96 is designed for high volume, broad-spectrum screening of raw, pasteurized,

homogenized or skim milk at or above European, Maximum Residue Levels (MRL) (Charm Sciences, 1995).

The results can be read by visual color comparison or optionally with a micro-plate reader. In a single assay, the Charm AIM 96 detects beta-lactams, sulfa drugs, tetracyclines, amino-glycosides and macrolides. This test has the advantage of running 45 samples (induplicate including controls) simultaneously in approximately 4 hours. However, the test cannot be used to test specific or individual anti-microbial families. Table 1 shows the sensitivities of charm Aim96 test for various anti-microbial agents.

Table 1: Detection levels of Charm Aim96 test compared to European Union/Codex Maximum Residue Limit (EU/CodexMRL).

Positive test

	Colour 4	**Colour5**	**EU/Codex MRL**
Penicillin G	3ppb	4ppb	4/4ppb
Amoxicillin	4pbb	5ppb	4ppb
Ampicillin	3ppb	4ppb	4ppb
Ceftiofur metabolite	100ppb	200ppb	100ppb
Cephapirin	10ppb	20ppb	10ppb
Cloxacillin	30ppb	50ppb	30ppb
Dicloxacillin	25ppb	30ppb	30ppb
Oxacillin	10ppb	20ppb	30ppb
Sulfamethazine	25ppb	50ppb	100/25ppb
Gentamicin	35ppb	50ppb	100/100ppb
Oxytetracycline	200ppb	300ppb	100/100ppb
Tylosin	25ppb	50ppb	50ppb

The Charm Farm Test is a broad screening assay for five families of drugs, including beta-lactams, sulfa drugs, tetracyclines, amino-glycosides and macrolides in raw, commingled bovine milk. The results can be read by visual color comparison or optionally with a PH meter and are stable for 8 hours after assay completion. Up to 12 tests can be run simultaneously and the assay can be completed in approximately 3.5 hours.

The Charm Rapid Inhibition Test (RITe), is an inhibition assay based on bacteria cultured in milk which generates acid resulting in colour change that is measured by visual color comparison or optionally with PH meter. The test can be completed in approximately 2 hours. The *Bacillus strearothermophilus* Tablet Disc Assay is used to detect penicillin- G, amoxycillin, ampicillin and cephapirin. The tablet format eliminates spore counting, glass ampoules and other disadvantages to the standard disc assays

Nouws *et al.* (1999) used a microbiological multi-plate system to detect anti-microbial residues in raw milk. The multi-plate system detected residues of beta-lactam antibiotics, tetracyclines, aminoglycosides, macrolides, sulphonamides, colistin and quinolones in raw milk. They found the system to be a reliable method that can be performed easily and cheaply in microbiological laboratories.

2.6.2. Chemical Analytical Methods.

Anti-microbial residues in milk have been assayed using chemical analytical techniques such as liquid chromatography (L.C.) with post column derivatization and fluorescence detection to determine tetracycline residues in milk (Pena *et al.*, 1999). The recoveries for tetracyclines (Oxy-tetracycline, tetracycline and chlortetracycline)

exceeded 80% at all levels and precision was good. Tetracycline residues in bovine milk have been determined by sensitive spectrofluorimetry (Croubels *et al.*, 1994). The detection limits were 1ng /ml for oxy-tetracycline, 2ng / ml for tetracycline and 4ng/ml for chlortetracycline. Boison et al., (1994) also used Liquid Chromatography to detect penicillin G residues in milk. In this case, the detection limit for the antibiotic was 3ppb in fluid milk.

Other methods used include conductimetric method used for penicillin G residues detection in milk (Chen and Chang, 1994) and metal chelate affinity chromatography for multiple tetracycline residues detection (Carson, 1993). Moats. (1990) Used high performance liquid chromatography with automated liquid chromatography clean up to determines penicillin G in milk. Recoveries of 92 % were achieved and a sensitivity limit of 2ppb was reported.

Haagsma and Mengerler (1989) used fluorimetry in the screening of chlortetracycline, oxy-tetracycline and tetracycline in pig meat. The complex aromatic structure of the tetracycline emits a golden yellow light when exposed to U.V light. This method does not discriminate between the individual tetracyclines. Ryan and Dupont (1974) and Markakis, (1996), used paper and thin-layer chromatography for separation of tetracyclines.

Radioimmunoassay has been used to detect oxy-tetracycline residues in milk (Anderson *et al.*, 1995 and Moats *et al.*, 1995). Spectrophotometer has been used in the determination and evaluation of tetracyclines (Salinas *et al.*, 1989, Vetuschi and Ragno, 1990, Efimovskiks and Anokhina, 1991, Nabi *et al.*, 1997 and Healy *et al.*, 1997). In this method, the sample solution absorbs electromagnetic radiations from an appropriate source the amount absorbed is related to the concentration of the analyte in the sample solution.

2.6.3. Other Assay Methods

Charm Sciences Inc. (1999a,b) introduced Charm SL beta lactam test and Charm SL tetracycline test for detection of beta lactams and tetracycline residues in milk respectively. The Charm SL beta–lactam test uses receptors that bind to the beta lactam ring (*See figure 1*) and detects beta lactam drugs at or below the U.S. tolerance level. The Charm SL tetracycline test uses antibodies that bind to tetracycline nucleus (*See figure 2*) and detects tetracyclines at or below U.S. safe levels (300ppb for chlortetracycline, oxy-tetracycline and tetracycline).

Enzyme immunoassay for the detection of isoxazolyl penicillin antibiotic residues in milk has been reported (Usleber *et al.*, 1994). Polyclonal antibodies were raised against isoxazolyl penicillin in rabbits after immunization with a cloxacillin – human serum albumin conjugates. The test detected cloxacillin and dicloxacillin residues in milk at 10 and 30ng/ml respectively. The average recoveries were 102% for cloxacillin and 84% for dicloxacillin. A test based on inhibition of beta-galactosidase enzyme biosynthesis in *Escherichia coli by* tetracycline residues in milk has also been reported, validated and applied to field samples contaminated with tetracyclines (D'Haese *et al.*, 1997).

Aureli *et al* (1996) identified sulfonamide and antibiotic residues in milk by using microbial inhibition test. The antibiotics identified were penicillin, cephalosporins and streptomycin. Tetracycline residues have been determined in bovine milk using capillary electrophoresis following treatment of dairy cows with the drugs (Chen and Gu, 1995). Zeng *et al.* (1996) also screened penicillin G and cephapirin residues in goat milk following treatment with drugs using Delvotest P, Penzyme test and *Bacillus stearethermophilus* var *calidolactis* disk assay (BsDA).

Penzyme test was found to be highly sensitive and specific that gives quick results within 20-25 minutes.

The detection levels in parts per billions (ppb.) of various assay methods for anti-microbial drugs are shown in Table 2.

Table 2: The detection levels in parts per billions (ppb) of various assay methods for anti-microbial drug residues in cow's milk.

Assay method	**Detection levels**	**Anti-microbial drug**
Microbiological assay based on *Bacillus cereus* test.	30ppb	Oxytetracycline (Nouws *et al.*, 1998)
	10ppb	Chlortetracycline (Nouws *et al.*, 1998)
	1ppb	Oxy-tetracycline (Croubels *et al.*, 1994)
Spectrofluorimetry	4ppb	Chlortetracycline (Croubels *et al.*, 1994)
High performance Liquid Chromatography (HPLC)	2ppb	Penicillin G (Moats, 1990)
Liquid Chromatographic method (LC)	5ppb	Oxy-tetracycline (White *et al.*, 1993)
	5ppb	Chlortetracycline (White *et al.*, 1993)
	5ppb	Tetracycline (White *et al.*, 1993)
Charm SL-tetracycline (ROSA) test	125-175ppb	Oxy-tetracycline (Charm Sc. Inc.1999b)
	225-275ppb	Chlortetracycline(Charm Sc. Inc.1999b)
	50-70ppb	Tetracycline (Charm Sc. Inc.1999b)
Charm SL-Beta Lactam (ROSA) test.	5ppb	Penicillin G (Charm Sc. Inc.1999a)
	6ppb	Amoxycillin (Charm Sc. Inc.1999a)
Delvo test	4ppb	Penicillin G (Zeng *et al.*, 1996)
Dutch tube test	150ppb	Oxy-tetracycline (Shitandi, 2000)
	1.5ppb	Penicillin G (Shitandi, 2000)
Charm II test	5ppb	Oxy-tetracycline (Anderson *et al.*, 1995)
Charm AIM96 test	3ppb	Penicillin G (Charm Sc. Inc.1995)
	150ppb	Oxy-tetracycline (Charm Sc. Inc.1995)

2.7 ANTIMICROBIAL DRUG RESIDUES IN ANIMAL FOODS.

Various anti-microbial drugs used in the treatment and control of animal diseases has been shown to occur in many animal foods such as milk, meat and eggs. The drug residues found in such foods are as result of oral or parenteral administration of the drug. For examples Britt *et al., (*1999) reported oxy-tetracycline residues in milk samples obtained from cows after treatment for papillomatous digital dermatitis. Sulphonamide residues have also been demonstrated in milk of dairy cows following intravenous injection (Roudant and Moreitain, 1990).

Suliman *et al.* (1990) reported the presence of antibiotic residues in milk following treatment of mastitis. In Kenya, Chewulukei (1978) demonstrated the presence of antibiotic residues in commercial milk received by the Kenya Co-operative Creameries from dairy co-operative societies for processing and subsequent marketing. In southern Ontario, one pasteurized milk (0.1%) and 50 raw milk samples (0.9%) were found to contain penicillin residues (Schiemann, 1976). Other workers have also reported the presence of antibiotic residues in meat (Mdachi and Murillla, 1991) and eggs (Yoshimura *et al.*, 1991). In this regard, FAO/WHO Codex Alimentarius have recommended the Maximum Residue Limit (MRL) for various anti-microbials in milk and other animal foods, which are shown in Table 3.

Table 3: The FAO/WHO Codex Alimentarius Maximum Residue Limit (MRL) for various anti-microbial residues in milk and other animal foods (FAO/WHO, 1999).

Anti-microbial drugs	FAO/WHO Codex-MRL
Penicillin G	4ppb
Oxytetracycline	100ppb
Sulfadimidine	25ppb
Spiramycin	100ppb
Streptomycin	200ppb
Neomycin	100ppb
Ceftiofur	100ppb
Chlortetracycline	100ppb

2.8. UNDESIRABLE EFFECTS OF ANTIMICROBIAL RESIDUES IN MILK

There has been concern about public health hazards from anti-microbial residues following treatment of animal diseases or intentional addition by market agents. The drugs can cause health problems when milk containing these residues is consumed.

2.8.1. Hypersensitivity.

Hypersensitivity reactions have been reported for many antibiotic families. Penicillin is associated with the highest rate of hypersensitivity. These reactions can be fatal if they are severe (Anderson, 1968,and Olson & Sanders, 1975).

2.8.2 Resistance Problems.

Microbial resistance to anti-microbial drugs may develop following exposure of microorganisms to sub-therapeutic levels of drugs or after consumption of animal food products containing the drug residues. Hyper-production of the chromosomal class C beta- lactamase and production of inhibitor-resistant TEM $_{(IRT)}$ enzymes by *Escherichia coli* have been found to be the most frequent mechanisms of resistance to amoxicillin-clavulanate (Leflon-Guibout *et al.*, 2000). The resistance to quinolone antibiotics has been shown to be due to active efflux pump and mutations in the bacterial gyrA genes (Piddock, 1995). Nijsten *et al.* (1996) demonstrated antibiotic resistance among *Escherichia coli* isolated from fecal samples of pig farmers. The resistant human *E. coli* isolated was resistant to streptomycin. Other studies have shown that cross-resistance exists within the tetracycline group of antibiotics. This means microorganisms that are resistant to one of the tetracyclines are frequently resistant to other compounds in this class (Brown, 1988, Sande and Mandell, 1990, Chopra *et al.*, 1992 and Roberts, 1996).

2.8.3. Gastrointestinal Disturbances.

In humans, after oral administration, tetracyclines can cause irritation of gastrointestinal tract. The seriousness depends on the dosage and on the type of tetracycline. The clinical signs include nausea, vomiting, abdominal discomfort and epigastric burning. Super infections can also occur following tetracycline administration, since they suppress the growth of the resident micro flora thus enhancing the proliferation of antibiotic resistant microorganisms such as *Candida albicans* (Sande and Mandell, 1990 and Corpet and Brugare, 1996).

2.8.4 Miscellaneous - Effects.

Schultz *et al.* (1963) reported susceptibility to liver damage in pregnant women following use of tetracycline. Tetracyclines, especially oxytetracycline have been reported to cause yellowing or browning of teeth and dental hypoplasia (Moffit *et al.*, 1974). Other effects include the implication of oxy-tetracycline in nephrotoxicoses of feedlot calves (Lairemore *et al.*, 1984). Non-dose related aplastic anemia caused by chloramphenicol in man and carcinogenesis caused by nitroimidazole are other potential hazards of antibiotic contamination in food. (Prescott and Baggot, 1988c)

2.9 USE OF ANTIMICROBIAL DRUGS IN VETERINARY MEDICINE IN KENYA.

Anti-microbial drugs have been used worldwide in the treatment and prevention of animal diseases. In Kenya, it has been shown that the major families of anti-microbials used to treat animal disease treatment are beta-lactams, tetracyclines, aminoglycosides, nitrofurans, quinolones and sulphonamides/trimethoprim combination. Others include tiamulin and macrolides (Mitema *et al.*, 2001). The mean anti-microbial consumption (Kg) per year in food producing animals in Kenya during the 1995 to 1999 period are given in the Table 4

Table 4: The mean anti-microbial consumption (Kg) per year in food producing animals in Kenya during the 1995 to 1999 period.

Anti-microbial family	Mean anti-microbial Consumption (Kg)
Tetracyclines	7975.38
Sulfonamides/Trimethoprim	3477.97
Nitrofurans	1125.96
Aminoglycosides	1081.58
Beta-lactam	904.76
Quinolones	93.76
Macrolides	34.56
Others (Tiamulin)	23.56

[Source: Mitema *et al.*, 2001]

CHAPTER THREE:

3.0 MATERIALS AND METHODS

3.1 SEASONAL SURVEY AND MILK SAMPLING.

The study was a part of a broader project carried out by MOARD/KARI/ International Livestock Research Institute/ Small holder Dairy (SDP) Project and Department of Veterinary Public Health, University of Nairobi to assess public health hazards associated with marketed milk.

3.1.1. Selection of study area

Respondents were randomly selected with the assistance of enumerators from the Central Bureau of Statistics offices in Nairobi and Nakuru. The selection was done within production system (extensive and intensive) and human population density (urban, peri-urban and rural) strata. Nakuru and Narok districts represented extensive production systems and low population density (also medium market access). Nairobi and Kiambu districts represented intensive production systems and high population density (also high market access). The characteristics of the selected areas showing Agro-Ecosystem Zone (AEZ) potential, animal population, farming system and type of animal kept are shown in Table 5.

Table 5: The characteristics of the selected areas showing Agro-Ecosystem Zone (AEZ) potential, animal population, farming system and type of animal kept

District	AEZ Potential	Market access	Cattle (Dairy) Pop ('000) [b]	Farming system
Kiambu+ Nairobi	High	High	262	Intensive
Nakuru (Njoro)	High	Medium	196	Extensive
Narok (North)	Medium	Low	65	Extensive

[b] Estimates derived from MALDM (1993), except for Kiambu, which is derived from MALDM/KARI/ILRI (1996)

These are a sub-set of sites selected for dairy systems characterization on the basis of AEZ and market access

3.1.2. Sampling of milk

3.1.2.1 Consumer level

Milk samples were collected between January 1999 and January 2000 from 212 and 172 raw (non-pasteurized) milk-consuming households in the dry and wet seasons from Nairobi and Nakuru districts. Out of the samples collected, 176 and 172 milk samples were tested in the dry and wet seasons respectively.

3.1.2.2 Market level

At the market, level 262 and 246 informal market agents were interviewed and milk samples collected from them during the two respective seasons. Out of the samples collected, 180 and 178 were tested in dry and wet seasons respectively. The informal market agents that were sampled included dairy co-operatives, milk bars, milk shops and mobile traders on foot, bicycle or motorized transport. Attempts were

made during the wet season to sample the same agent sampled in the dry season. Where this was not possible, substitution was done within the same locality.

One hundred and ten formally (pasteurized) marketed milk samples from Nairobi and Nakuru were also tested. The samples were transported in a cool box containing ice to the laboratory at the Department of Veterinary Public Health, University of Nairobi and stored at –20°C until analyzed.

The numbers of samples collected and analyzed in each study level are shown in Tables 6 and 7

Table 6: The numbers of samples targeted, collected and analyzed at consumer study.

(A) Consumer level

District	**Targeted HHs**		**Sampled HHs**		**Samples analyzed**	
	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2
Nairobi	53	53	50	52	42	45
Nakuru	199	199	162	170	130	131
Total	252	252	212	222	172	176

Key: HH represent households

Table 7: The numbers of samples targeted, collected and analyzed at market level.

(B) Market study

District	Target market agents		Sampled market agents		Samples analyzed	
	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2
Nairobi/Kiambu	173	170	162	170	160	151
Nakuru/Narok	89	76	87	76	74	73
Total	262	246	249	246	234	224

3.2 SCREENING OF FIELD MILK SAMPLES USING CHARM AIM96

The frozen milk samples stored in plastic tubes were allowed to stand in lukewarm running tap water for about 2 hours in order to thaw. The thawed milk samples were shaken for about 30 seconds to mix the separated cream with the rest of whey and screened for anti-microbial agents using a microbial inhibition assay described by Charm Sciences, Inc (1995). Briefly, the Charm AIM96 block was placed on a level position away from air circulation and all the heating block holes were filled with distilled water until water overflowed into the gutter while ensuring that each hole had a convex water level.

Fifty microlitres of each milk sample was dispensed into the wells of a 96 flat-bottomed microtitre plate by means of standard pipette. 50µl of negative control and positive controls consisting of 4ppb penicillin G, 50ppb sulfamethazine and 300ppb oxy-tetracycline were also dispensed into the wells. A vial of lyophilized medium and *B. strearothermophilus* spore tablet was dissolved in 22mls of distilled water, thoroughly mixed, and 200µl dispensed into each of well.

The plate was sealed with the special sealing tape and placed onto Charm AIM 96 incubator, and covered by a clear lid (supplied with multi well plate). The cover was secured by tightening the screws until finger tight and then unscrewed half turn. The Charm AIM96 incubator was turned on. The test was complete after 4 hours. The plate was removed and sealing tape carefully peeled off after drying the bottom of the plate with a paper towel.

The results were read by observing the colour of samples under cool white fluorescent light. Control and sample colours were compared with reference colours supplied with Charm AIM 96. The reference colours were given scores of 1,2,4 and 5. For valid tests, (positive control matching colours 4 or 5 and negative control matching colours 1 or 2) yellow colour was given a score of 1 or 2 and was negative for anti-microbial residues. Purple bluish colour was given a score of 4 or 5 and was positive for the residues.

Forty-five milk samples were analyzed in duplicate in a 96 flat-bottomed microtitre plate on each run. Fifty parts per billions of sulfamethazine reconstituted with 5mls of anti-microbial drug free milk determined using *Micrococcus lutea* inhibition assay and 4ppb of penicillin G reconstituted with 10mls of anti-microbial drug free milk in vials were used as positive controls. One tablet of negative control was dissolved in 0.5ml of de-ionized water and was used as negative control.

3.3. EVALUATING CHARM AIM 96 AND CHARM SL TESTS.

Charm AIM96 and Charm SL tests were evaluated before analyzing raw field milk samples for anti-microbial agents. This was achieved by testing raw milk samples collected from lactating cows treated with oxy-tetracycline and

beta lactam based drugs administered by intra-muscular and intra-mammary routes.

3.3.1 Treatment Of Experimental Animals.

Eight lactating adult cows in their late lactation stage were selected for the study. The cows were housed at the University of Nairobi, Veterinary Faculty farm. Milk samples were collected from the eight lactating cows on day zero before treatment with antibiotic drugs. The cows were there after injected once with therapeutic doses of oxy-tetracycline and penicillin G as shown in Table.8

Table 8: Treatment of cows with therapeutic doses of 10% oxytetracycline and penicillin G drugs

Cow's ID. No.	Breed	Drug administered	Administration route
234	Guansey	10% oxytetracycline injection	Intramuscular
173	Arshire	10%oxytetracycline injection	Intramuscular
228	Guansey cross	Penicillin G & Streptomycin injection	Intramuscular
092	Guansey	Penicillin G & Streptomycin injection	Intramuscular
166	Fresian	Oxytetracycline based intramammary (Multimast®)	Intramammary
076	Arshire/Guansey cross	Oxytetracycline based intramammary (Multimast®)	Intramammary
073	Guansey	Penicillin G based intramammary (Multiject®)	Intramammary
024	Fresian	Penicillin G based intramammary (multiject®)	Intramammary

Fifty millilitres of milk samples were collected after treatment from all the eight cows at 24-hour interval for a period of 120 hours (5 days) and transported in a cool box to the Department of Public Health, Pharmacology &Toxicology, Faculty of Veterinary Medicine for analysis.

3.3.2. Charm AIM 96 Screening and Charm ROSA Analysis.

Fourty eight milk samples collected from eight experimental lactating cows in six days were screened for antibiotic residues. A procedure outlined by Charm Science Inc (1995) and described in section 3.2 was used to test the samples for penicillin G and oxy-tetracycline residues in the milk samples collected.

All the 48 milk samples collected from the eight experimental cows were also tested for both oxy-tetracycline and penicillin G using Charm SL tetracycline and Charm beta lactam tests as described in Sections 3.5.1 and 3.5.2 (Charm Sciences, inc.1999). Agreements between the two tests were then determined.

3.4. DETERMINING THE DETECTION LEVELS OF CHARM SL TESTS.

The detection levels (sensitivity) of Charm SL tetracycline and charm SL beta lactam tests were determined in the laboratory. This was achieved by testing raw milk samples spiked with various dilutions of 20% oxy-tetracycline, benzyl-penicillin G (100,000i.u) and amoxycillin (150mg/ml) antibiotics.

Raw bovine milk samples were bought from the University of Nairobi, Faculty of Veterinary Medicine farm and tested using charm SL tests for the presence of anti-microbial residue. The anti-microbial free raw milk samples were spiked with oxy-tetracycline (200mg/ml), penicillin-G (100,000i.u) and amoxycillin trihyrade (150mg/ml) as follows;

Oxy-tetracycline (200mg/ml) solution was added into milk samples to obtain various dilutions of the drug ranging from 50ppm to 31.2ppb. A volume of 0.1ml(20mg) of 20% oxy-tetracycline solution was spiked into 39.9mls of antibiotic free raw milk to make 500ppm and shaken thoroughly before being allowed to stand for a few minutes. Milk sample containing 500ppm of oxytetracycline was further diluted in antibiotic free raw milk tenfold for three consecutive dilutions to obtain samples with 50ppm, 5ppm, and 500ppb of the antibiotic. The milk sample containing 500ppb of oxy-tetracycline was doubly diluted further in antibiotic free milk to get milk containing the following antibiotic concentrations; 250ppb, 125ppb, 62.5ppb and 31.2ppb.

Amoxycillin trihydrate (150mg/ml) was spiked into antibiotic free raw milk to get various dilutions of the drug ranging from 500ppm to 3.125ppb. A volume of 0.1ml (15mg) of amoxycillin trihydrate (150mg/ml) was spiked into 30mls of antibiotic free milk to make 500ppm. A sample containing 500ppm of the drug was serially diluted tenfold for four consecutive dilutions with milk (antibiotic free) to obtain dilutions containing 50ppm, 5ppm, 500ppb and 50ppb of the antibiotics. The sample containing 50ppb of the drug was again diluted with antibiotic free milk in doubling dilutions to give four consecutive dilutions containing 25ppb, 12.5ppb, 6.25ppb and 3.125ppb of the antibiotic.

Benzyl penicillin (100,000i.u, intra-mammary formulation) was spiked into antibiotic free milk to obtain various dilutions of the drug ranging from 16.7iu/ml to 0.001iu/ml of the drug. 0.1g(1.2mg) of the intra-mammary formulation was reconstituted with 12mls of antibiotic free milk to get 167ius/ml of the antibiotic. This was diluted ten fold with antibiotic free milk to obtain 16.7iu/ml of the drug. The sample was further diluted hundred fold and then ten

fold with antibiotic free milk to get 0.167iu/ml and 0.0167iu/ml of the antibiotic respectively. The samples containing 0.0167iu/ml of the antibiotic was diluted double fold for four consecutive dilutions to obtain milk samples containing 0.008iu/ml, 0.004 iu/ml, 0.002iu/ml and 0.001iu/ml.

Seven raw milk samples fortified with various oxy-tetracycline concentrations (50ppm, 5ppm, 500ppb 250ppb, 125ppb, 62.5ppb and 31.2ppb) including negative control were tested using charm SL tetracycline test (Charm Sc. Inc, 1998). For milk samples spiked with amoxycillin trihydrate and penicillin G, charm SL beta lactam test was used to test the spiked milk samples. For amoxycillin, milk samples containing concentration ranges of 50ppm, 5ppm, 500ppb, 50ppb, 25ppb, 12.5ppb, 6.25ppb, 3.125ppb and 1.56 ppb were tested. Spiking for penicillin G included the following range of concentrations 16.7 iu/ml, 0.167iu/ml, 0.0167 iu/ml, 0.008 iu/ml, 0.004 iu/ml, 0.002iu/ml and 0.001iu/ml.

3.5. ANALYSIS OF POSITIVE MILK SAMPLES USING CHARM SL TESTS.

The positive milk samples on Charm AIM 96 test were screened for both tetracyclines and beta lactam drugs using a procedure described by Charm Sciences, Inc. (1999). The frozen milk samples were thawed, centrifuged for 3 minutes at 1200g (see Appendix 6) and skim portion used for the test. The Charm ROSA incubator was placed at level position and temperature set at 56 degrees centigrade (temperature indicator green) before analyzing the milk samples. Five parts per billion of penicillin G and 300ppb of oxy-tetracycline standards were reconstituted with 10mls and 8mls of antibiotic free milk respectively. The reconstituted standards were used as positive controls for performance monitoring of Charm SL tests.

Tetracycline and beta lactam negative raw milk was used as negative control for the tests.

3. 5.1. Charm-SL-Beta-Lactam Test.

Four SL beta lactam test strips were placed in ROSA incubator with flat side facing up. The sample pad compartment for each of the strips were exposed by peeling the tape to edge of white label using the tab. Three hundred micro-litres of the positive milk samples was pipetted into either side well of the sample pad compartment. For each test strip, the tape was resealed over sample pad by pressing. The incubator lid was closed and latches tightened. The samples were incubated for at least 8 minutes and the test strips removed from incubator and the results visually inspected before reading the results using Rosa-Reader.

3.5.2. Charm SL Tetracycline Test.

Each of the milk samples were diluted 1: 2 with milk diluent buffer consisting of antibiotic free powdered milk and then mixed thoroughly. Three hundred microlitres of diluted milk samples were dispensed into either side well of the sample pad compartment. The sample pad compartments of the test paper strips were resealed. The incubator lid was closed and latches tightened. The samples were incubated for 4 minutes and the tetracycline test strips removed from incubator, and the results read visually by comparing control line and test line. The readings were further confirmed using Rosa-Reader.

3.5.3 The Criterion for Reading Results

For positive samples, the test line (T) was clearly lighter than the control line(C), or the test line (T) was partially coloured or test line (T) was absent. For

negative samples, the test line (T) was darker than the control line (C) or the test line (T) was the same as the control line(C). When the control line was absent or irregularly formed, then the test was considered to be invalid and was repeated. Samples that were visually positive were read using ROSA- reader.

3.6. STATISTICAL ANALYSIS

The data was entered into the spreadsheet editor of the Intercooled Stata 6.0 package (Stata Corporation 702 University Drive East College Station, Texas 77840 USA). Descriptive statistics of variables selected were determined by using the Stata intercooled package. The proportions of positive milk samples from rural and urban household were tested statistically for any significant difference at 95% confidence level by performing Pearson chi-square test.

A Logistic regression model was fitted using Stata Intercooled Package to assess the relationship between outcome variable and explanatory (independent) variables. The outcome variable was the proportion of milk samples with detectable levels of anti-microbial agents. The explanatory (independent) variables were season, farming systems (intensive/non-intensive), sales volume, trader type, bulking number, and market channels. Milk sources and trader types were used to establish various market channels. Market channels with less than two observations were dropped and therefore not regressed

CHAPTER FOUR:

4.0 RESULTS

4.1 RESULTS OF SCREENING FIELD MILK SAMPLES

4.1.1 Consumer survey

In consumer survey, 41 samples (11.8%) of the 348 milk samples had detectable levels of anti-microbial residues when analyzed using charm AIM96 test. Five samples (5.7%) from Nairobi and 36(13.9%) from Nakuru had anti-microbial residues above FAO/WHO Codex MRLs and therefore tested positive. In season 1, 4(7.8%) and 15(13.6%) milk samples collected from Nakuru urban and Nakuru rural households respectively, tested positive. In season 2, all the milk samples from Nakuru and Nairobi urban households tested negative for anti-microbial residues. However, 17(16.2%) samples from Nakuru rural households had detectable levels of anti-microbial residues. The proportions of milk samples collected from urban and rural consumer households containing detectable levels of anti-microbial residues were found to be significantly different ($P < 0.05$).

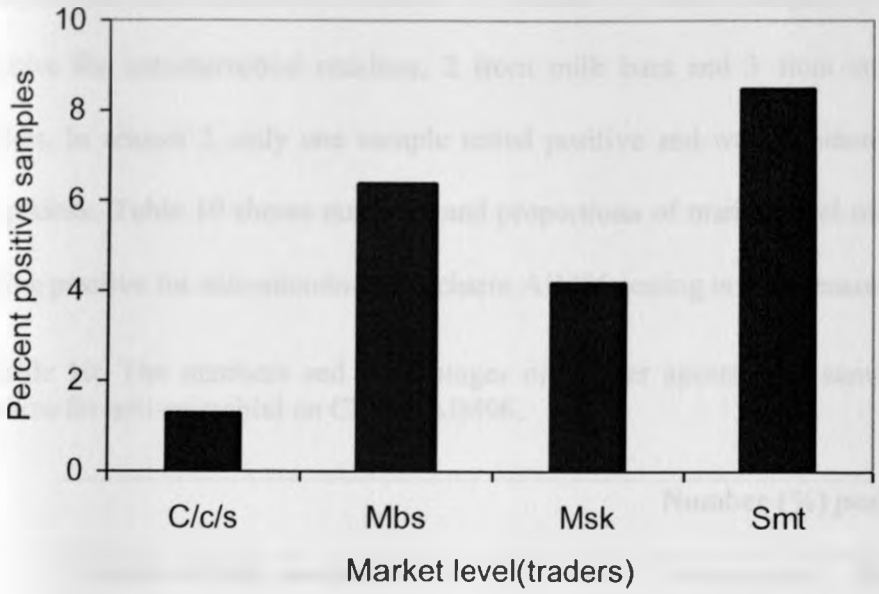
Thirty-six (13.8%) milk samples and 5 (5.7%) others analyzed in both seasons from Nakuru and Nairobi households respectively, tested positive for anti-microbial residues. These proportions were significantly different ($P=0.04$). The proportions of positive milk samples collected in Season1 (wet) were not statistically significantly different from samples collected in Season2 (dry) ($P=0.278$). Table 9 summarizes the results of milk samples analyzed for anti-microbial residues in milk samples from consumer households.

Table 9: The results of milk samples analyzed for anti-microbial residues in consumer survey in two seasons.

Area of study	(n) % Positive	
	Season 1	**Season 2**
Nairobi - Urban	(5) 11.4	(0) 0
Nakuru - Urban	(4) 7.8	(0) 0
- Rural	(15) 13.6	(17) 16.2

4.1.2 Market survey

Four hundred and fifty eight milk samples were collected from market agents in Nairobi, Kiambu, Nakuru and Narok districts, and screened for anti-microbial agents. Overall, 25 samples (5.5%) of all the milk samples had detectable levels of anti-microbial drugs. None of the samples collected from cooperatives and self-help groups tested positive for anti-microbial residues in all the four districts. One sample from a collection centre in Kiambu tested positive for anti-microbial residues. Milk bar and milk shop kiosks had 10 samples (6.4%) and 5 samples (4.2%) collected from them testing positive respectively, while small mobile traders had 9(8.5%) of samples collected from them testing positive. Figure 3 shows, the proportions of positive milk samples along various market levels in Nairobi Kiambu, Nakuru and Narok.



Key. C/c/s: Co-operatives/collection centres/self help group,

Mbs: Milk bars, Msk: Milk shop/ kiosks and Smt: small mobile trader.

Figure 3: The proportions of positive milk samples along various market chains in Nairobi, Kiambu, Nakuru and Narok in both seasons.

Three hundred and eleven samples from milk market agents in Nairobi and Kiambu: 159 in season 1 and 152 in season 2, were analyzed. In season one, 4 samples (2.5%) were positive, 2 from milk bars and one each from milk shop/ kiosk and a small mobile trader. In season two, 15 samples (9.7%) were positive, 6 from milk bars, 3 from milk shop/ kiosks and 5 from small mobile traders. One sample was positive from collection centre.

One hundred and fourty seven milk samples from informal market agents in medium market access and extensive production area (Nakuru & Narok) were analyzed for anti-microbial residues. In season 1, 5milk samples (6.3%) were positive for anti-microbial residues, 2 from milk bars and 3 from small mobile traders. In season 2, only one sample tested positive and was obtained from milk shop/kiosk. Table 10 shows numbers and proportions of market level milk samples testing positive for anti-microbials on charm AIM96 testing in both seasons.

Table 10: The numbers and percentages of market agents milk samples testing positive for anti-microbial on Charm AIM96.

	Number (%) positive	
Source of milk sample	**Season one**	**Season two**
1) Nairobi/Kiambu (High market access and intensive production area)		
a) Cops/coll. Centres/self help groups	0(0)	1(2.2)
b) Milk bars	2(4.0)	6(5.7)
c) Milk shop kiosks	1(2.2)	3(4.3)
d) Small mobile traders	1(4.0)	5(6.2)
2) Nakuru/Narok (Medium market access and extensive production area).		
a) Cops/coll. Centres/self help groups	0(0)	0(0)
b) Milk bars	2(8.0)	0(0)
c) Milk shop kiosks	0(0)	1(5.3)
d) Small mobile traders	3(11.3)	0(0)

Amongst the various market agents, small mobile traders had significant proportions of milk samples (n=9) containing detectable levels of anti-microbial residues (OR=6.48, P=0.08) at 90% confidence interval. However, traders such as milk bars and milk shop kiosks do not contribute to the presence of anti-microbial residues in milk (P> 0.1). Table 11 shows the odds ratio, standard error and P-values of logistic regression model of positive milk samples collected from milk bars, milk shop kiosks and small mobile traders.

Table 11: The odds ratio, standard error and P-values of logistic regression model of positive milk samples collected from milk bars, milk shop kiosks and hawkers

Drugs	Odds ratio	Standard Error	P-value
Milk bar	4.47	4.75	0.160
Milk shop/kiosks	2.94	3.25	0.330
Small mobile traders	6.48	6.91	0.080

4.1.3 Proportions of Positive Milk Samples along Various Market Channels.

Market agents obtained milk for sale from three major sources, individual farms, dairy cooperatives and small mobile traders. Fifteen samples (5.1%) obtained from individual farms tested positive for anti-microbial residues, while cooperatives and small mobile traders had 9 (6.1%) samples and 5(7.9%) samples testing positive for anti-microbial residues, respectively.

Eleven milk-trading channels were identified. Sixteen of the positive milk samples from milk bars, milk shop/kiosks and small mobile traders were obtained

from individual farms. However, only 8 positive milk samples were obtained from other sources such as cooperatives and mobile traders. None of the milk samples from cooperative agents obtained from individual farms tested positive for anti-microbial residues. Bulking of milk at cooperatives explains the absence of residues in milk. Sixty two percent of the positive milk samples from various market agents were obtained from individual farms while 21% were obtained from mobile traders. The rest of the samples (17%) were from dairy cooperative societies. Table 12 shows the proportions of various milk samples with detectable level of anti-microbial residues (positive) from various market channels.

Table12: The proportions of various milk samples with detectable level of anti-microbial residues (positive) a long various market channels.

		Trader			
		Number (percent) positive milk samples			
		C/c/s	Mbs	Msks	Smts
Source	Individual farms	0(0.0)	7(10.0)	2(3.3)	7(7.9)
	Dairy co-op society	-	1(4.3)	1(4.8)	1(14.1)
	Small mobile traders	-	1(4.5)	2(8.7)	1(25.0)

Key: *C/c/s:* Co-operatives/collection centres/self help group,

Mbs: Milk bars, *Msk*: Milk shop/ kiosks and *Smt*: small mobile trader.

The P-values for a logistic regression model shown in table 13 indicates that season, farming systems, sales volume, farm to milk bar and farm to small mobile trader channels do not contribute to the presence of anti-microbial residues in milk (P>0.05). Table13 shows the likelihood of detecting anti-microbial residues in milk samples (odds ratio) and respective P values for the independent variables such as season, farming systems sales, volume and market Channels.

Table 13: A Logistic regression model of risk factors associated with anti-microbial levels above FAO/WHO Codex MRLs for 309 samples collected from Nairobi, Kiambu, Nakuru and Narok districts.

Drugs	Odds Ratio	Std Error	P-value	95% C.I
Season	1.41	0.677	0.473	0.55124, 3.6141
Farming system	1.68	0.855	0.305	0.6219, 4.5553
Sales volume	0.99	0.004	0.166	0.9854, 1.0025
Farm-Milk bar	2.27	1.362	0.172	0.70057, 7.3580
Farm-mob. trader	2.26	1.371	0.178	0.6898, 7.4176

4.2. RESULTS OF CHARM SL (ROSA) TESTS ANALYSIS OF POSITIVE FIELD MILK SAMPLES.

Out of the 75 milk samples (from informal market & formal market) positive for anti-microbial residues on screening, no sample tested positive for tetracyclines and beta-lactam antibiotics when analyzed using Charm SL tetracycline and Charm SL beta lactam tests.

4.3. EXPERIMENTAL AGREEMENT BETWEEN CHARM AIM 96 AND CHARM SL TESTS

4.3.1 Charm AIM96 tests

No antibiotic residues were detected in all the milk samples collected from the eight cows on day one (before treatment) when analyzed using Charm AIM96 test. The following day (after treatment) and 48hours thereafter Charm AIM96 detected penicillin G and/or oxy-tetracycline residues in all the milk samples collected. By 120 hours, both drugs were detected in seven out of eight milk samples. However, after 48 hours penicillin G residues were not detected in a milk sample collected from one cow treated with the antibiotic by intra-muscular route.

4.3.2. Charm ROSA Tests

Similarly, the Charm ROSA tests did not detect antibiotic residues in all the pre-treatment milk samples collected from eight cows on day one. The Charm SL test detected oxy-tetracycline residues in all the milk samples collected 24, 48, and 72 hours after treatment. However, Penicillin G residues were detected using charm SL beta-lactam test in milk samples from all cows up to 48 hours only post-treatment. Beyond 48 hours, penicillin G residues were not detected in milk samples collected from one cow injected through the intra-muscular route.

Ninety-six hours after treatment, two milk samples tested positive for oxy-tetracycline and penicillin G when tested using Charm SL tetracycline and charm SL beta lactam tests respectively. By day five (120 hours after treatment), only one milk sample tested positive for penicillin G residues on Charm SL beta lactam test. There were no oxy-tetracycline and penicillin G residues detected in the other seven milk samples using charm SL tests. The number of Charm Rosa and Charm AIM96

positive milk samples collected from lactating cows after treatment with antibiotics are shown in Figure 4

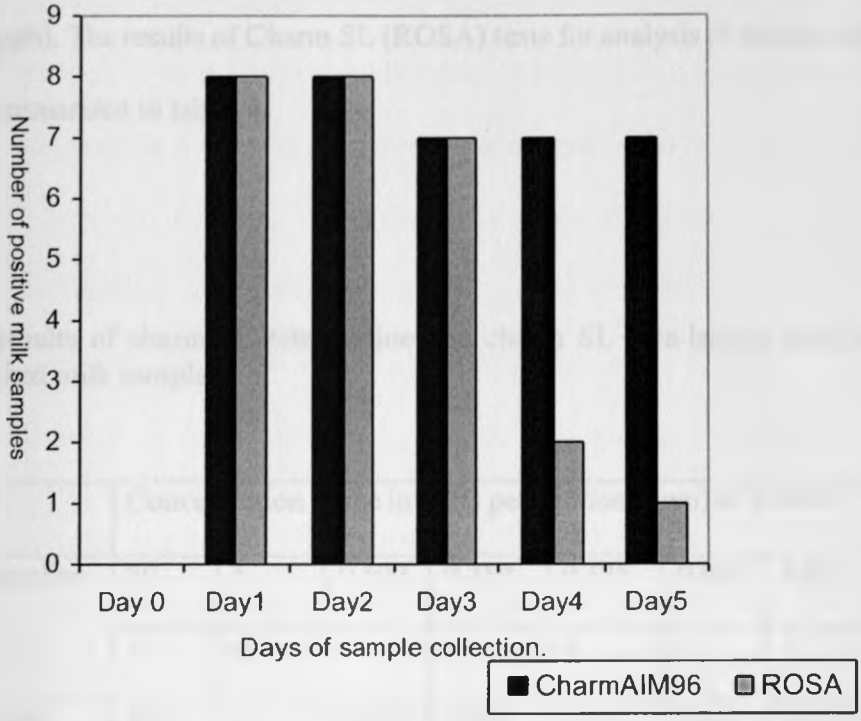


Figure 4: Number of ROSA positive and CharmAIM96 positive milk samples collected from eight lactating cows treated with 10% oxy-tetracycline and Penicillin G (100,000 ius/5g) based drug formulations.

4.4. DETECTION LEVELS OF CHARM SL (ROSA) TEST.

Results of milk samples spiked with oxy-tetracycline showed that the drug residues were detected in milk at concentrations above 62.5ppb. However, antibiotic concentrations below 62.5ppb were not detected in milk when analyzed using charm tetracycline test.

Amoxycillin tryhyrade was detected in milk at concentrations of above 3.1ppb only. The detection level was 3.1ppb when analyzed by Charm SL beta-lactam test. For samples spiked with penicillin G, the residues were detected in milk at concentrations above 0.008iu/ml. Concentrations below 0.008iu/ml were not detected in milk. Hence, the detection level for penicillin G residues in milk was 0.008iu/ml (5ppb). The results of Charm SL (ROSA) tests for analysis of spiked milk samples are summarized in table14.

Table 14: Results of charm SL tetracycline and charm SL beta-lactam tests for analysis of spiked milk samples.

Drugs	Concentration range in parts per million (ppm) or IUs/ml							
10% Oxytetracycline	50	5	0.500	0.250	0.125	0.062	0.031	
	+	+	+	+	+	-	-	
15% Amoxycillin	50	5	0.500	0.050	0.025	0.012	0.006	0.003
	+	+	+	+	+	+	+	+
Penicillin-G	16.70	.167	0.016	0.008	0.004	0.002	0.001	
(100,000ius/5g)	+	+	+	+	-	-	-	

Key; + (positive) represent antibiotic residues detected.

- (Negative) represent antibiotic residues not detected.

Units for Penicillin G are given in International units per millilitre (IU/ml)

CHAPTER FIVE:

5.0. DISCUSSIONS

5.1. RESULTS OF FIELD MILK SAMPLES

5.1.1 Consumer Survey

Rural households from consumer survey, had significantly high proportion of milk samples with detectable levels of anti-microbial residues than urban households ($P<0.05$). Rural household consumers usually get milk from individual neighbours' farms or from their own animals. The higher proportion of samples from rural households with residues may reflect non-observation of withdrawal periods following treatment with antibiotics where as the lower proportion of samples from urban households seems to imply that bulking may be reducing some residue levels to undetectable limits.

5.1.2 Market Survey

Milk samples from cooperative societies in Nairobi, Kiambu, and Nakuru districts had no detectable levels of anti-microbial residues. This result was consistent with study done by Ombui, (1994) who detected no anti-microbial inhibitors in milk received by dairy cooperatives societies in Kiambu district in Kenya. However, this finding differs from that of Chewulukei, (1978) who found 15.6% of samples of milk supplied to Kenya cooperative creameries from Kiambu and Nairobi areas to contain antibacterial inhibitors.

The observed lack of anti-microbial residues in milk from dairy cooperatives may also be attributed to bulking of milk at dairy cooperatives. This may lead to dilution of the anti-microbial agents to un-detectable levels as explained above. Other market agents like milk bars, milk shop/kiosks and hawkers through a

questionnaire indicated that they do not bulk milk from different sources so that they can claim compensation from the origin of the milk in case it gets spoilt. In addition, more than half (62%) of the total milk samples with detectable levels of anti-microbial agents were obtained by market agents from individual farms. This is further evidence that residues could have resulted from failure to adhere to withholding periods following therapy at farm level.

On the other hand, the increasing level of detection of anti-microbial residues as the milk moves up the market chain indicates possibility of addition of anti-microbial agents in the complex informal marketing chain. Milk samples collected directly by traders from dairy cooperatives did not have detectable levels of anti-microbial residues. However, milk samples collected from milk bars, milk shop/kiosks and small mobile traders had detectable levels of anti-microbial residues yet the traders obtained milk from dairy cooperative societies which were reported to have milk that was free of anti-microbial residues. This further suggests the possibility of addition of anti-microbial drugs to milk by these market agents to prolong the self-life of marketed milk.

5.2 EVALUATION OF CHARM AIM96 AND CHARM SL TESTS.

The Charm SL (ROSA) tests detected oxy-tetracycline and penicillin G residues in milk from 0 to 96 hours after treatment. This was a slight deviation from a study done by Dinsmore *et al.* (1996) who found that the detection duration of oxy-tetracycline residues in milk after the last treatment with the drug ranged from 0 to 144 hours using high performance liquid chromatography (HPLC) method.

The lower detection level of HPLC of 2ppb of oxy-tetracycline explains the relatively longer detection period for antibiotic residues in milk by HPLC analysis

than by the Charm SL tests. Charm SL tests have a detection level of 125-175ppb for oxy-tetracycline and 5ppb for penicillin G. This means that the Charm SL tests could not detect lower concentrations of the drugs that can be detected by HPLC method. The concentration of drug residues in milk decreases with time as the drugs are metabolized and excreted by the body, resulting in failure to detect the drugs beyond 96 hours.

However, CharmAIM96 detected antibiotic residues in milk sampled from most cows up to duration of 120 hours after treatment. This compared well with HPLC method of analysis (Anderson *et al.*, 1995). Charm AIM96 test was able to detect antibiotic residues in milk samples that were negative on Charm SL tetracycline test and Charm SL beta lactam test, implying that the screening tests are based on the drug with lowest MRLs.

In the study administration of oxy-tetracycline and penicillin G antibiotics by intra-muscular route to lactating cows resulted in residues of both drugs in milk. This was in agreement with a study done by Anderson *et al.* (1995) in which they used Charm II test and HPLC method to test oxy-tetracycline residues in milk after treating lactating cows with the drug. They found that oxy-tetracycline administered to lactating cows by the intra-venous or intra-muscular route had the potential to cause oxy-tetracycline residues in market milk.

Milk from six cows contained oxy-tetracycline residues above 30ppb, the Food and Drug Administration Safe Level, by 120 hours after oxy-tetracycline administration. This implies that the milk samples could have not tested positive on charm SL tetracycline test that has detection range of 125-175ppb. This means that the residues in milk samples from cows treated with oxy-tetracycline by intra-

muscular route in this study could not be detected by charm SL tetracycline test by 120 hours after treatment.

This study also found that penicillin G residues were detected in milk after both intra-muscular and intra-mammary administration. However, by 96 hours after treatment, penicillin G residues were not detectable in three out of four milk samples. This means that by 96 hours, penicillin G residues in milk were generally below the detection level of charm SL beta lactam test that has a detection level of 5ppb. The finding was consistent with the findings of Anderson *et al.* (1996) who found that amoxycillin residues in milk less than 10ppb were not detected beyond 96 hours. By this time, the antibiotic has been absorbed, distributed, metabolized and then excreted in urine and milk resulting in undetectable residue levels. This is consistent with the high metabolism rate for penicillin G reported.

Although milk samples from most cows did not contain antibiotic residues that could be detected by charm SL (ROSA) tests by 120 hours, milk sample from one cow still had detectable level of penicillin G residues. The inconsistent finding can be explained by the fact that some animals metabolize drugs differently than others thus delaying the excretion of the drug hence unusually longer duration of detection in milk.

5.3 DETECTION LEVELS OF CHARM SL (ROSA) TESTS.

Milk samples fortified with various dilutions of 10% oxy tetracycline showed that the residues in milk were detected up to a level of 125ppb when analyzed using charm SL tetracycline test. The finding coincided with the lower side of detection level range for oxy-tetracycline (125-175ppb) indicated by Charm Sciences Inc. (1999, the manufacturers of the kit. The value was below United States tolerance

level for oxy-tetracycline residues in milk, which is 300ppb. However, the detection level (125ppb) was slightly above the European Union Maximum Residue Level that is 100ppb for oxy-tetracycline residues in milk.

The detection limit of Charm SL tetracycline test (125ppb) achieved in this study was higher than that achieved by White *et al.* (1993) when they analyzed milk samples for tetracycline residues using LC method (5ppb). This inconsistency can be explained by the fact that the Charm SL tetracycline test is based on binding of antibodies to tetracycline nucleus while LC method is based on response of specific detectors to the aromatic structure of tetracycline nucleus. At very low concentrations of the antibiotic, limited binding of antibodies to tetracycline nucleus is achieved resulting in failure to detect low residue levels. In the case of LC method, the specific detector response can be attained even at low level of the antibiotic residues hence the high sensitivity of 5ppb.

The stringent milk sample preparation in LC method that involves deproteinization, filtration, partitioning and washing of inorganic layer with de-ionized water before analysis eliminates chances of interference at retention times of the residues resulting in higher sensitivity (lower detection level). In the Charm SL tetracycline test the sample is merely double diluted in milk dilution buffer and then analyzed hence possibility of interference during analysis resulting in relatively lower sensitivity (higher detection level).

Milk samples spiked with various concentrations of 100,000 i.u of penicillin G and tested using charm SL beta- lactam test, had a detection level (sensitivity) of 5ppb, which was consistent with sensitivity range established by Charm Sciences Inc. (1999)(4 -5ppb). However, Moats, (1990) reported a lower detection level of 2ppb for penicillin G residues in milk using HPLC.

The improved sensitivity can be attributed to extensive milk sample preparation that involve deproteinization, extraction, concentration, cleanup and elusion of sample before analysis. This effectively separates penicillin G from interferences resulting in lower detection limit (higher sensitivity). In the Charm SL beta-lactam test, the milk sample is dispensed directly into the sample pad compartment of test paper strips without prior preparation. Hence, there is possibility of interference with the binding of beta-lactam receptors to beta-lactam ring of the drug resulting in relatively higher detection limits of 5ppb (lower sensitivity).

CHAPTER SIX:

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the findings of this study, several conclusions concerning validation and determination of the sensitivities of charm SL (Rosa) tests as well as results of field milk samples analysis by Charm AIM96 and Charm SL tests were made.

1. Anti-microbial residues detected in field milk samples were more likely to have originated at farm level because of poor market handling practices. This is likely to be due to failure to adhere to withdrawal periods after chemotherapy.

2. Season, farming systems, sales volume and market access and channels were not significant risk factors associated with the presence of anti-microbial residues in marketed milk sampled from study areas.

3. On experimentation, the sensitivities of Charm ROSA tests obtained in this study were in agreement with that given by Charm sciences the manufacturers of Charm ROSA kit.

4. Charm AIM96 and charm ROSA tests detected penicillin G and oxy-tetracycline residues at levels above 5ppb and 125ppb respectively in post-treatment milk samples for the first 3 days. However, the detection agreements between the two tests were inconclusive for milk samples collected beyond the third day post-treatment.

6.2. Recommendations

1. Public education/awareness of farmers and market agents on the public health hazards of residues in milk.

2. Causal relationships need to be defined at the farm level in order to devise appropriate measures to curb the high proportion of samples with anti-microbial residues. A starting point should be a farm level investigation to determine the magnitude of contamination of milk by anti-microbial agents.

CHAPTER SEVEN:

7.0. REFERENCES AND APPENDICES

7.1. REFERENCES

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7.2. APPENDICES

Appendix 1: A part of questionnaire used in collection of information on milk procurement.

Source Area	Source type	Organization of collection	Unit of measure	Amount purchased	Maximum number bulked from each source	Size of handling cans
_____	a [ ]	[ ]	[ ]	_____	_____	_____
_____	b [ ]	[ ]	[ ]	_____	_____	_____

Source type	Organization of collection	Unit of measure
1= Individual farmer(s)	1=Farmer(s) deliver to a collection point	1=Litre
2=Dairy co-op. Society		
3=Private processor	2=Traders deliver to a cooperative	2=Kilogram (Kg)
4=Self help group		
5=Traders/hawkers	3=Farmer(s) deliver to trading premises	3=Treetop (750ml)
6= Own farm		
7=Others (specify)	4= Trader(s) deliver to trading premises	4=Soda bottle (300ml)
	5=Buyer collects at coop/collection point	5=Small cup (350ml)
	6=Buyer collects at farmstead	6= Large cup (500ml)
	7=Cooperative delivers to trading premises	7=Others (specify)
	8=Others (Specify)	

Appendix 2: A part of questionnaire used in collection of background information.

District	Division	Sex of respondent	Trade type	Position in Business	Prevailing weather condition	Source of financing
_____		[ ]	[ ]	[ ]	[ ][ ]	[ ]

Codes

Type of trade
1=Cooperative
2=Self help group
3=Milk bar
4=Milk shop/kiosk
6=Small mobile trader (hawker)
7=Raw milk processor
8=Other (specify)

Position in business
1=Proprietor
2=Employee
3=Others (specify)

Sex of respondent
1=Male
2=Female

Source of financing
1=Savings
2=Credit (Specify creditor)
3=Others (specify)

Weather condition
1=Hot
2=Cold
3=Dry
4=Wet

Appendix 3: A part of questionnaire used in collection of information on milk handling prior to sale.

Is milk still Kept separate After receiving	Longest period milk stays before sale (Hrs)	Do you process milk?	Major sales Products	Quality control measures prior to sale	Method of preservation
[ ]	_____	[ ]	[ ]	[ ][ ]	[ ][ ]

Codes

Is milk still kept separate after receiving?

1=No
2=Yes

Process milk

1=No
2=Yes

Major products types

1=Raw fresh milk
2=Mala/Lala
3=Yoghurt; own processed
4=Yoghurt; not own processed
5=milk shake
6=Cream
7=Ice cream
8=Tea
9=Others (specify)

Quality control measures prior to sale

1=None
2=lactometer
3=Odour test
4=Visual check
5=match check
6=Alcohol test
7=Thermometer test
8=Boiling

Method of milk preservation

1=Not treated
2=Boiling
3=Refrigerating/chilling
5=Antibiotic added
6=hydrogen peroxide
7=Lactoperoxidase
8=Other additives (Specify)

Appendix 4: Intercooled Stata 6.0 package output of chi-square test for independence of anti-microbial presence in milk and sampling regions (urban/rural)

Result	Region 1	2	Total
0	168	139	307
	54.72	45.28	100.00
	94.92	81.29	88.22
1	9	32	41
	21.95	78.05	100.00
	5.08	18.71	11.78
Total	177	171	348
	50.86	49.14	100.00
	100.00	100.00	100.00

key; Region 1 represent urban
Region 2 represent rural

Result 1 positive
Result 0 negative

Pearson chi2(1) = 15.5430 Pr = 0.000 ($p<0.05$)

Appendix 5: Results of milk samples collected from lactating cows after treatment with 10% oxy-tetracycline and penicillin G when analyzed by CharmAIM96 and ROSA tests.

Day (Hrs)	Test/Cow's ID. No	166	076	234	173	073	092	228	024
1	CharmAIM96	2	2	2	2	2	2	2	2
	ROSA	-	-	-	-	-	-	-	-
2	CharmAIM96	5	5	4	5	4	5	5	5
	ROSA	+	+	+	+	+	+	+	+
3	CharmAIM96	5	5	4	5	5	5	5	5
	ROSA	+	+	+	+	+	+	+	+
4	CharmAIM96	5	5	4	4	5	4	1	5
	ROSA	+	+	+	+	+	+	-	+
5	CharmAIM96	5	5	4	5	5	4	1	5
	ROSA	-	+	-	-	+	-	-	-
6	ChramAIM96	5	5	5	5	5	5	1	5
	ROSA	-	-	-	-	+	-	-	-

Key:

+ (Positive) represent anti-microbial residues detected

- (Negative) represent anti-microbial residues not detected

For CharmAIM96 scores 4 &5 are read as *positive* while scores 1&2 are read as *negative* results

Appendix 6: Calculation of speed of centrifugation (g)

$$G = \frac{(RPM \times 0.147)^2}{980} \times \text{Radius (cm)}$$

Where ***RPM*** is the Revolutions per minute

Radius is the radius of the rotor arm to the bottom of the tube (cm)

g is the speed of centrifugation

RPM = 5000 rev/min

Radius = 7.2cm

$$\frac{(5000 \times 0.147)^2}{980} \times 7.2 \text{ (cm)} = 2000g$$