Abstract

Shitandi, A. 2004. *Risk factors and control strategies for antibiotic residues in milk at farm level in Kenya.* Doctoral dissertation.
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Antibiotic residues in milk in Kenya constitute a problem and there is strong interest to enhance milk safety. The research sought to establish whether the prevention of residues in milk was viable at the farm level. It also evaluated implications of non-restricted use of antibiotics in terms of resistance among *Staphylococcus aureus* isolates from the small and large-scale farms, which are the two main producer categories in the country.

The research demonstrated the possible use of a low cost microbiological screening assay (two-tube test) for detection of antibiotic residues in a local dairy. The verified limits of detection for selected antibiotics were below or near established Codex Alimentarius standards. During 2000–2001, 14.9% of the analysed 1109 herd milk samples caused inhibition in the microbial test. Eleven percent (n=118) of the samples had ß-lactam type residues in concentrations $\geq$ 10µg/kg exceeding established limits two fold and the contamination differed significantly ($p<0.001$) among the two types of producers.

Circumstantial evidence was provided between rising incidence of antibiotics and prevalence of drug resistant *S. aureus* isolates from mastitis milk. The isolates were tested for susceptibility to five antibiotics from different families. The overall mean prevalence of multidrug resistance from 402 isolates was 34.3% for small and 18.0% for large-scale farms ($p < 0.05$).

Five characteristics were identified based on Hazard Analysis Critical Control Point principles, which predicted residue contamination on farms and were associated with a higher risk ($p < 0.05$). The characteristics were used to develop a simple risk assessment tool, which was validated in local and remote cohort farms. The tool was then applied in a single blind randomised control study on small-scale farms (42 cases and 42 controls) alongside the two-tube test. A 52% reduction in incidence of antibiotic residue violation was observed in the treatment group compared to the control farms ($p < 0.05$).

The risk assessment tool and the two-tube screening assay were concluded to offer a viable strategy to minimize violative antibiotic residues in farm milk within a control program. The results suggest that this approach could also be implemented on other farms in low-income countries.

Key words: Food safety, risk based strategy, control program, Maximum residue limits, Veterinary drugs, *Bacillus stearothermophilus* var *calidolactis*

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"Youth fades; love droops, the leaves of friendship fall; A mother's secret hope outlives them all."
-- Oliver Wendell Holmes--

To Nerima, my mother!

Contents

Appendix

The thesis is based on the following studies, which will be referred to in the text by their Roman numerals (I - IV)

Papers I-IV

I. Shitandi, A. & Sternesjö, Å. 2001. Detection of antimicrobial drug residues in Kenyan milk. *Journal of Food Safety, 21,* 205-214.

II. Shitandi, A. & Sternesjö, Å. 2004. Factors contributing to the occurrence of antimicrobial drug residues in Kenyan milk. *Journal of Food Protection, 67*. 2. 399-402.

III. Shitandi, A. & Sternesjö, Å. 2004. Prevalence of multi-drug resistant *Staphylococcus aureus* in milk from large and small scale Producers in Kenya. (Submitted).

IV. Shitandi, A. & Kihumbu. G. 2004. Development and evaluation of a risk assessment tool for control of antimicrobial residues in milk. *Journal of Food Safety* (in press).

List of abbreviations

ADI	Acceptable Daily Intake
AI	Artificial Insemination
CAC	Codex Alimentarius Commission
CBS	Central Bureau of Statistics Kenya
CCPs	Critical Control Points
CMT	California Mastitis Test
DRSK	Dairy Recording Service of Kenya
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GDP	Gross Domestic Product
GOK	Government of Kenya
HACCP	Hazard Analysis Critical Control Point
IDF	International Dairy Federation
KARI	Kenya Agricultural Research Institute
LOD	Limit of Detection
MOA	Ministry of Agriculture, Kenya
MRL	Maximum Residue Limit
NGO	Non Governmental Organisation
NCCLS	National Committee for Clinical Laboratory Standards
SCC	Somatic Cell Count
WHO	World Health Organisation

Introduction

Kenyan dairy sector

Dairy production in Kenya is one of the most developed in Sub-Sahara Africa whose dairy cattle population is estimated by the Kenyan Ministry of Agriculture (MOA, 1997) to exceed 3 million dairy cattle. The breeds in the sector are mainly *Bos Taurus* and their crosses with *Bos indicus* and their average milk yields are estimated at 1300 litres per lactation (Omore *et al.*, 1999). According to the Kenyan central bureau of statistics (CBS, 1999), the dairy sector contributes to almost one-third of the national agricultural Gross Domestic Product (GDP). Dairy production thus provides a platform for poverty reduction in rural areas for many of the impoverished populace (Olaloku *et al*., 1990; GOK, 1999).

The dairy cattle are concentrated in the Central and Rift Valley provinces, which form part of the Kenyan highlands with a cool temperate climate (Borton, 1989). Rift Valley province with about 50% of the total dairy population leads in milk production followed by Central province 30%, Eastern Province 9%, Nyanza province 4.5% and Western 3.5% respectively (CBS, 1999).

Figure 1. Map of Kenya showing the Rift valley highlands.

Two major dairy cattle production systems, i.e. large and small scale, are found in Kenya (Omore *et al*., 1999) whose typical characteristics are summarised in Table 1. The small-scale farms are family operations and are very diverse where dairy production is carried out using intensive stall-fed (”zero-grazing”) systems. In this type of production system, land sizes varied between 0.1 and 4 hectares (Staal *et al*., 1999) and most of the family income is wholly dependent on the farm. For units close to city centres this is particularly intensive and a popular system of management where the animals are confined in the backyard.

Table 1. *Characteristics of large and small dairy farmers in Kenya*

	Production System				
	Large Scale		Small Scale		
Production parameter	Extensive (Zebu)	Extensive (exotic/cross)	Semi-intensive Zebu	Semi-Intensive (exotic/cross)	Intensive (exotic/ cross)
Farms (ha) & (herd size)	Communal (>100)	>100 (>50)	20 (30)	15(20)	4.5 (1-10)
Breeding & (Grazing management)	Bull (Free range - pastoral)	Bull/AI (Free, agro-pastoral)	Bull (Free, agro-pastoral)	Bull/AI (Semi-zero)	Bull/AI (Intensive stall fed)
Forage source	Communal pastures	Planted + Natural	Natural	Planted + Natural	Planted + Natural
Adult mortality (%/yr)	6	4	6	5	5
First calving (Weaning) ages (days)	4 (>200)	3 (90)	3.5 (>200)	3 (90)	3 (90)
Pre-weaning calf mortality (%/yr)	20	10	20	15	15
Level of technology	Low	Medium to high	Low	Low-medium	Low-Medium
Inputs & extension	Very low	High, Cooperatives & private	Very low	Medium - public & self help groups	Public & self help groups
Marketed milk production (litres/cow/year)	0	2000	10	900	1500
Average gross margin (USD/cow/yr)	0	500	200	300	350
Service categories	minimal or not available or expensive.	extension, marketing, credit, feed supply, AI, veterinary services	minimal or not available or expensive	minimal or not available or expensive	minimal or not available or expensive

Note: 1 ha = 2 acres.
(Source: Ingram, 1994; Staal *et. al.,* 1999; Omore *et al.,* 1999; CBS, 2002)

The overriding concerns of the small-scale farmers are with sustaining the home and their family rather than production for growth and profit (Staal & Shapiro, 1994; Ndegwa, 2002). These farms are small, but they often sustain more than half, and up to 90 percent, of the population in some regions (Olaloku *et al., 1990;* GOK, 1999). Financial inputs go towards the purchase of improved crossbred animals, production of farm-grown fodder where land is available, purchase of supplementary feeds and payment for veterinary services and hired labour (Staal *et al.,* 1994). Labour is provided by family members, although hired labour is utilized as well. Labour is required mainly for feed procurement and distribution, as well as animal house sanitation. These units flourish in many parts of the country, and have been described as one of the major development success stories occurring in sub-Saharan Africa (Staal & Shapiro, 1994). In this category over 600 000 smallholders produce about 70 percent of Kenya's marketed milk (Omore *et al*., 1999). They account to a large extent to the low level of Kenya's dairy imports compared to other sub-Saharan African countries (Olaloku *et al*., 1990).

About 36% of the produced milk is consumed by the household or fed to calves and the remaining 64 % percent is marketed through cooperatives, processors and directly to consumers (CBS, 1999). Approximately 80 percent of the marketed milk is sold raw in the unregulated informal market, leading to public concerns about hygiene and safety (Omore *et al*., 1999). The potential for the rural poor to contribute to these market opportunities given the increasing demand for livestock products expected in the future is thus threatened by ever increasing fears regarding milk quality and safety.

The typical characteristics of large-scale farms are illustrated in Table 1. In contrast to small-scale, the large-scale farms are extensive, non-fenced under free range and on large commercial professionally operated enterprises (Omore *et al*., 1999). The average size > 163 acres and rely largely on grazing, with some supplementary feeding of concentrates. The cattle are relatively well taken care of and in general, get more attention than small livestock. This applies to all types of inputs. Cattle holders give their animals for instance veterinary drugs and feed supplements, while improved breeds/artificial insemination and feeding with crop residues is also very common. Assistance for is much more common for large compared to small-scale farms and is mostly provided by an extension officer. Cooperatives and private companies serve as mediators between farmers and the competitive market but also as marketing channels.

Antibiotics in dairy production

Antibiotic residues are remnants of antibiotic drugs or their active metabolites that are present within tissues or products e.g. meat, milk and eggs from treated animals (IDF, 1995; CAC, 1998). Levels of the drug and their metabolites may persist at unacceptable levels and consumers can be exposed to them (CAC, 1998). In dairy cows, the drugs are administered for treatment of mastitis through intramammary or intravenous infusions (Blood *et al.,* 1989). The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage (Ivona & Mate, 2000).

Many drugs are also retained in the animal body for longer times than indicated by label discard times (Seymour *et al.,* 1988b; Bishop *et al.,* 1992).

In Kenya, the quantities and types of antibiotics, which are used in food animals, are not well documented although Mitema *et al.,* (2000) indicated penicillins, cephalosporins, erythromycin, and tetracyclines to be the main types imported into the country. It is evident from several studies (Faraj & Ali, 1981; Omija *et al.,* 1994; Shitandi & Sternesjö, 2001; Odero, 2002; Muriuki *et al*., 2001; Shitandi & Sternesjö, 2004) that many animal derived foods in Kenyan market have unacceptable high levels of drug residues. Education on prudent use of antibiotics has been observed to be particularly lacking amongst dispensers and prescribers of antibiotics in the country (Okeke, *et al*., 1995; Shitandi & Sternesjö, 2004). There is particularly limited information on the consequences of residues in terms of public health implications and bacterial resistance. Also, due to prevailing harsh economic conditions farmers are known to allow only a 1-day withdrawal period for milk regardless of the type of antibiotic used (Keyyu *et al.,* 2003).

There are several formal and informal suppliers of veterinary services in Kenya. The formal service had in 1994, more than 1,012 registered veterinarians (MOA, 1998). Eleven percent of all veterinarians worked in private practice while the remainder were employed by the state veterinary services (69%) and about 20% in teaching and research establishments and in other industries (MOA, 1998). Small-scale farmers often experience difficulties in paying for veterinary services and are more likely to use informal sources. These include the services of traditional healers (Bollig, 1995), pharmacists (direct sales without prescriptions), businessmen and even fellow farmers. They have often carried out animal treatments for many years and established a loyal clientele. Government-employed veterinarians in a bid to earn extra money (as the prevailing salaries are low), also sell their services to farmers either within or outside of government hours. As civil servants they have few or no overheads and can easily undercut the fees of private veterinarians. Antibiotics are thus availed at very low costs and easily available in the country. The ease in which antibiotics can be sourced in the country is indicative of a weak regulatory authority

In order to safeguard human health, the World Health Organisation (WHO) and the Food Agriculture Organisation (FAO) have set standards for acceptable daily intake and maximum residue limits in foods (FAO, 1995; CAC, 1995a). Regulatory limits for antibiotic residues have been imposed on the dairy industry in many countries (CAC, 1995a; EU, 1999; FDA, 1996; Folly & da Machado, 2001). However, Kenya has no established specifications for residue limits in raw milk and the dairy industry has not adopted any control program to ensure the safety of raw milk (GOK/FAO, 1992). These limits, which apply to both the parent drug or chemical and its metabolites, need to be enforced within Kenya. The limits and guidelines would then serve as a basis for therapeutic decisions.. The practical realities faced by farmers and health advisors however still require that cows with mastitis be treated.

Mastitis

Mastitis is an inflammation of the mammary glands of dairy cows accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Blood *et al.,* 1983; Blood & Radostits, 1989; Deluyker *et al.,* 1993). Over 100 different microorganisms (both Gram-positive and negative) can cause mastitis, and these vary greatly in the route by which they reach the cow and in the nature of the disease they cause (National Mastitis Council (NMC), 1999; Hish & Zee 1999). *S. aureus* is one of the major pathogens causing the severe form of mastitis (NMC, 1999; Ma *et al.,* 2000; Murinda *et al.,* 2001). The disease, which is common in dairy cows, causes significant losses to the dairy industry and affects milk hygienic and sanitary features (Harmon, 1994; Heeschen & Reichmuth, 1995). The clinical form of mastitis is readily observed as it is characterized by visible abnormalities in the udder and/or milk (Blood *et al.,* 1983; Barkema *et al.,* 1998; Hish & Zee, 1999). Mastitis is of technological significance in milk processing as valuable components like casein are decreased while undesirable components like ions and enzymes are increased (Kitchen, 1981; Blood & Radostits, 1989; Heeschen & Reichmuth, 1995).

Bovine mastitis is the most frequent disease in Kenyan dairy herds (Hamir *et al.,* 1978; Ogaa, 1980; Odongo & Ambani, 1989) and particular problematic in small-scale dairy cattle (Omore *et al.,* 1997). Omore *et al.,* (1996), estimated the prevalence of sub-clinical mastitis to be 71% with an average somatic cell counts (SCC) of 620, 000 cells/ml of milk on small-scale dairy farms in Kenya. The same study reported the incidence of clinical mastitis to be 13.3% per annum. Other studies (Lauerman *et al.,* 1973; Hamir *et al.,* 1978; Ngatia, 1988) estimated the prevalence of sub-clinical mastitis to be 49 %, 48% and 55% respectively. The types of mastitis, the severity and duration of disease, the major risk factors and the extent of resistant strains in the country are however not well documented (Hart & Kariuki, 1998). These need clarification, as the knowledge may be useful to build up control methods.

Antibiotics have been the class of drugs most often advocated in clinical practice and research as a therapy for mastitis (Dodd *et al.,* 1970; Bramley & Dodd, 1984; Sol *et al.,* J., 1990; Honakanen - Buzalski, 1995; Hish & Zee, 1999). However, because of the limited therapeutic dosing of these drugs that is usually employed due to economic and residue avoidance concerns, effective inhibitory concentrations are often not maintained (Ziv, 1980; Sol *et al.,* 1997). The key variables that influence the approach to formulating a treatment protocol are causative agent, drug selection and cow immune status (Ziv, 1980; Bramley & Dodd, 1984; Anderson, 1989). Since many farmers do not submit clinical specimens in low-income countries (Hart & Kariuki, 1998) the therapeutic regimens are unlikely to be adhered to, with possible implications.

Implications of non-restrictive use of antibiotics in dairy cows

Public health aspects

Human health problems that may result from intake of sub chronic exposure levels include allergic reactions in sensitive people, toxicity, carcinogenic effects (Neftel & Cerny, 1992; Lee *et al.,* 2000; Phillips *et al.*, 2000) although the validity of some of the reactions is sometimes debated. Penicillins especially, as well as other ß-lactam antibiotics such as cephalosporins and carbapenems could cause allergies if high levels of residues persist in milk consumed by penicillin-allergic persons (Neftel & Cerny, 1992; Kindred & Hubbert, 1993; Phillips *et al.,* 2000). Tetracyclines residues also have the potential to stain teeth of young children (Wilson *et al.,* 1982).

The non-restrictive usage of antibiotics in animal rearing may lead to problems due to the presence of harmful residues in foods and raw materials of animal origin (Cernilglia, 1995; Zijpp & Van der, 1999; Klaus, 2000; Boor, 2001). Development and spread of antibiotic resistance represents a serious threat with potential public health implications (WHO, 2000; Lee *et al.,* 2000). Dissemination of resistance traits could narrow the line of defence against bacterial infections to only a few antibiotic agents and could increase health care costs (Lee *et al.,* 2000).

A close relationship seems to exist between the rate of development of resistance development and the quantities of antibiotics used (2000; Lopez-Lozano *et al.,* 2000). There is, however, still no agreement on the significance of antibiotic use in animals and on the development and dissemination of antibiotic resistance among bacterial pathogens (Wierup, 1997; WHO, 1997; Stewart, 1999). Contributing to the controversy is the isolation of bacterial pathogens of animal and human origin that are increasingly resistant to most frontline antibiotics, including third-generation cephalosporins, aminoglycosides, and even fluoroquinolones (Wall *et al.*, 1996; WHO, 1997; Tollefson *et al.,* 1998). Recent studies have demonstrated that the majority of these multiple antibiotic resistant phenotypes are obtained by the acquisition of external genes that may provide resistance to an entire class of antibiotics (De Oliveira *et al.,* 2000; Martel *et al.,* 2000).

The development of antibiotic resistance in bacteria is mediated by both selective pressure due to antibiotic use and the presence of resistance genes (Tenover & Hughes, 1996; McCormick, 2003). Resistance to antibiotics is by four major mechanisms: i) alterations in the target site of the antibiotic, such as changes in penicillin binding proteins ii) drug degradation and enzymatic inactivation of the antibiotic (e.g. penicillinases), iii) changes in cell wall permeability that prevent access to antibiotics and iv) increases in the activity of efflux pumps in the cell wall which prevent accumulation of antibiotic within the cell (Mazel & Davies, 1999; Hawkey, 2000).

There are several factors which are thought to influence the development of resistance and this include drug concentration, long-term exposure, organism type, antibiotic type and host immune status (Sjogren *et al.,* 1992). Low-level, long-term exposure to antibiotics may in particular have a greater selective potential

than short-term, full-dose therapeutic use (Lopez-Lozano *et al*., 2000). The judicious use of these drugs is thus of great global concern (Martel *et al.,* 2000; WHO, 2000).

Technological aspects

The dairy starter cultures currently used in the Kenyan dairy industry for the primary acidification of the milk belong mainly to the genera Lactococcus, Streptococcus, Leuconostoc and Lactobacillus. These starter cultures are mainly lactic acid bacteria used in the production of a range of fermented milk products, including cheese, yoghurt, cultured butter and cultured milks. The primary role of starter cultures in cheese manufacture is the production of lactic acid from lactose at a consistent and controlled rate. The consequent decrease in pH affects a number of aspects of the cheese manufacturing process and ultimately cheese composition and quality (Broome *et al.,* 2002).

Antibiotic residues in milk are undesirable from a manufacturing perspective, as they can interfere with starter culture activity and hence disrupt the manufacture process (Mäyrä-Mäkinen, 1995; Mitchell *et al.,* 1998; Katla *et al.,* 2001; Broome, *et al.,* 2002). The concentrations of antibiotics which would cause such effects is however often higher than would be found inherent as residues in milk (Katla *et al.*, 2001). Total inhibition of the starter culture has been observed to occur at approximately 60 μg/kg penicillin G, (Schiffmann *et. al.,* 1992). The sensitivity of starter cultures to antibiotic substances present in milk also varies considerably (Katla *et al.,* 2001). Even within the same species of culture strain, differences in sensitivity are evident (Packham *et al.,* 2001). Further, the response of starter cultures to residual antibiotics in milk destined for cheese or yoghurt manufacture can also be affected by the presence of other natural potential inhibitors (Egan & Meaney, 1984; Carlsson & Bjorck, 1987; Carlsson & Bjorck, 1989; Packham *et al.,* 2001).

Environmental aspects

Active metabolites of antibiotics may be excreted by animals through urine and faeces and reach the soil and water (Strauch, 1987). The most prevalent antibiotics found in the environment (surface waters) belong to the macrolide and the sulfonamide groups (Heberer, 2002). Tetracyclines, penicillins or fluoroquinolones have only been found in some cases and at low concentrations (Hirsch *et al.,* 1999; Heberer, 2002; Kolpin *et al*., 2002). Zuccato *et al.,* (2000) identified some commonly used antibiotics, such as erythromycin, cyclophosphamide, sulfadimidin, and tetracycline as antibiotics, which persist in the soil and remained in surface waters and soils for over a year.

Antibiotic metabolites have also been found to be able to be transformed back to their original active substances once in the environment (Hirsch *et al.*, 1999). Since most antibiotics are water-soluble, up to 90% of a dose can be excreted in urine and up to 75% in animal faeces (Halling-Sørensen *et al.,* 2001). It has however been difficult to ascertain whether the residues are caused by waste water management or if they are due to inputs from agriculture (Halling-Sørensen, 1998) It is thus generally felt that dairy animals have a low influence on the input of antibiotics into the acquatic environment (Heberer, 2002).

Residue detection methods and control strategies

Analytical methods

There are several methods available for screening of raw milk for the presence of antibiotic residues (Cullor *et al.,* 1992; Sischo & Burns, 1993; IDF, 1997). Table 2 gives examples of commonly used commercial screening tests.

Table 2. *Examples of antibiotic screening tests used commercially,*

Test name	Manufacturer	Antibiotics	Matrices	Type/Analyte principle
SNAP ß - lactam Test Kit	IDEXX Laboratories, Inc.	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	bulk tank bovine milk	Screening test/Bacterial growth inhibition
Delvotest SP	DSM Foods	amoxicillin, ampicillin, cephapirin, ceftiofur	bulk tank bovine milk	Screening test
Penzyme milk and Penzyme III	UCB Biopdts / CHR. Hansen, Inc.	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	Bulk tank bovine milk	Screening test/Enzymatic
LacTek™ CEF & LacTek™ B-L	Idetek (Sunnyvale, CA)	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	bulk tank bovine milk	Screening test/Competitive enzyme system
Parallux™ ß-Lactam Assay System	IDEXX Laboratories, Inc.	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	bulk tank bovine milk	Screening test/solid-phase fluorescence immune receptor
New SNAP ß -Lactam Assay	IDEXX Laboratories, Inc (Westbrook, ME)	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	raw commingled, whole bovine milk	Screening test/Antibiotic-antigen capture system
Charm II sequential and Charm I cowside	Charm Sciences, Inc. (Malden, MA)	ampicillin, cephapirin, ceftiofur, penicillin, amoxicillin	commingled, whole bovine milk	Screening test/competitive binding; ^{14}C penicillin displacement

Source: AOAC, (2003).

The common tests include microbial growth inhibition assays, which involve a standard culture of a test organism seeded in an agar or liquid growth medium

(Seymour et al., 1988a; Crosby, 1991, IDF, 1991; Sischo, 1996). Milk sample is added and the test is incubated for periods up to several hours. In the absence of inhibitory substances, the organism grows. This can be detected visually either by opacity of the agar growth medium or typically by a colour change resulting from acid production (IDF, 1991). In the presence of an antibiotic, or any other inhibitor, the organism fails to grow and a zone of inhibition or lack of a colour change is observed. Microbiological inhibitor tests are generally reliable, have high capacity and are cost-effective. They have a broad detection pattern, which on the other hand makes them unspecific. Their main disadvantage is perhaps the required typically incubation for several hours before the result can be evaluated.

Other types of methods, which can be used for routine screening of residues include immunoassays, receptor assays and enzymatic assays (Nakazawa *et al.,* 1992; Deshpande & Rocco, 1994; Bremner & Johnston, 1996; Anderson, 1996). These methods can also be applied for a preliminary identification of classes of antibiotics (Sternesjö & Johnsson, 1998a; Mitchell *et al.,* 1998). The majority of these tools are quite expensive, and require instrumentation and technical skills but have the advantages of reliability, automation and fast readings of results. They are specific and typically they have poor capacity.

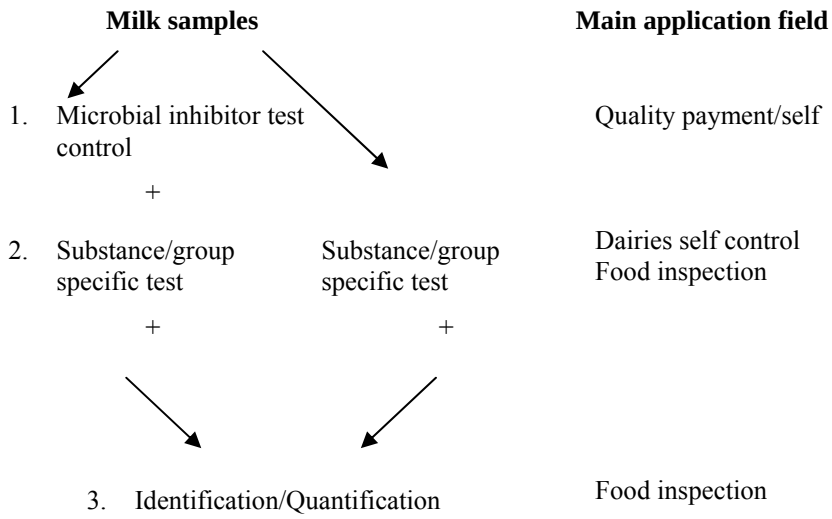


Figure 2. *Flow chart of an integrated system in raw milk (Heeschen &Suhren, 1996)*

A control program for antibiotic residues in milk is usually performed in two steps where a microbial, enzymatic or receptor-based method is used for initial screening (IDF, 1995). The samples found positive are usually confirmed by a chemical method. Since there are no tests that satisfy all requirements with respect to detection pattern and limits, an integrated control system as illustrated in Figure

2 would be the ideal (Heeschen & Suhren, 1996) to protect both consumers and producers.

A confirmatory method has to be able to identify the molecule present in the sample and to quantitate it. High-pressure liquid chromatography coupled with UV detector (HPLC-UV) is the technique often adopted as a confirmatory method for antibiotic residues (Riediker & Stadler, 2001; Marchetti *et al.,* 2002). This technique has some limitations in a low sensitivity and selectivity; therefore many purification steps are needed (Ito, *et al.,* 2001; Marchetti *et al.,* 2002). Other techniques used for confirmation of residues include, spectrophotometric, thin-layer chromatographic and bioautographic, gas chromatographic, mass spectrometric, and immunochemical methods (Kennedy *et al.,* 1995; Elliott *et al.,* 1998).

Maximum Residue Limits

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 CAC and Table 3 gives some examples of those, which have been set for milk from veterinary cows.

Table 3. *CAC Residue Limits of common veterinary drugs (µg/kg) set for milk*

Antimicrobial	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 & 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 Kenya and 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.

Control strategies and HACCP

A food control system is an official institutional set up, at national and sub-national level, responsible for ensuring the quality and safety of the food supply (Heeschen & Suhren, 1996). It includes the relevant food legislation and regulation, food inspection, food analysis, food import/export inspection and certification and food control management (FAO, 1998).

The World Health Organization has initiated the promotion and acceptance of integrated risk assessments to foster more holistic assessments of health and risks (WHO, 2001). FAO defines the food chain approach as recognition that the responsibility for the supply of food that is safe, healthy and nutritious is shared along the entire food chain, by all involved with the production, processing, trade and consumption of food (Herrman, 1995). The holistic approach to food safety differs from previous models in which responsibility for safe food tended to concentrate on the food processing sector. The Codex Alimentarius Commission (CAC) recommends a Hazard Analysis Critical Control Point (HACCP) approach wherever possible to enhance food safety (CAC, 1998). A hazard in the context of food safety refers to the inherent capacity of an agent to cause an illness. The agent that may enter the food chain could be biological (e.g. antibiotic resistant pathogens), chemical (veterinary drugs residues) or physical (e.g. pieces of glass).

HACCP is a process control system where a specific system is evaluated for food safety risks. This is by implementing the seven basic principles of HACCP, as illustrated in Table 4. HACCP is an effective food safety tool that has been recognised internationally and is mandatory for companies exporting most food products to the EU countries and the USA. Currently (2004), regulations are most comprehensive in Europe and North America, while in African countries statutory directives may not even exist.

Table 4. *The principles of HAACP*

Principle	Characteristics
Principle 1	Listing of all potential hazards, assessing their likelihood of occurrence and identifying the preventive measures for their control.
Principle 2	Determining the critical control points (CCPs)
Principle 3	Establishing critical limits(s)
Principle 4	Establishing a system to monitor control of the CCP
Principle 5	Establishing the corrective action to be taken when monitoring indicates that a particular CCP is not under control
Principle 6	Establishing procedures for verification to confirm that the HACCP system works efficiently
Principle 7	Establishing documentation concerning all procedures and record appropriate to these principles and their application.

Source CAC, (1995b); WHO, (2001).

Residue control strategies in low-income countries such as Kenya can actually attain those of international standards, but the lack of technical and institutional capacity to control and ensure compliance essentially makes the standards less effective (Oboegbulem, 1996). Inadequate technical infrastructure - in terms of food laboratories, human and financial resources, national legislative and regulatory frameworks, enforcement capacity, management and coordination - weakens the ability to confront these challenges. Such systemic weaknesses may not only threaten public health but may also result in reduced trade access to global food markets.

Risk assessment

People's concerns about risks of technology have been present since the time of the industrial revolution (McClellan, 1999). However, it has been noted that in recent times that these concerns have become much more pronounced (Covello, 1983; Slovic, 1987; Bayer, 1995). Covello & Merkhofer (1994), define risk as a combination of something that is undesirable and uncertain. Similarly, risk has been described as "the probability of an adverse outcome, and uncertainty over the occurrence, timing or magnitude of that adverse outcome" (Hattis & Silver, 1993). In many instances complete information on risks is not known and risk assessment is then used as a diagnostic tool to help professionals make decisions (McClellan, 1994; WHO, 2001).

Risk assessment is a discipline that can be traced back to 3200 B.C (Covello & Mumpower, 1985; Perera, 1987; Paustenbach, 1995; Samuels, 1997). A risk analysis framework based on the risk analysis model for communication has been commonly described in literature (Frewer *et. al.,* 1998; Lundgren & McKakin, 1998; Bennett & Calman, 1999; Coles, 1999; Coote & Franklin 1999). It has been adopted by several international organizations including the CAC & the Canadian food inspection agency (FAO, 2003). This model as illustrated in Figure 3, sets out three specific components. They include risk assessment (being the determination of the degree of risk involved), risk management (establishing if and what measures are required to mitigate risk) and risk communication (ensuring that all stakeholders are involved in the process).

In food safety, the risk assessment process provides an estimate of the probability and severity of illnesses attributable to a particular hazard related to food. To carry out the assessment, risk factors are determined. In the context of food safety the factors can be described as environmental, demographic, behavioural, or biological factor usually in prevalence studies, which if present, directly increase the probability of contracting the illness (FAO, 2003). Their absence or removal will reduce the probability of the hazard to cause an illness.

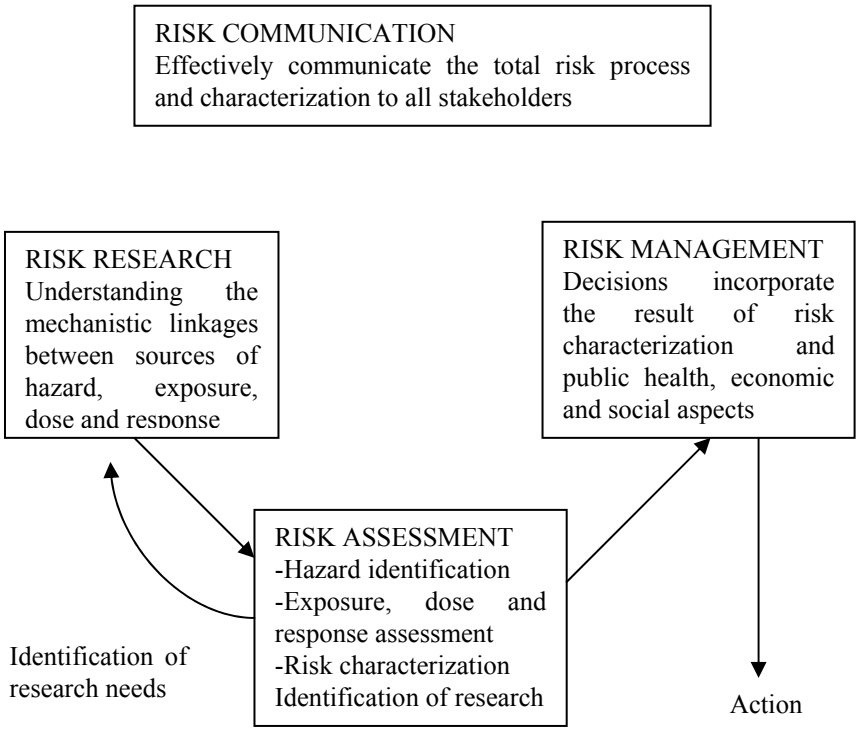


Figure 3. Paradigm for risk research, assessment and management (McClellan, 2003)

A risk-based approach to the management of food safety hazards by definition implies risk analysis (FAO, 1997). Since risk factors are part of the causal chain, a risk strategy can then be used, as a preventive method in, which effort is focussed on a selected group of individuals at risk. Food control resources are thus directed to those hazards posing the greatest threat to public health and where the potential gains from risk reduction are large relative to resource use.

Objectives

The project sought to establish whether the prevention of residues in milk at the farm level was viable and also evaluated the implications of non-restricted usage of antibiotics in terms of bacterial resistance among *S. aureus*.

The study hypothesized that the occurrence of residues in farms could be reduced by using a risk strategy in which prevention would focus on a selected group of producers at high risk. It aimed at identifying contributing risk factors, utilizing the characteristics to develop a simple risk assessment tool which could then be used alongside a low cost screening assay in a residue control program. The underlying rationale was that an affordable innovation, tailored to local conditions, would be readily adopted by resource-poor farmers.

The specific aims were

1. To evaluate the possibility of utilizing a low cost microbiological method in screening for antibiotic residues and determine the prevalence of drug residues in milk samples (Paper I).
2. To investigate whether there were any differences in the frequency of antibiotic residue contamination of milk between the main producer categories and to find underlying reasons contributing to the contamination (Paper II).
3. To determine the prevalence of drug resistant *S. aureus* in milk from large and small-scale producers in the Rift Valley highlands (Paper III).
4. To assess whether a developed simple risk assessment tool could be used amongst small-scale farms to reduce the incidence of antibiotic residues in milk in a voluntary control program (Paper IV)

Materials and methods

Herds and animals

Data was obtained from the dairy herds located in Rift Valley highlands of Kenya. The population in the study was divided into two different groups based on farm type. The concept of typical farms was used and herds were chosen to either represent small or large scale dairy production systems. Small-scale farms are family operations with an average farm size of 4.5 acres, herd size < 10 with an average of 3 milking cows and the grazing system consists of intensive stall-fed (”zero-grazing”) systems. The large-scale farms are commercial operations with an average farm size of 163 acres while herd sizes average 100. They have an average of 40- 50 milking cows at any point in the season and the grazing system consists of free range pasture typically unfenced and permanent grazing systems.

Systematic random samples were obtained by selecting one unit (farms, lactating cows or milk samples) on a random basis from each of the large and small farms, which constituted the population of the research area. Additional units were selected at evenly spaced intervals until the desired number of units was obtained. Data collection was simplified by selecting every 5th unit (farm, cow milk) after the first unit had been chosen randomly. This sampling method was considered appropriate for the study and satisfied observer statistical independence (Patton, 1990). It was also not time consuming as large numbers of samples were handled.

The project was carried out at the department of Dairy & Food Science, Egerton University Njoro, which was established as a farmer training institution and is now the main agricultural university in Kenya. The department located in the Rift Valley highlands, has also a commercial dairy processing plant (Guildford dairy plant) with a maximum daily intake of 3,000 litres. Small-scale dairy farmers are the main suppliers of milk to the plant from an approximate radius of 100 Kilometres and the department thus formed a good research base for the study.

Applicability of the two tube test (Paper I)

Two- tube test

The improved Dutch tube diffusion test consists of two test tubes, A (pH 7.0) and B (pH 8.0). The two tubes contain agar medium with bromocresol purple as pH indicator, inoculated with *B. stearothermophilus* spores. The tubes differ with respect to pH and synergistic antibiotics added to the medium. The normal growth of the bacteria results in acid production, which alters the pH indicator from purple to yellow. If the bacteria growth is inhibited by antimicrobials present in the milk sample the colour of the medium remains purple. Table 5, illustrates the detectable concentrations (μg/kg) in the two-tube test in comparison with EU (MRLs) as claimed by the developers of the method (Rikilt–dlo, 1998a).

The principle of verification of the LODs followed the IDF guidance for the standardized description of microbial inhibitor tests (IDF - Group E 503, 1997). From the antimicrobial stock solutions, appropriate working solutions were

prepared from the stock solutions. Samples were pipetted to tubes A and B, and left to allow the milk to diffuse into the medium. After decanting the remaining milk, the tubes were covered and heated in a water bath to activate the growth of the spores. The tubes were then incubated in a water bath.

Table 5. *Detectable concentrations (μg/kg) in the two-tube test in comparison with EU MRLs*

Antimicrobials	EU-MRL	LOD Tube A	Tube B LOD
ß -lactams			
Benzylpenicillin	4	1	1
Ampicillin	4	2	2
Cloxacillin	30	10	10
Cephalosporines			
Ceftiour	1000	25	10
Cephalexin	100	40	50
Cephapirin	60	5	3
Cephalothin	20	5	5
Cephacetrile	125	15	15
Aminoglycosides			
Dihydrostreptomycin	200	125	40
Neomycin	500	100	15
Kanamycin	-	100	50
Gentamycin	100	25	10
Tetracyclines			
Oxytetracycline	100	35	700
Tetracycline	100	30	600
Doxycycline	100	35	600
Chlortetracycline	100	40	3000
Macrolides/Lincosamides			
Erythromycin	40	40	10
Spiramycin	200	3000	100
Tylosin	50	90	10
Oleandomycin	-	900	50
Lincomycin	150	300	50
Quinolones			
Enrofloxacin	100	1300	400
Flumequine	200	4000	3500
Sulphonamides			
Sulphamethazine	100	4000	60
Sulphadiazine	100	5000	30
Sulphanilamide	100	3500	150
Other antibiotics			
Dapsone	0	600	1
Rifamycin	50	900	50
Trimethoprim	50	1000	900
Novobiocin	-	1200	1500
Colistin	-	2500	700
Chloramphenicol	0	1500	6000

Source: Nouws *et al.* (1995); Rikilt –dlo, (1998a; 1998b); Nouws *et al.* (1999).

Survey samples with a result indicating the presence of inhibitory substances were subjected to penicillinase (penase) treatment for a preliminary identification of ß-lactams. The penase used was a *Bacillus cereus* 569/H9 lactamase presented as a freeze-dried powder containing buffer salts. Each vial contained 3,300 IU of activity (1 unit of enzyme activity will hydrolyse 1.0 µmol of benzylpenicillin to benzylpenicilloic acid per minute pH 7.0 and at 25°C). The preparation is known to successfully inactivate a range of penicillins. The enzyme preparation was used to inactivate susceptible ß-lactam antibiotic preparations. A negative reaction in the two-tube test after penase treatment confirmed the presence of a ß-lactam antibiotic.

ß-lactam plate assay

Milk samples that were found to contain ß-lactam residues by the penicillinase (penase) test were also analysed with the ß-lactam plate for estimation of the level of contamination. Inhibition zones of milk samples spiked with different concentrations of penicillin G were determined and the relationship between penicillin G concentrations and the sizes of their corresponding inhibition zones were used to construct a logarithmic standard curve. This was then utilized for quantification of ß-lactam equivalents in milk samples.

The Delvo test

The Delvotest (DSM Food Specialities, The Netherlands) was used as a reference method in this study and performed according to the manufacturer's instructions. The test is primarily designed to detect ß-lactams. The target organism, *B. stearothermophilus*, is encapsulated in an agar medium containing a pH indicator The test is capable of detecting a wider spectrum of substances, notably sulphonamides, but also has increased sensitivity to tylosin, erythromycin, neomycin, gentamicin, trimethoprim and other antibiotics. It has a sensitivity to penicillin G of 1-2 µg/kg.

Antibiotic residues and contributing factors (Paper II)

Milk was sampled from collection centres randomly selected for the study. The raw milk was transported to the laboratory and samples were tested using the two-tube diffusion test. Milk samples that were found to contain ß-lactam residues by the penicillinase test were also analysed with the ß -lactam plate for estimation of the level of contamination. For statistical analysis a Chi-square test (Minitab, 2000) was used to determine whether any observed difference in frequency of contamination between small and large-scale producers was statistically significant

A semi-structured questionnaire was developed and used for data collection in the study areas. Smallholder dairy farms were individually interviewed regarding antibiotics and treatment practices in use on their farms. Additional information was collected from the extension office of the Ministry of Agriculture in the respective district.

Drug resistant *Staphylococcus aureus* (Paper III)

The California Mastitis Test

The California Mastitis Test (CMT) test was conducted on quarter milk samples obtained from lactating dairy herds in the study area. The test is based on the quantity of deoxyribonucleic acid (DNA) in the milk and hence the number of leukocytes and other cells present. A squirt of milk from each quarter of the udder is placed in each of the four shallow wells (cups) in the CMT paddle. An equal amount of commercial CMT reagent is added to each cup. A gentle circular motion is applied to the mixture in a horizontal plane and a positive gelling reaction occurs in a few seconds with the positive samples. The consistency of the gel (i.e. the heavier the gel the higher the somatic cells in the milk and vice versa) is indicative of the leukocyte count (Quinn *et al.,* 1994).

Isolation and identification of Staphylococcus aureus isolates

The isolation and identification of *S. aureus* isolates was performed according to the National Mastitis Council recommendations on examination of quarter milk samples (NMC, 1999). After delivery, the milk samples were inoculated on agar plates and streak lines were made on the agar. Samples were incubated for and examined for bacterial growth. Pure cultures were further examined for morphological, staining and cultural characteristics, and for biochemical reactions according to standard keys. Staphylococci were studied in particular for haemolysis and coagulase production. To determine the number of *S. aureus*, the milk sample was inoculated at the appropriate dilutions on agar and incubated before colony counting. Typical colonies identified as *S. aureus* were stored in cryogenic vials containing trypticase soy broth and glycerol for prolonged storage.

Antibiotic Susceptibility Testing

Staphylococcus aureus isolates, were selected from each of the two producer categories so that each isolate represented a single strain from a single herd. Prior to antibiotic susceptibility testing, the isolates were revived by sub-culturing on agar base and then incolutated into a broth medium. The suspension was adjusted to the McFarland standard and agar plates inoculated by swabbing the inoculum over the surface of the plate. Antibiotic discs were placed on the agar surface aseptically and the isolates were tested for their susceptibility to antibiotics selected from different families. The plates were examined after incubation and the zones of inhibition measured. The interpretive breakpoints for resistance were used to categorize the isolates as suceptible, intermediate or resistant.

Statistical analysis

For statistical comparison, results were classified into two categories: sensitive and resistant. The resistant category from the susceptibility testing included isolates with a result of either intermediate susceptibility or resistant. Chi-square analysis was used to compare zone diameters for all antibiotics. Odds ratios and confidence intervals were computed to determine the associations between the prevalence of resistant *S. aureus* in milk and the producer categories. The

significance of differences in resistance was evaluated using Minitab software, 2000. The Chi test with Yates correction was performed on the cross-tabulated categories of the presence of the resistant/susceptible isolates.

Semi-structured interview

Interviewing was carried out as adapted from Veldhuizen *et al.,* (1997). In this type of interview a rigid questionnaire was not used but one with predetermined questions. This had an open-ended format that was presented to all respondents in the same manner. The facilitator prepared a checklist of important points and exercises to be covered which allowed the interview to be flexible and permit the respondents to express their thoughts in their own words within their own conceptual frameworks.

A risk tool for control of antibiotic residues on farm (Paper IV)

Risk assessment tool

In study IV, a four-phase process for identifying high-risk farms was conducted. The first phase determined identifiable risk factors associated with antibiotic residue violation on farms. The second phase then developed a risk assessment tool that would distinguish effectively between farms at high risk and those at low risk. The third phase assessed the milk producer farms for the factors contained in the risk tool to predict the risk of milk contamination on each farm. The final phase consisted of an intervention with the provision of the prevention regimen to the farms.

Initially a case-control study was carried out to identify the risk factors that were significantly associated with residue contamination of milk delivered to the dairy. Control producers were selected and matched to residue violator farms (cases) whose milk had recently failed the two-tube and Delvo tests. Potential risk factors were identified in follow up visits where the two groups of producers were interviewed. The risks factors identified among producers whose milk had failed (positive result violation) the screening tests were compared statistically with those of producers whose milk had passed. The risk factors found to be significantly and independently associated with residue violations were used to develop a risk assessment tool.

To validate the risk tool, an investigation was performed, among both local suppliers of the research dairy and a remote cohort of farms away from the research base. The tool was used on farms to predict the farms most at risk of having antibiotic residues in their milk and comparisons made with milk samples taken from the farms.

A voluntary control program for small-scale milk producers to promote rational protocols, directed at managing and reducing the use of antibiotics on the farm was designed using the developed risk tool. The experimental design was a single blind randomised control study, which consisted of case-control farms matched in pairs as closely as possible to control farms. Facilitators followed up farms, at weekly intervals and on treatment farms they jointly used the risk assessment tool

with producers to identify specific risks and develop a plan to avoid risks that were specific to the farm.

On control farms, the facilitator provided the risk assessment tool to the farmer but did not detail on how to use it (placebo) to draw any risk avoidance plan. Compliance with the study treatment was assessed by reviewing the records on farms and management judgements. To assess the effects of the treatment allocation on antibiotic incidence, milk samples were taken from each farm and analysed for inhibitory substances. The treatment and control groups were compared for any resulting changes in antibiotic residues violations during the intervention and on completion.

Statistical analysis

To compare the two groups of farms, logistic regression for categorical data was used (SAS, 2001). The proportions of violators and non-violators correctly identified on farms were compared and the specificity and sensitivity of the tool determined. The overall hypothesis tested was that farms with the control risk assessment tool (placebo) might commit more residue violations compared to farms with treatment risk tool.

Results and discussion

Applicability of the two tube test (Paper I)

The detection of antibiotic residues in food requires screening methods sensitive at antibiotic concentrations close to the MRL and the first investigation evaluated the applicability of the two-tube test as a screening method under small-scale dairy conditions. The detection levels for selected drugs in the two-tube test were initially verified by construction of concentration - response curves (Figure 4). For all the antibiotics tested a characteristic sigmoid response curve was observed. This meant that as the antibiotic concentration increases, there was also a corresponding increase in percent positives until a concentration plateau was attained after which all samples were positive (Fig 4). The LOD, was defined as the concentration giving 95 percent positive results with 90 per cent confidence (IDF, 2002).

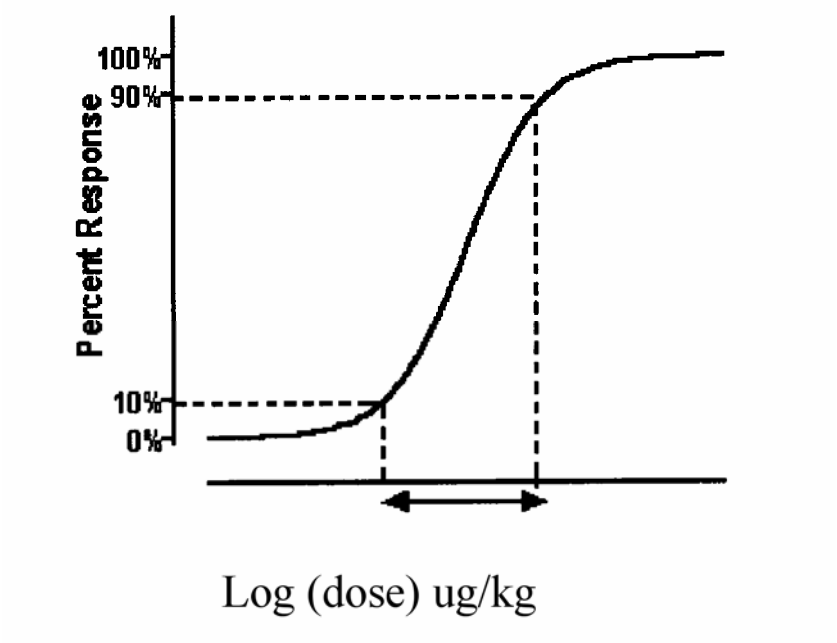


Figure 4. Illustration of the concentration plateau attained in the verification of LODs

For the tested antibiotics, the LODs for three antibiotics (penicillin, spiramycin and dihydrostreptomycin) were below established CAC MRLs (Table 6). The other four antibiotics (oxytetracycline, dapsone, sulfamethazine and spiramycine) LODs of the test developers could not be verified.

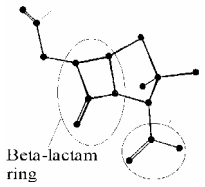
Table 6. *Results of limits of detection of the two-tube test using milk standards fortified with selected antibiotics*

Antibiotic	Present study (µg/kg)	Claimed detection limit (µg/kg)	CAC limit
Penicillin G (Tube A)	1.5	1	4
Penicillin G (Tube B)	1.8	1	4
Oxytetracycline	140	35	100
Dapsone	1.25	1	0
Sulfamethazine	225	60	100
Trimethoprim	150	20	50
Spiramycin	125	100	200
Dihydrostreptomycin	100	40	200

Despite the method detecting some of the antibiotics at slightly higher levels than established MRLs, the method offered advantage in its ability to detect a broad spectrum of antibiotics. Validation of screening tests is essential to determine the most appropriate testing option in different regions. This is necessary as tests may differ in their ability to predict for the presence or absence of antibiotics in the milk due to various factors, which interfere with the screening

assays. These components may include somatic cells, lactoferrin, lysozyme, microbes and free fatty acids (Carlsson *et al.,* 1989; Cullor *et al.*, 1992; Sischo & Burns, 1993).

In a later study (Shitandi & Kihumbu, 2004) the applicability of the test was tested for detection of a wider range of the ß-lactam family. This group consist of antibiotics which are the used widely for mastitis and dry-cow treatment. A common feature of the family is the ß-lactam ring which confers antibiotic activity. The ß-lactam ring is fused to a 5-membered thiazolidine ring and in the case of penicillins it contains a 6-aminopenicillanic acid (6-APA) nucleus (Fig 5). Although the 6-APA nucleus has little antibacterial activity itself, it is a major



structural requirement for antibacterial activity of penicillins.

Figure 5. 6-aminopenicillanic acid (6-APA) nucleus, composed of a ß -lactam ring fused to a 5-membered thiazolidine ring

Test parameters studied were: practicability; LODs compared to MRLs and repeatability and the findings are summarized in Table 3. The LODs established for the test were: penicillin G, 2µg /kg; ampicillin, 2µg /kg; amoxicillin, 2µg/kg; oxacillin 30µg/kg, cefalexin 100µg /kg, cephapirin 60µg /kg and ceftiofur 100µg/kg all within CAC MRLs. The cost per ten samples using the improved tube test was ≤ 1 USD per compared with 5 USD for the Delvo test.

Table 7**.** *Summary of the results observed with the improved two-tube test and Delvo test*

Characteristic	Improved two tube	Delvo Test SP
Quantitative/Qualitative	Semi-qualitative	Semi-qualitative
Sample throughput	>70/day	>500/day
Analyst	Semi-skilled	Semi-skilled
Sensitivity at 4µg/kg	96%	92%
Specificity at 4 µg/kg	92%	96%
Positive predictive value	94%	96%
Negative predictive value	95%	91%
Agreement strength measured by Kappa	Good	Good
Cost per 10 samples	< 1 USD	≥ 5 USD

Source: Shitandi & Kihumbu, (2004)

Sensitivity (the probability that the test gives a positive result for a truly positive sample) and specificity (the probability that the test gives a negative result for a truly negative sample) are recognised as standard measures of performance. The results showed that the strength of agreement between the analysts in two laboratories tested was good. The determined performance characteristics

(sensitivity, specificity, and reproducibility) of the two-tube test at the penicillin G cut off limit of 4 µg/kg were good (Table 7).

The two-tube method was able to detect residues of the most commonly utilised ß-lactam family in local laboratories (Shitandi & Kihumbu, 2004). Minimal use of capital equipment is needed for the test, as the main requirements include an incubator, refrigerator and a water bath. Although exact quantification of antibiotic concentrations cannot be assessed with the tube test method, a semiskilled analyst can reliably classify milk samples as positive or negative for common members of the ß-lactams at levels exceeding established MRL. Low cost and ease of performance are considered to be key criteria when tests are used in food industries (Gardner, 1997) and this applies in particular to dairies in low-income countries. The improved tube test could thus be a useful method for analysis of raw milk in dairies in low-income countries.

The two-tube diffusion test was consequently utilized in a prevalence study (Paper I) to determine the prevalence of antibiotic residues in raw milk within the Nakuru district. Between 2000 –2002, 14.6 % of the field samples (n = 1109) were tested of which 22.0 % (n = 244) were found to be suspect positive in the test. Identification of ß-lactam antibiotics was accomplished by means of the *B. stearothermophilus* plate based on the relationship inferred between known concentrations in fortified milk samples and inhibition zone sizes along a plotted regression curve. The identification procedure confirmed 165 samples to contain ß- lactams of which 37.6 % (n = 56) contained levels ≥ 10 µg/kg, thus exceeding established CAC limits by two fold. The high antibiotic content of raw milk was found to constitute a problem in local dairies.

Antibiotic residues and contributing factors (Paper II)

To identify the factors contributing to the occurrence of antibiotic residues in milk evident in study I, the frequency of contamination was further investigated in paper II. A comparison study was performed amongst small and large-scale dairy producers to determine if there were differences between the two types of producers.

Table 8. *A comparison of reported antibiotic residue prevalence in consumer milk*

Country	Year	Reported Prevalence of antibiotic residues	Control program in local dairies	Reference
Brazil	2000 & 2001	4.3-50%	None	Borges *et al.,* 2000; Folly & Machado, 2001; Do Nascimento *et al.,* 2001
India	1995	9%	None	Sudershan & Bhat, 1995
Poland	1995	13-22%	None at time of study	Rybinska *et al.,* 1995
Sweden	1998 & 2003	0.26-0.08%	Present	Sternesjö & Johnsson, 1998; 2003
Nine European countries	1990	1.2 – 1.5%	Present	Suhren *et al.,* 1990.

Of the 1600 samples tested with the improved two-tube test, 13% contained ß-lactams at levels exceeding the established CAC MRL of 4µg/kg for penicillin G. These results were in agreement with those found in paper I, where 10.6% of the survey samples contained ß-lactam antibiotics at levels exceeding MRLs.

Eighteen percent (18%) of the samples from small-scale producers in the study area were ß-lactam positive compared to 8% from the large-scale producers and this difference in contamination was significant ($p<0.001$). Other independent studies within Kenya have shown that many animal products in Kenyan market have an unacceptable high level of drug residues (Faraj & Ali, 1981; Omija *et al.*, 1994; Odero, 2002; Muriuki *et al*., 2001).

A comparison of reports of incidence of antibiotic residues in milk between low and high-income countries shows a distinct difference (Table 8). While these results may be influenced by several factors such as the type of analytical method and milk sample, the difference in incidence of antibiotic residues in milk between low and high-income countries is evident. The presence of a control system in regions with reported low prevalence rates of antibiotic residues is likely to be the main contributing factor.

To explain why the higher frequencies (69%) of the violations in paper II occurred among small-scale milk producers, a qualitative analysis of the small-hold milk producers (n=220) was carried out in several districts. The use of a qualitative method was considered useful from the recognition that some variables could not be accommodated in the experimental design and should allow opportunities for these variables to be identified in the research process. To obtain unbiased information about attitudes a semi-structured interview was used.

According to Kadushin, (1990) there are many advantages with asking open-ended questions in a semi-structured interview. One advantage is that open-ended questions allow the participants to introduce significant material that the interviewer may not have thought about as they give the participant the freedom to answer in a variety of ways. Open-ended questions are more likely than closed-ended questions, which are used in quantitative analysis, to provide, "information about the interviewee's feelings and intensity of feeling and are more likely to provide information about the interviewee's explanation of his attitudes and behaviour (Kadushin, 1990).

The results suggested a lack of knowledge on antibiotic management aspects and the absence of a control program as main risk factors. Only 9% of the producers were familiar with a quality assurance program and only 11% of the producers made some use of written treatment records. None of the farms used on-farm screening tests for antibiotics. This was mainly attributed to the high costs and lack of incentives to reward quality milk within the dairy industry. The farmers also indicated their unwillingness to discard milk from recently treated cows due to economic difficulties.

Drug resistant *Staphylococcus aureus* (Paper III)

The main part of antibiotics used in therapeutic treatment of mastis in Kenya (Njau & Kundy, 1985; Odongo & Ambani, 1989) and many other regions in the world are for treatment of *S. aureus (*Roberson *et al.,* 1994; Pyörala & Pyörala, 1997; Sol *et al.,* 1997*)*. It was thus hypothesized that if there were any differences in adherence to recommended regimens of antibiotic usage it would be manifested in the resistance profile of *S. aureus* isolates from the two producer categories. Study III thus evaluated the prevalence of multi-drug resistant *S. aureus* in Kenyan milk and investigated any differences between the main producer categories in the country.

Table 9. *Susceptibility of S. aureus n(%) isolates from quarter milk samples in large (n = 201) and small-scale (n = 201) herds*

	Categories of farms						
	Large-scale farms			Small-scale farms			
Antibiotic tested	R	I	S	R	I	S	P value
Penicillin 30µg	33 (16.4)	5 (2.5)	163 (81.2)	62 (30.8)	6 (3.0)	133 (66.2)	0.001
Tetracycline 30µg	11 (5.5)	0 (1.0)	190 (94.5)	27 (13.4)	1 (0.5)	173 (86.1)	0.004
Chloramphenicol 10µg	11 (5.5)	26 (12.9)	164 (81.5)	16 (8.0)	8 (4.0)	177 (88.1)	0.071
Erythromicin 15µg	18 (9.0)	29 (14.4)	154 (76.7)	39 (19.4)	23 (11.4)	139 (69.2)	0.092
Trimethoprim /Sulfamethazine 1.2/23.8µg	5 (2.5)	4 (2.0)	192 (95.5)	6 (3.0)	1 (0.5)	194 (96.5)	0.610

R-Resistant; I-Intermediate; S-Susceptible. The overall distribution difference between large and small scale farms was significant ($p<0.05$), as determined by Chi-square test.

The prevalence of *S. aureus* in milk in paper III, was 30.6 %, which was lower than in the studies by Lauerman *et al.,* (1973), Hamir *et al*., (1978) and Ngatia (1988) who observed prevalence's of 49 %, 48 % 55 %, respectively. The previous studies were, however, concentrated around a single area and the findings from this study indicate a wider distribution geographically. Differences in antibiotic sensitivities amongst isolates from the two producer categories were found for penicillin G and tetracycline from the five types of antibiotics tested (Table 9).

There was an overall mean prevalence difference of 7.6 % between isolates from the small and large farms and the overall distribution of the susceptibility pattern was significant ($p < 0.05$). Among the small farm isolates, susceptibility to penicillin G was lowest with susceptibility to trimethoprim/ sulfamethazine being highest. For large-scale farms the lowest susceptibility was to erthromycin and highest to sulfamethazine/trimethoprim. The difference in overall mean susceptibilities between the farm types was 4.7 %.

From Table 10, among the 402 isolates from the two types of farms, multiple resistance defined as lack of susceptibility to at least two antibiotics from different families was observed in 69 (34.3%) of the isolates were from the small-scale

farms compared to 36(17.9%) from the large-scale farms. The difference in overall prevalence of multi drug resistant *S. aureus* isolates between the two producer categories was significant ($p < 0.05$).

Table 10. *Categories of antibiotic resistant S. aureus, isolated from milk in small and large dairy herds (n=402)*

Number of antibiotics to which isolates were susceptible	Category of farm	
	Large scale farms isolates	Small-scale farms isolates
5	132(65.7%)	122(60.6%)
4	33(16.4%)	10(5.0%)
3	10(5.0%)	37(18.4%)
2	16(8.0%)	27(13.4%)
1	9(4.5%)	4(2.0%)
0	1(0.5%)	1(0.5%)
Total	201	201

Staphylococcus. aureus has developed multi-drug resistance in many regions of the world (De Oliveira *et al*., 2000; Martel *et al*., 2000; World Health Organization, 2000) although reported prevalence rates indicate that wide variations exist regionally and even from herd to herd (Hinckley *et al.,* 1985; Myllys *et al.,* 1998; Owens, *et al.,* 1988; Anderson, 1989; Waage *et al*., 2002). The increasing numbers of antibiotic resistant bacteria being isolated in milk and milk products (Abbar & Kadder, 1990; D'aoust *et al.,* 1992; Diaz de Aguayo *et al.*, 1992, Jayarao & Oliver, 1992; Adesiyun *et al*., 1995) is of particular worry. In Kenya, there is no formal system for monitoring or surveillance of antibiotic resistance in animal bacterial isolates and previous mastitis studies have not addressed the issue, a common trend in many low-income countries (Hart & Kariuki, 1998).

The observed differences in paper III may be suggestive of a difference in selection pressure between the two categories of producers. There is, however, a limitation in that, the amounts of antibiotics applied is unknown due to lack of control at the individual farms. There was in particular little detailed information regarding the actual use of drugs and treatment regimens on the different farms and no clear-cut relationship could be established between antibiotic usage and resistance development. It would be thus of future interest to investigate in Kenya, the effect of both antibiotic duration of exposure and concentration, especially concentrations below the minimum inhibitory concentration, on the rate of resistance selection.

The producers were interviewed about their usage of antibiotics and their attitudes towards education in related fields. There was an evident difference between producers in their documentation of antibiotic usage, small-scale farms being less inclined to keep treatment protocols. A safe and unequivocal identification of each animal is the basis for all further controls on the farm. This requires the marking of each animal a farm register and a careful control and documentation of all antibiotics administered. Between the two types of producers, the difference in documentation of antibiotic usage was significant

($p<0.001$), suggesting that farm category had a strong influence on the decision by the farmer to keep records.

The lack of documentation in itself does not indicate non-usage. There could also be other risk factors such as inappropriate dose and/or combination of drugs, which affect treatment regimens even where documentation is present. Treatments in most small-scale farms have been observed to depend on availability of money and drugs and not the epidemiology of the disease (Keyyu *et al.,* 2003).

The farmers (n = 234) who were interviewed indicated a need for more information in five areas. These were preventive management (34.0%); affordable tests to control residues in milk (22.8%); preparation of antibiotics (20.0%); public health concerns (11.2%); disposal of surplus antibiotics (7.8%) and antibiotic persistence in milk (4.2%).

A risk tool for control of antibiotic residues on farm (Paper IV)

The final phase of the experimental study (IV) was devoted address the need for an affordable control strategy. Since the decisions associated with use of antibiotics to lactating dairy cows occur at the farm level, it was felt that an effective residue control program must be characterized by management practices that will assure safe use of a drug. Study IV thus focussed on development of an on farm control tool, which could be readily used to determine the risk status of producers in terms of antibiotic residues violation.

A simple risk assessment tool (Table 11), was developed that predicted the chance of antibiotic residues in milk from small-scale farms based on the HACCP principles

The use of semi-structured interviews in study II and III were exploratory in nature and served-as prelude to the quantitative study carried out in study IV. The results from the interviews (paper II and III) identified the risk factors of interest that formed the basis of the case-control study.

Using such a control strategy it is necessary to carry out risk assessment so as to assess the significance of each identified risk factor in order to build in appropriate control mechanisms (McClellan, 2003). In study IV, on comparisons of each risk factor, only five were found to be significantly ($p < 0.05$) and independently more prevalent among residue violators than controls and were selected to be used in the risk tool

These factors apart from showing significant differences in univariate analysis could be readily monitored by producers based on their day-to-day observation of their farms and are performed easily. This conferred the advantage of generating a pragmatic risk assessment tool and requiring no formal measurements, additional training, or equipment. The use of case indices likely to contribute to antibiotic residues from the farms follows the general principles of HACCP (WHO, 2001), where specific hazards are assessed, and the points to control them defined.

Table 11. *Indices selected to develop antibiotic risk assessment tool (CHRAV*)

No.	Interview prompts & Check list
1	Is there a lack of written communication to milkers on treated animals as assessed by whether the treated cows are unlabelled? (Yes=1, No=0)
2	Does the producers have a previous history (last 1 month) of ≥ 3 residue violations in milk supplies to local dairy? (Yes=1, No=0)
3	Is there a lack of records documenting treatment protocols, culture tests, SCC and disease events? (Yes=1, No=0)
4	Is their lack of knowledge on how to use antibiotics rationally? (Yes=1, No=0)
5	Does the milk producer lack a disease prevention program from a registered veterinarian? (Yes=1, No=0)
	Total score

During a 16 week, intervention period on farms utilising the risk tool, the treatment group had a mean rate of 1 antibiotic residue violation per producer. The control group had a mean rate of 1.875 residue violations per producer, a difference of 30.4 %. The success of the developed risk assessment tool was further gauged (Table 12), where it was observed that the intervention group had an overall residue violation of 0.119 compared to 0.381 per producer for the control group. This was a difference of 52.4% in residue violations after the intervention and the overall difference was significant ($p < 0.05$).

Table 12. *Prevalence (%) of residue violations per farm after the intervention with the risk tool on 84 farms*

Numbers of residue violations per producer	Controls (n=42)	Intervention (n=42)
None	26(62%)	37(88.0)
One	6(14.2%)	2(4.8%)
Two	4(9.5%)	2(4.8%)
Three or more	6(14.3)	1(2.4%)

The purpose of the risk assessment tool was not to primarily diagnose residues in milk but to identify producers and risk factors contributing to residue violations. The five indices (Table 11) that were selected and the risk tool derived appear to predict high-risk producers well. The main finding is thus that by undertaking an educational training on farm to address the issues highlighted by the risk tool, the incidence of antibiotic residues decreased in milk. Many low-income countries do not have the resources to support extension programs and there is a decline of governmental support for conventional means of extension (Oboegbulem, 1996). There is thus a recognised need for alternative methods for disseminating technologies (Scarborough *et al*., 1997).

Conclusions

The thesis addressed the need for cost-effective, sustainable control program in the local dairy industry through the use of simple approaches. The results provided evidence that implementation of an affordable control program at farm level, which would result in a reduction of antibiotic residues in milk, is possible in Kenya. This can be attained through the use of a risk-based strategy in, which prevention through an extension resource persons would focus on selected high risk milk farms. While the results may not be a reflection of the whole Kenyan dairy industry the approach can be used in other areas.

The study recommends the use of a low cost microbiological method in local dairies for screening of antibiotic residues in bulk milk. The possible use of such a method was demonstrated in this study using the two-tube test microbiological ssay. Dairy processing plants could easily adopt the technique.

The project was able to contribute to data in terms of identifying the type of antibiotics frequently present in contaminated Kenyan milk, which was lacking. It was evident that antibiotic contamination of raw milk occurred more frequently on small than large farms. The major risk factors were identified and the results reinforce an urgent need for education of farmers with respect to risks associated with the administration of antibiotics. Economical incentives may also be necessary to convince producers of the importance of food safety.

The study identified perceived needs of farmers to be addressed and suggests that regulatory policies are required to be put in place by the governmental authorities to address the problems of farmers. These alone cannot solve the inherent residue problems. Control strategies that focus on implementing on-farm measures to reduce the risk for contamination of milk should be more sustainable.

Training of producers and other holders is therefore a key point to improve quality. Any control strategy as the one suggested in this thesis should be implemented alongside educational measures to producers on prudent antibiotic use and adoption of sound management practices. The safety and the quality of dairy products will improve as people realize that

- healthy animals are more profitable, which encourages them to pay attention to diagnosis and treatment of diseases.
- prevention costs less than cure
- image of quality can increase the attractiveness of milk on the market.

Methods and tools described in this thesis can only support the collective efforts to improve food safety and quality. Adopting a holistic food chain approach to food safety recognises that primary responsibility for supplying safe milk lies with all those involved in dairy production. The major outcome of this initiative is that it has shown how such a control program can be applied within the context of low-income dairies where cost is a major disincentive to setting up control systems. The findings and recommendations in this thesis are not specific to Kenya and could be used elsewhere in low-income countries.

Suggestions for future research

There is still a lack of standardised data from both the veterinary and medical sectors in many countries on the susceptibility of zoonotic bacteria and the presence of resistance determinants in indicator bacteria transmitted from animals to humans (WHO, 1997, WHO, 2000).

In Kenya, the role of antibiotics in the food chain in relation to the emergence of resistant bacterial pathogens of public health significance needs to be further clarified and quantified. To achieve a better estimation of the risks posed, more accurate and quantifiable risk assessment studies are needed. It would be thus of future research interest to investigate:

* The rate of transfer of medically relevant resistance genes and resistant bacteria from food animals to humans.
* The rate of development of resistance in bacteria used in starter cultures of importance in food-production.
* The effect of both duration of antibiotic exposure and concentration, especially concentrations below the minimum inhibitory concentration, on the rate of resistance selection.
* The stability of important antibiotics used on food animals and their metabolites in the environment.

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