STUDIES ON ANTIMICROBIAL CONSUMPTION AND OCCURRENCE OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* IN FOOD ANIMALS IN KENYA AND PUBLIC HEALTH IMPLICATIONS

BY

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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

Dedicated to my parents Jotham Ibrahim Mapenay Ole Kikua and Sarah Naneu Kikua; my wife Dr. Monica Maichomo-Mapenay and Children-Milanoi, Lemayian and Nashipae and the entire Kukuo family at large.

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ABSTRACT

Antimicrobial agents are commonly used in the food animal industry for the treatment of bacterial and protozoal infections, prophylactic use in intensive animal rearing or for growth promotion purposes. Increasing levels in antimicrobial resistance have renewed interest in obtaining relevant antibiotic consumption data from humans and food animal industry. These data are essential for risk analysis and planning and can be timely in interpreting antimicrobial surveillance data. In an effort to generate baseline data on the magnitude of antimicrobial resistance (AMR) for risk assessment analysis in Kenya, this study was carried out, with the following broad objectives:

a) To monitor antimicrobial consumption patterns for food animals in Kenya during the 2-year period, 2000-2001; b) to determine the prevalence of antimicrobial resistance in *Escherichia coli* (as an indicator bacteria) isolates from some food animals in Kenya during the same period; c) to characterize genotypic resistance of multi-drug resistant *E. coli* using polymerase chain reaction; and d) to study associations between AMR and antimicrobial consumption in food animals using regression and correlation analysis.

Data on antimicrobial usage in animals was determined based on importation figures recorded by the pharmaceutical industry. These data, representing the volume of drugs imported from overseas as well as those locally manufactured, was collected from the official records of the Pharmacy and Poison's Board (PPB) registry of the Ministry of Health. Data including the name, quantities (in vials or packs), potency of pharmaceutical preparation imported for sale or manufactured for local use were recorded for each

antibiotic, antimicrobial class or combination. In addition, data on dosage form and route of drug administration, amount or quantities (kilogrammes) of active ingredients of each antimicrobial class, intended use of antimicrobial agent and target species were recorded for each antimicrobial agent.

Antimicrobial agents were classified in accordance with the international classification system for drug consumption studies, Anatomical Therapeutic Class (ATC) code maintained by the WHO Collaborating Centre for Drug Statistics and Methodology. For each antimicrobial group, total amounts (in kilograms of active ingredients) were calculated for specific variables like intended use, route of administration and target species.

During the study period the annual increase of total antibacterials for systemic use was approximately 5400 kg. In 2001, the quantity of antimicrobials used in food animals increased by 54.7 % to an estimated 15234 kg active compound. The usage of fluoroquinolones increased by 2500 %, from 200 kg in 2000 to 5204 kg in 2001 while usage of tetracyclines increased by 6.4 %, from 8481 kg in 2000 to 9020 kg in 2001. The usage of sulfonamides decreased by 21 %, from 148 kg to 117 kg in 2001, while aminoglycosides consumption declined by 60 %, from 318 kg to 127 kg in 2001. The consumption of penicillins (including Extended Spectrum β-lactams) marginally increased by 4 %, from 520 kg in 2000 to 540 kg in 2001.

Tetracycline was the most prescribed antibiotic class during the study period. A mean total of 8751 kg (69.8 %) was consumed per year. The mean consumption per year for the fluoroquinolones (2702 kg) represented 21.6 % of total antimicrobial consumption while the mean annual consumption for the β-lactams was 530 kg (4.2 %) of antimicrobial

consumption during the study period. The mean consumption per year for the aminoglycosides was 222 kg (1.8 %). With regard to sulfonamides, the percentage mean total consumption per year during the study period was approximately 1 %.

Approximately 9232 kg (74 %) of antimicrobials was intended for consumption by large animals (cattle, shoats and goats) while 2886 kg (23 %) and 422 kg (3%) was meant for large animals and poultry and poultry only categories, respectively. About 98 % (12300 kg) of total antimicrobial use was meant for specific therapy while approximately 2 % (208 kg) was used for prophylactic purposes. Of the total antimicrobial consumption meant for systemic use, 76 % (10371 kg) was for parenteral use while 24 % (3355 kg) was for oral administration.

In the second part of this study, a random sampling of cattle, swine and chicken was carried out during slaughter between 2000 and 2001. Fecal samples and carcass swabs were collected from 55 cattle and 116 pigs while cloacal swabs were collected from 69 chicken at various slaughterhouses in Kiambu and Nairobi. These samples were inoculated into Stuart's transport medium, and transported to our laboratories for isolation. In the laboratory the samples were cultured in enrichment media (peptone water) for 24 hours at 37°C and thereafter cultured in selective media (Eosin methylene blue agar) for a further 24 hours at 37°C. *E. coli* isolates were identified biochemically using Indole Methyl red Vogues-Proskauer and Citrate (IMViC) reactions and morphologically using microscopy and gram stain.

Escherichia coli isolated from cattle, chickens and pigs during slaughter were characterized according to their antimicrobial resistance profiles to eight antimicrobial agents. Antimicrobial susceptibility was carried out using disc diffusion technique. The

antimicrobial agents tested were ampicillin, tetracycline, sulfamethoxazole, streptomycin, co-trimoxazole, chloramphenicol, kanamycin and gentamycin.

Antimicrobial resistant *E. coli* isolates identified phenotypically were further characterized using genotypic methods. The distribution of five resistance genes covering three antimicrobial classes (beta-lactams, tetracycline and sulfonamides) and Class 1 integrons were assessed using Polymerase Chain Reaction (PCR). *In vitro* conjugation tests on antimicrobial resistance were performed on multi-drug resistant *E. coli* isolates using recipient *E. coli* K12F-(nalidixic acid resistant). The antimicrobial susceptibility testing and genotypic characterization of the transconjugants were also determined.

E. coli was isolated from 70.8 % of the animals that were sampled. However, the proportion of *E. coli* recovered differed among species i.e. 57.3 %, 76.8 % and 80.2 % in cattle, poultry and swine, respectively.

Approximately 38 % of all *E. coli* isolates from food animals were antibiotic-resistant with 26 % of them being multi-drug resistant (resistant to two or more antimicrobials). Resistance to ampicillin, tetracycline and sulfonamides was found to be the most frequent, but resistance to gentamycin was absent. Overall, 58.6 % of all isolates resistant to a single antimicrobial agent were resistant to ampicillin while 20.7 % and 17.2 % were resistant to tetracycline and sulfonamides respectively. A higher prevalence of AMR was observed for *E. coli* from swine and poultry compared to cattle. Some 27.1 % of the *E. coli* isolates from pigs and 29.2 % from poultry were multi-drug resistant. In contrast, only 2.4 % of the bovine isolates were multi-drug resistant.

Among cattle and swine isolates a higher proportion of *E. coli* isolates from carcasses (68.4 %) were multi-drug resistant compared to 31.6 % of fecal isolates ($P > 0.05$). A

significantly higher proportion of isolates from carcass were resistant to ampicillin (65 % vs 44%, P = 0.04) compared to fecal isoates, but not significantly higher for tetracycline (56 % versus 44 %, P = 0.25), streptomycin (63 % vs 35%, P = 0.24) and sulfonamides (55 % vs 45%, P = 0.41). Similarly, higher resistance rates of fecal isolates compared to carcass isolates to chloramphenicol (75 % vs 25 %) and kanamycin (67 % vs 35 %) were not significant (P > 0.05).

Examination of the linkages among different resistance phenotypes determined statistically showed a significant degree of positive associations between oxytetracycline and chloramphenicol resistance as well as oxytetracycline and kanamycin resistance.

The prevalence of AMR and the Multiple Antibiotic Resistance (MAR) index in *E. coli* from food animals from the Rift Valley, Central and Eastern province were used to compare and contrast antibiotic resistance levels in these regions. The prevalence of AMR and MAR index were highest in Eastern Province and lowest for Rift Valley Province. These findings indicate that 17 %, 28 % and 44 % of *E. coli* isolates from Rift Valley, Central and Eastern Province, respectively came from high risk sources (MAR index > 0.20). However, these differences could not be substantiated since data on antimicrobial consumption from the different regions was unavailable.

Approximately, 93 % (n = 27) of the 29 ampicillin resistant *E. coli* isolates tested expressed the *TEM*- β-lactamase genes. Significant differences in the distributions of tetracycline [*tet*(A) and *tet*(B)], and sulfonamide (*sulI*, *sulII*) resistance genes were observed during the study period. Tetracycline resistance was encoded by the presence of a single gene, either *Tet(A)* or *Tet(B)* in 52 % of tetracycline- resistant isolates. Similarly Sulfonamide resistance was encoded by the presence of either, *SulI* and /or *SulII* genes in 95 % of

sulfonamide-resistant isolates. Three out of 20 (15 %) multi-drug resistant isolates possessed large (2.0 - 2.5 kb) Class 1 integrons. All three isolates possessing class 1 integrons were also phenotypically resistant to sulfamethoxazole.

In vitro conjugation (mating) studies demonstrated a plasmid-mediated transfer of genes encoding resistance to ampicillin, tetracycline and sulfonamides.

Ten (58.8 %) out of 17 isolates transferred at least one resistance marker to recipient *E. coli* K12. Of these, 64.3 % of the tetracycline resistant isolates transferred resistance to tetracycline, 45.5% of the sulfonamide resistant *E. coli* isolates transferred resistance to sulfamethoxazole and co-trimoxazole while 42.9 % of ampicillin-resistant isolates transferred resistance to ampicillin. In addition, seven (53.8 %) of the 13 multi-drug resistant isolates transferred resistance to two or more resistance markers to recipient *E. coli* K12.

In the final study, associations between AMR in *E. coli* isolates from food animals and Non-typhoidal *Salmonellae* [NTS] from humans and antimicrobial consumption in food animals were examined. An ecologic study design was used, which allows measurement of the total and individual antimicrobial agents exposure on antimicrobial resistance. AMR surveillance data in Kenya for the period from 1996 to 2001, obtained from published data, was included in this study. National antimicrobial consumption data for food animals from 1996 to 2001 were obtained from both published information and also from the results generated from the present study. Least-square linear regression analysis was used to determine the relationship between antimicrobial use (expressed in tonnes) and the prevalence of resistance (R). Spearman Rank correlation coefficient was used to determine the degree of correlation between antimicrobial use and the prevalence of AMR.

Total antimicrobial consumption in food animals was positively correlated with the prevalence of multi-drug resistance ($r = 0.203$) but negatively correlated with sulfonamide resistance ($r = -0.239$). However, both associations were not statistically significant ($p > 0.05$). Total antimicrobial consumption was significantly associated with the prevalence of tetracycline resistance ($r = 0.878$; $p < 0.05$). Moreover, the prevalence of tetracycline resistance was significantly associated with tetracycline consumption ($r = 0.953$; $p < 0.05$) while β-lactam usage was correlated with ampicillin resistance ($r = 0.717$; $p = 0.062$) although the association was not significant.

Among NTS isolates from humans, total antimicrobial consumption was positively correlated with tetracycline resistance ($r = 0.801$; $p > 0.05$). β-lactam consumption was positively correlated with ampicillin resistance ($r = 0.489$; $p > 0.05$) while aminoglycoside consumption was positively correlated with streptomycin resistance ($r = 0.265$; $p > 0.05$). However, nalidixic acid resistance was negatively correlated with total antimicrobial consumption ($r = -0.512$; $p > 0.05$) and fluoroquinolone consumption in food animals ($r = -0.136$; $p > 0.05$).

In conclusion, the study found increasing levels of fluoroquinolone consumption in food animals in Kenya and therefore proposes the use of the " precautionary principle" as a risk reduction measure in the mitigation of AMR in food animals. In addition, the study concludes that healthy food animals carry transmissible multi-drug resistant *E. coli* and may pose a public health risk to humans. The data presented in the ecologic analysis suggest an important association between antibiotics (tetracyclines and β-lactam) usage in food animals and occurrence of resistance among *E. coli* isolates from food animals. Therefore, the study recommends the restriction of tetracycline usage and prescription of

extended spectrum β-lactams (ESBL's) in food animals in line with international guidelines on the prudent use of antibiotics in food animals in order to reduce AMR in Kenya. The study also proposes the planning and implementation of a national action plan for the appropriate use of antimicrobial agents in Kenya, whose effectiveness should be monitored and evaluated through prospective and continuous surveillance of antimicrobial resistance and antimicrobial consumption data.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

The use of antimicrobials for treatment and prophylaxis in both human and veterinary medicine has resulted in marked improvements in the control of bacterial infections (Espinasse, 1993). However, the indiscriminate and widespread use of antimicrobial agents has accelerated the emergence and spread of antimicrobial resistance (Mazodier and Davies, 1991). According to the infectious-disease report released by the World Health Organisation in 2000, antimicrobial resistant organisms have become prevalent worldwide (WHO, 2000).

Increasing antimicrobial resistance (AMR) presents a major threat to public health because it reduces the efficacy of antimicrobial treatment leading to increased morbidity, mortality and healthcare expenditure (Coast *et. al.*, 1996). Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of AMR in microorganisms (Witte, 1998). Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals (animals and humans) or populations (van den Bogaard, 1997; Piddock, 1996).

Antibiotics are used in animals as in humans for therapy and control of bacterial infections. In intensively reared food animals, antibiotics may be administered to whole flocks rather than individual animals. In addition, antimicrobial agents may be continuously fed to food animals as antimicrobial growth promoters (AMGP). Therefore the antibiotic selection pressure for resistance in bacteria in food animals is high and consequently their faecal flora contains a relatively high proportion of

resistant bacteria (van den Bogaard and Stobberingh, 1999). However, most AMGP commonly used in developed countries until recently are effective mainly against Gram-positive bacteria. Therefore, most resistance in faecal enterobacteria of food animals is attributed to antibiotics used on veterinary prescription (van den Bogaard, 1997).

Escherichia coli is one of the most predominant bacterial species that form part of the normal flora in the gut of humans and other animals (Atlas, 1984). Although most strains are non-pathogenic, some have acquired a diarrhogenic ability and cause infections in humans (Honda, 1992). Pathogenic strains of these bacteria are important cause of bacterial infections. In humans, these strains are the foremost cause of urinary tract infections (Falagas & Gorbach, 1995), as well as a major cause of neonatal meningitis (Klein et. al., 1986), nosocomial septicemia and surgical site infections (Thielman and Guerrant, 1999) and travellers' diarrhoea (Ericsson, 2003). In food animals, *E. coli* strains are the main aetiological agents for mastitis in cattle, air sacculitis and perihepatitis in chicken as well as newborn and post weaning diarrhoea in swine (Cheville and Arp, 1978; Kariuki, 1996; Sarff *et al.*, 1975; Bischoff *et al.*, 2002). *E. coli* due to their ubiquitous nature are considered to be a good indicator of the selective pressure of antibiotic usage.

The transfer of resistant bacteria has been shown to occur among different animal species, between humans, and from animals to humans and vice versa (Rollard *et al.*, 1985; Marshall *et al.*, 1990). These resistant bacteria may colonize the human intestinal tract and may also contribute resistance genes to human endogenous flora. The mechanism of spread of antibiotic resistance from food animals to humans

remains controversial. Colonization of the intestinal tract with resistant *E. coli* from chicken has been demonstrated in human volunteers (Linton *et al.*, 1977a). Spread of an antibiotic resistance plasmid, pSL222-6, in *E. coli* from chickens to human handlers was described by Levy *et al.* (1976a,b). Linton *et al.* (1977a,b) found the same O serotype in chickens from a commercial rearing centre, in oven-ready birds and in humans. Ojeniyi (1985; 1989) described direct transmission of *E. coli* resistant to streptomycin, sulphonamides and tetracycline from poultry to poultry attendants in Nigeria. Bass *et al.* (1999) described a high incidence of integrons encoding multidrug resistance among chicken isolates as part of transposon Tn*21*. In addition, they described the dissemination of Tn*21* among pathogenic poultry isolates and suggest that Tn*21* may transfer between pathogenic microorganisms in humans as well as in poultry.

In contrast, others have concluded that human and poultry isolates belong to two distinct pools of resistant *E. coli*. Smith (1969) concluded that the antibiotic resistance transfer between animals and humans was limited and that animal strains colonized the alimentary tract less readily than human ones. He stated that in view of the high prevalence of antibiotic resistance in humans, animals are not an important source of resistant *E. coli* in man. Shooter *et al.* (1974) serotyped animal and human *E. coli* isolates using 150 O antisera. Of the animal strains, 289 (36%) of 798 could be serotyped, whereas only two of 1580 human isolates could not be typed (Shooter *et al.*, 1974). They concluded that O serotypes of animal origin may differ from those of humans. Similarly, differences in chloramphenicol and streptomycin resistance between *E. coli* isolates from poultry and those from their attendants in North India

has been described (Kapoor *et al.*, 1974). In female poultry workers exposed to resistant microorganisms of animal origin but who had not received antibiotics, urinary tract infections were infrequently caused by poultry strains. A more detailed analysis using restriction enzyme analysis of plasmid DNA showed that none of the plasmids from human isolates appeared to be related to any of the poultry isolates (Parsonet and Kass, 1987). Caya *et al.* (1999) compared the phenotypes and genotypes of *E. coli* isolates from sick broilers in abattoirs in the province of Quebec with human isolates from hospitalized patients living in the same locality as the abattoir. A higher prevalence of resistance was found among the poultry isolates especially to gentamicin, spectinomycin, tetracycline and sulphamethoxazole. Only two poultry isolates demonstrated a possible relationship with human strains. Similar observations were reported between poultry and poultry farmers in Netherlands (van den Bogaard *et al.*, 2001). Comparing *E. coli* from a poultry processing plant in Kenya and isolates from children with diarrhoea living in close contact with poultry, Kariuki *et al.* (1997) observed differences in antibiotic resistance patterns and in the levels of multidrug resistance. The authors concluded that human and poultry isolates carry two distinct pools of resistance plasmids. Similar conclusions were made by Niljsten *et al.* (1996) while comparing resistance patterns of faecal *E. coli* isolates of pig farmers and their pigs.

Other reports have suggested the use of antimicrobials to be a major factor in the emergence and dissemination of antimicrobial-resistant *E. coli* (Meng *et al.*, 1998; Stephan and Schumacher, 2001; Teshager *et al.*, 2000). However, few studies have been carried out to determine the prevalence of antimicrobial resistance in Kenya.

Earlier, multi-drug resistant *E. coli* had been reported in childhood diarrhea in Kenya (Chunge *et al.*, 1992; Kariuki *et al.*, 1997) as well in poultry and food products (Bebora *et al.*, 1994; Ombui *et al*, 1994). However, a relative paucity of information exists regarding antimicrobial resistant *E. coli* from non-clinical sources, especially from healthy food animals. Healthy food animals are a constant source of resistant bacteria and resistance genes and can be considered to be a suitable population to study with regard to the possibility of resistance transfer from animals to humans. Resistant bacteria from the intestinal flora of food animals contaminate carcasses of slaughtered animals and reach the intestinal tract of humans via the food chain. Moreover, in Kenya few studies have been directed toward the characterization of the genotypic resistance profiles of *E. coli* strains isolated from food animals. Therefore, in this study the prevalence of resistance in faecal and carcass *E. coli* was analysed in the following populations: beef animals, chickens and pigs.

1.1 OVERALL OBJECTIVE

The main objective of the study was to examine the phenotypic and genotypic characteristics of AMR of *E.coli* from healthy food animals and to evaluate the relationship between AMR and antimicrobial use in food animals in Kenya.

1.2 SPECIFIC OBJECTIVES

1. To determine antimicrobial consumption patterns for food animals in Kenya during the 2-year period between 2000-2001.
2. To determine and compare the prevalence of antimicrobial resistance phenotypes among *E. coli* isolates from healthy food animals in Kenya.

3. To determine the prevalence of tetracycline [*Tet(A)* and *Tet(B)*], sulfonamide (*SulI* and *SulII*) and ampicillin resistance (Bla_{TEM}) genes among multi-drug resistant *E. coli* using a polymerase chain reaction (PCR) assay.
4. To investigate the occurrence of Class 1 integrons among multi-drug resistant *E. coli* isolates from food animals using PCR.
5. To study associations between AMR and antimicrobial consumption in food animals using correlation analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Escherichia coli*

2.1.1 History

Escherichia coli was first isolated in Germany by a German paeditrician, Theodor Escherich, as a cause of infantile diarrhoea (Sussman, 1985). The earliest indication of a specific virulence factor associated with *E. coli* was reported by De *et al.* (1956). They showed that certain strains of *E. coli* were associated with diarrhoeal diseases which caused secretions of fluids and electrolytes into ileal loops of rabbits. Although *E. coli* forms part of the normal microflora of the gut of humans and animals it has been found to be potentially pathogenic for both animal and humans (Dupont *et al*, 1982, Mundell *et al.*, 1976, Field, 1979).

2.1.2 Morphology and biochemical characteristics

Escherichia coli belongs to the family enterobacteriaceae and is the only member of the genus Escherichia. Members of this family ferment glucose (Atlas, 1984) and they are also oxidase negative and most reduce nitrates to nitrites. Morphologically, *E. coli* is a short gram-negative, non-spore forming and usually motile by peritrichous and fimbriate bacillus (Holts *et al.*, 1994). *E. coli* is a facultative anaerobe and most strains frequently ferment lactose although this fermentation may be delayed or even absent in some strains (Holts *et al.*, 1994). The main biochemical characteristics associated with *E. coli* are shown in Table 1.1.

Table 1.1: Main biochemical characteristics of *Escherichia coli*

Optimum growth temperature	37°C
Catalase	+
Oxidase	-
D-galactosidase	-
Gas from glucose at 37°C	+
KCN (growth on)	-
Nitrate	+
Carbohydrates (acid from)	
Lactose	+ or X*
Maltose	+
Mannitol	+
Sorbitol	+
Sucrose	d*
Inositol	-
Dulcitol	d*
Arabinose	+
Xylose	d*
Other carbon sources	
Citrate	-
Malonate	-
d-Tartrate	+
Methyl red reaction	+
Voges-Proskauer reaction	-
Protein utilization	
Arginine	d*
Gelatin hydrolysis	-
H_2S from Triple sugar iron medium	-
Indole production	+ /–
Lysine decaeboxylation	+
Ornithine	d*
Urea	-
Glutamic acid	-
Phenyl alanine	-

Key: + means positive, - means negative; d* refers to different reactions by different serotypes; X* refers to late and irregular positive (mutative).

Source: Holts *et al.*, 1994 In: Bergey's Manual of determinative bacteriology (9th edition)

2.1.3 Serology

E. coli have been classified serologically based on antigenic properties of various structures of the bacterium (Kauffman, 1966). Antigens used in the serological classification of *E. coli* include the O or the somatic antigen, which denote the polysaccharide moiety of the cell wall, the K or capsular antigen, generally an acidic polysaccharide, and the H or flagellar antigen which are proteinaceous in nature. Currently 171 different O antigens (designated O1 to O171), 55 H antigens (H1 to H55) and 133 K antigens have been identified with the recognition and establishment of new antigens being co-ordinated by the WHO Centre for Reference and Research on *E. coli* in Copenhagen (Guinee *et al.*, 1980).

2.1.4 Ecology and Pathogenicity

The primary habitat of *E. coli* is the gastro-intestinal tract of mammals and birds. This habitat has enabled the extensive use of *E. coli* as an indicator micro-organism of fecal contamination of water and foods (Atlas, 1984). *E. coli* may show opportunistic pathogenicity by causing enteritis, urinary tract infections and neonatal meningitis and mastitis in cows (MacDonald *et al.*, 1970, Sarff *et al.*, 1975). The most important of these diseases is enteric diseases in children and young animals.

2.1.5 Pathogenicity mechanisms

The different pathogenic groups of *Escherichia coli* involved in enteric infections have been classified in humans (Honda, 1992) as follows;

1. Enteropathogenic *E. coli* (EPEC)

2. Enterotoxigenic *E. coli* (ETEC)
3. Enterohemorrhagic *E. coli* (EHEC)
4. Enteroinvasive *E. coli* (EIEC)
5. Enteroaggregative *E. coli* (EAEC)

2.1.5.1 Enteropathogenic *E. coli* (EPEC)

The varieties of *Escherichia coli* that cause diarrhea are classified into pathogenic groups (pathotypes) according to their virulence determinants (Nataro and Kaper, 1998). The specific nature of these virulence determinants imbues each pathotype with the capacity to cause clinical syndromes with distinctive epidemiologic and pathologic characteristics (Robins-Browne and Hartland, 2002). For example, enterotoxigenic *E. coli* causes watery diarrhea in children in developing countries and in travelers to those countries, whereas enterohemorrhagic *E. coli* (EHEC) may cause hemorrhagic colitis and the hemolytic uremic syndrome because of the production of Shiga toxins. Enteropathogenic *E. coli* (EPEC) shares several key virulence determinants with the most common varieties of EHEC but does not produce Shiga toxins nor cause hemorrhagic colitis or hemolytic uremic syndrome. Instead, it causes nonspecific gastroenteritis, especially in children in developing countries (Robins-Browne, 1987; Trabulsi *et al.*, 2002). EPEC also differs from other pathotypes of *E. coli* in that it typically carries an EPEC adherence factor plasmid (pEAF). This plasmid encodes a bundle-forming pili (Bfp), which promote bacterial adherence to epithelial cells and are an essential virulence determinant (Bieber, *et al.*, 1998), and a transcriptional activator, Per, that upregulates genes within a chromosomal

pathogenicity island, termed the locus for enterocyte effacement (Frankel *et al.*, 1998; Gomez-Duarle and Kaper, 1995). This pathogenicity island encodes a number of essential virulence proteins, including the surface protein intimin (the product of the *eae* gene), which is required to produce the attaching-effacing lesions that are a key feature of EPEC-induced pathology. However, a subset of EPEC known as atypical EPEC, do not carry pEAF and hence do not produce Bfp or Per (Trabulsi *et al.*, 2002).

Despite evidence from reports of outbreaks of diarrhea attributed to atypical EPEC (Viljanen *et al.*, 1990; Yatsunagi *et al.*, 2003), the role of atypical EPEC in disease is controversial. In several reports, atypical EPEC strains have been identified in children with acute diarrheal disease in Iran, Norway, Peru, Poland, South Africa, the United States, United Kingdom and Australia (Afset *et al.*, 2003; Okete *et al.*, 1997; Bouazi et al., 2000; Galane and Le Roux, 2001; Knutton *et al.*, 2001; Nataro *et al.*, 1985; Pacicorek, 2000; Kukurocovic *et al.*, 2002). Atypical EPEC has also previously been reported in association with prolonged diarrhea (Afset *et al.*, 2004).

Atypical EPEC may have an innate propensity to persist longer in the intestine than varieties of *E. coli* which cause diarrhea that is more transient in nature. EPEC adheres tightly to epithelial cells and disrupts normal cellular processes (Chen and Frankel, 2005) and evidence suggests that atypical EPEC may retard apoptosis of intestinal epithelial cells (Heczko et al., 2001), possibly because of the lack of Bfp (Melo et al., 2005). These features may favor prolonged intestinal colonization by atypical EPEC compared with other intestinal pathogens.

2.1.5.2 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of pediatric diarrhea in the developing world and is also an important cause of diarrhea in adult travelers to these regions (Bern *et al.*, 1992; Black, 1990; Peruski *et al.*, 199). On an annual basis, ETEC strains have been estimated to cause 400 million episodes of diarrhea in children under age 5, resulting in 700,000 deaths (Todd, 1997). As a cause of traveler's diarrhea, it has substantial impacts in terms of both morbidity and economic consequences (Savarino and Bourgeois, 1993).

Two virulence attributes that characterize ETEC are the capacity to adhere to the small intestinal surface and to secrete enterotoxins. Over 20 distinct, human-specific ETEC adhesins or colonization factors (CFs) have been described, most of which constitute surface-exposed fimbriae or fibrillae (Cassels and Wolf, 1995; Gaastra and Svernnerholm, 1996). In many geographic areas, the most common CFs individually expressed by ETEC strains are CF antigen I (CFA/I), CFA/II, which is composed of coli surface antigen CS3 alone or in combination with CS1 or CS2 (Cravioto *et al.*, 1982; Symth, 1984), and CFA/IV, which is composed of CS6 alone or in combination with CS4 or CS5 (Thomas *et al.*, 1982). Besides CFs, ETEC strains elaborate one or both of two well-defined enterotoxins: a heat-labile toxin (LT), which is structurally and functionally similar to cholera toxin (Clements, 1978), and a heat-stable toxin (ST), which is a low molecular-weight, poorly immunogenic peptide (Cohen and

Giannella, 1993; Frantz and Robertson, 1981). ETEC strains also express somatic (O) and flagellar (H) antigens on the cell surface, differentiation of which forms the basis for serotype classification of *E. coli* (Orskov and Orskov, 1992).

2.1.5.3 Enterohemorrhagic *E. coli* (EHEC)

EHEC bacteria were first discovered in 1977 by the production of cytotoxin, verotoxin (VT), lethal to Vero (African green monkey) cells, which led to these pathogens being called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk *et al.*, 1977). Later, O'Brien *et al.* (1987) observed a striking similarity with the structure and biological activity of the verotoxin and Stx toxin produced by *Shigella dysenteriae* type 1. Subsequently it was called Shiga-like toxin (SLT). It was also observed that there were two major classes of SLT toxins: SLT-I and SLT-II known also as VT1 and VT2. Currently, these toxins also designated as Stx1 and Stx2, have been found to include numerous variants (Nataro and Kaper 1998; Paton and Paton, 1998; Kaper *et al.*, 2004).

For the first time, EHEC emerged as human pathogens in USA in the early 1980s during large-scale outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS) caused by shigatoxigenic, non-sorbitol fermenting (NSF) strains of *E. coli* O157:H7 (Karmali *et al.*, 1983a,b; Riley *et al.*, 1983). Ever since, strains of NSF *E. coli* O157:H7 have been epidemiologically, microbiologically, and clinically important worldwide. In addition, a new lineage, a sorbitol-fermenting (SF) nonmotile (H^-) O157, was identified as the cause of outbreaks of Hemolytic Uremic Syndrome (HUS) in Germany in 1988 (Ammon *et al.*, 1999).

Recently, strains of this phenotype have emerged in several other European countries (Karch and Bielaszewska 2001), and in Australia (Bettelheim *et al.*, 2002). Currently, among all known Stx producing *E. coli*, over 450 O:H serotypes of human and non-human origin have been detected (Blanco *et al.*, 2004). Of these non-O157 serotypes, strains of O26:H11/H$^-$, O103:H2/H$^-$, O111:H2/H$^-$, and O145:H28/H$^-$ have been involved in outbreaks of severe illnesses. Currently about 450 O:H serotypes of shigatoxigenic *E. coli* have occurred (Duffy *et al.*, 2001, Blanco *et al.*, 2004).

EHEC bacteria have acquired several chromosomal and plasmidal virulence factors. However, production of Stx1 and/or Stx2 or their variants encoded by phage-mediated chromosomally located *stx* genes is considered a main virulence factor (O'Brien *et al.*, 1983a,b; Duffy *et al.*, 2001). Adhesion mechanisms or production of other toxins, such as enterohemolysin (Ehly) or CDT, have been identified as augmenting virulence factors (Kaper *et al.*, 2004). However, the nomenclature of EHEC and the Stx family is a complex issue (Acheson *et al.*, 1999).

The principal reservoir of EHEC O157 and non-O157 bacteria is considered to be the gastrointestinal tract of healthy cattle and other ruminants like sheep and goats. In addition, domestic animals such as cats, dogs, and rabbits (Garcia and Fox, 2003) have been sources of EHEC. Human infections or outbreaks have also been associated with a wide variety of food items; undercooked ground beef, hamburgers, dry fermented sausage, unpasteurized milk or surface contaminated fruit and vegetables. In addition, untreated or contaminated drinking water or swimming water have been causative agents for the EHEC infection, and also person-to-person transmission has been observed (Nataro and Kaper, 1998; Jaeger and Acheson, 2000;

Kaper *et al.*, 2004). Nosocomial and laboratory-acquired infections have also been reported (Coia 1998a, b). In addition, human EHEC O157 (Lahti *et al.*, 2002) and non-O157 infections (Heinikainen *et al.*, 2004) have been traced to cattle.

2.1.5.4 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *Escherichia coli* (EIEC) is a pathogenic form of *E. coli* that can cause dysentery (Nataro and Kaper, 1998). Making a distinction between EIEC and *Shigella* spp. has been known for a long time to be difficult and depends on a very limited number of characteristics. *Shigella* spp. have been shown to be clones of *E. coli* by sequencing of housekeeping genes (Pupo *et al.*, 1997; Pupo *et al.*, 2000).

Historically, EIEC was first described in 1944, when it was called paracolon bacillus, but it was later identified as *E. coli* O124. In the 1950s, another group of *E. coli* strains was found to cause experimental keratoconjunctivitis in guinea pigs by the Serény test—a trait common with *Shigella*. These strains were initially classified under *Shigella* as *Shigella manolovi*, *S. sofia*, *Shigella* strain 13, and *S. metadysenteriae* and were later placed in the *E. coli* subgroup EIEC as *E. coli* O164 (Bando *et al.*, 1988; Maurelli *et al.*, 1998; Rowe *et al.*, 1977). EIEC and *Shigella* spp. bear remarkable phenotypic likeness, with a reduction in the number of substrates utilized relative to commensal *E. coli* strains. These similar phenotypes may be attributed to the fact that these organisms spend much of their lifetime within eukaryotic cells and have a different nutrient supply from most *E. coli* strains (Lan

and Reeves, 2002). Most EIEC strains are Lac$^-$, non-motile and lysine decarboxylase negative (Farmer *et al.*, 1985; Silva *et al.*, 1980).

A limited set of O antigens are found in EIEC strains, including O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, and O173 (Cheasty and Rowe, 1983; Ewing, 1986; Gross *et al.*, 1983; Guth *et al.*, 1989; Orskov *et al.*, 1991). Three of these EIEC-associated O antigens are identical to O antigens present in *Shigella* spp. (Cheasty and Rowe, 1983), namely, O112ac, O124, and O152, with *Shigella* O antigens of *S. boydii* serotype 15/*S. dysenteriae* serotype 2, *S. boydii* serotype 3, and *S. dysenteriae* serotype 12, respectively.

EIEC strains can be distinguished from other *E. coli* strains by testing their invasion capacity by the Serény test or by identification of bacterial invasion-associated proteins or genes via specific tests. EIEC and *Shigella* strains harbor a common 220-kb plasmid, collectively termed pINV, although specific names were given for some pINV plasmids, for example, pWR100 of *S. flexneri* 5, pMYSH6000 of *S. flexneri* 2a, and pSS120 of *S. sonnei*. However, differences in pINV may play a role in virulence. There are also pINV genes that are variably present in *Shigella* and EIEC (Lan *et al.*, 2003) strains, including *sepA*, encoding serine protease, the major secreted protein of *S. flexneri* 2a, and *ospD3* (*senA*), encoding an enterotoxin, which is found in only 75% of EIEC strains and 83% of *Shigella* strains (Nataro *et al.*, 1995).

2.1.5.5 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *Escherichia coli* (EAEC) is emerging as a significant diarrheal pathogen in multiple population groups. Although most commonly associated with pediatric diarrhea in developing countries, EAEC is also linked to diarrhea in adults including HIV-positive patients and travelers and has been a cause of food-borne outbreaks in the industrialized world (Vila *et al.*, 2000; Piva *et al.*, 2003). The pathogenic mechanisms of EAEC infection are only partially understood and are most consistent with mucosal colonization followed by secretion of enterotoxins and cytotoxins. However, there appears to be significant heterogeneity of virulence among EAEC isolates, in part due to lack of specificity in the HEp-2 adherence assay. The HEp-2 cell adherent assay allows identification of EAEC's characteristic aggregative or "stacked brick" adherence pattern. A few studies have evaluated the prevalence of EAEC virulence genes in clinical isolates (Elias *et al.*, 2002; Okeke *et al.*, 2000; Piva *et al.*, 2003; Vila *et al.*, 2000).

The plasmid-borne genes encoding aggregative adherence (AA) fimbriae (*aggR*, *aggA*, *aafA*, *agg-3a)*, plasmid encoded toxin (*pet)*, dispersin protein (*aap)*, AA probe associated protein (*aatA*), and Cap locus protein (*capU*) and the chromosomal *Shigella* enterotoxin 1(*set*) gene are responsible for the expression of adherence and cytotoxin production (Nataro *et al.*, 1992; 1994; Elias *et al.*, 1999; Nishi *et al.*, 2003). Recently, the *aap* gene was reported to encode a secreted 10-kDa protein that appears to coat the bacterial surface and thereby promote the dispersal of EAEC on the

intestinal mucosa. It has been shown that this protein is immunogenic in a human EAEC challenge model (Sheikh *et al.*, 2002).

2.2 Antimicrobial resistance

The increasing prevalence of bacterial resistance to commonly prescribed antimicrobials, especially in developing countries poses a major concern in the management of infections (Shanahan *et al.*, 1993; Niljisten *et al.*, 1996). This has mainly resulted from extensive use, and often misuse of antimicrobials in both human and animal medicine (Kayser, 1993; Mitsuhashi, 1993). The widespread use of antimicrobial agents has accelerated the spread of genetic elements coding for resistance giving selective advantage to resistant bacteria (Mazodier and Davies, 1991).

Resistance genes originated as a protective mechanism for antibiotic producing micro-organisms (Bensevite and Davies, 1973), this is exemplified by the presence of aminoglycoside-modifying enzymes in aminoglycoside-producing strains of *Streptomyces spp* that are closely homologous to modifying enzymes found in aminoglycoside resistant bacteria (Thompson and Gray, 1983). The role of R-plasmids as mediators of resistance was recognized as early as 1960 (Akiba *et al.*, 1960). It had been postulated, however, that R-plamid mediated antibiotic resistance may have evolved long before the antibiotic era (Forster, 1983). The origin of R-plasmids and the development of resistance was first reviewed by Watanabe (1971) and subsequently expanded to include the role of transposons and integrons as mediators of resistance (Kopecko *et al.*, 1976; Maltinez, 1990).

2.2.1 Plasmids

Plasmids are double-stranded circular pieces of extrachromosal DNA usually from 2 to 200 kilobase, that can replicate and are independently inherited (Sambrook *et al.*, 1989). Plasmids carry genes conferring virulence or pathogenicity as well as antimicrobial resistance. The presence of plasmids in bacteria is non-essential for their survival except in the face of a hostile external environment such as antibiotics. This factor contributes to the stability of plasmid –mediated resistance in bacteria. The presence of different types of antimicrobial resistance genes on plasmids and their potential for transmission suggest that plasmids are major vectors in the dissemination of resistance genes through bacterial populations (O'Brien *et al.*, 1993). The R-plasmids carry genes that code for antibiotic resistance in many micro-organisms (Akiba *et al.*, 1960, Boyd; 1984; Atlas, 1984). The R-plasmids can be passed from one bacterial species to another, such as from *E. coli* to pathogenic strains of *Shigella* or *Salmonella* (O'Brien *et al.*, 1982), and as demonstrated by the *in vitro* transfer of Tn*916* from *E. faecalis* to *E. coli* (Poyart *et al.*, 1995).

2.2.2 Transposons

Transposons are individual or small clusters of resistance genes that are bound by invert or or direct repeat DNA sequences. These terminal repeats act as specific recognition sites for transposase enzymes that catalyse the movement of the transposon from one replicon (plasmid or chromosome) to another (Murray, 1982). Transposons cannot replicate independently, but they carry genes that are necessary

for the transposition into a functional replicon (Chopra, 1990). Transposons are more diverse in their interaction than plasmids. They have been known to transpose one or more resistance genes within one genus and even across different genera of bacteria (Davies, 1994).

2.2.3 Integrons

The integron is a type of mobile genetic element found either as part of a transposon within the Tn*21* or independently on several groups of broad-host plasmids, coding for a site specific integration system (Davies, 1994). They range in size from 0.75 to 2.7kb and consist of two conserved DNA segments and a variable segment (White *et al.*, 2001). Class 1 integrons possess two conserved segments separated by a variable region (VR) which includes integrated antibiotic resistance genes or cassettes of unknown function (Recchia and Hall, 1997). The 3' conserved segment contains the *qacE* and *sulI* genes and an open reading frame (ORF) called *orf5*. The *qacE* gene determines resistance to ethidium bromide and quaternary ammonium compounds while *sulI* gene confers resistance to sulfonamides (Recchia and Hall, 1997).

Individual genes or gene cassettes can be inserted or removed from or even shuffled within these conserved segments. Integrons make it possible for resistance genes from different locations on bacterial genomes to come together on mobile elements that could be incorporated into genetic replicons such as plasmids. Thus, resistance genes of diverse localities in bacteria (i.e. plasmids and/ or transposon may spread across bacteria on integrons (Davies, 1994). Several studies have shown that integrons are

widespread among isolates of the family *Enterobacteriaceae* (Stokes and Hall, 1989; Hall, 1994; Bissonette and Ray, 1991).

A basic role in the spread of antimicrobial resistance in *Enterobactereciae* has been attributed to class 1 (Sandvarg *et al.*, 1998) and class 2 integrons (Goldstein *et al.*, 2001). *Sul1* gene encoding resistance to sulfonamides is normally found in Class 1 integrons linked to other resistance genes like aminoglycoside adenyltransferase A2 (*aad A2*), which confers resistance to streptomycin and spectinomycin and dihydrofolate reductase XII (*dfr XII*), which confers resistance to trimethoprim (White *et al.*, 2001).

2.2.4 Types of antimicrobial resistance

The mechanism of antimicrobial resistance can be classified as innate (natural) or acquired (Courvalin, 1996).

2.2.4.1 Natural antimicrobial resistance

Some bacteria are naturally resistant to certain antimicrobial agents. Well known examples include the resistance of Mycobacteria and *Pseudomonas spp* to most antibiotics, gram-positive bacteria to nalidixic acid (Chopra, 1990) and enterobacteria to macrolides (Courvalin, 1996). This form of resistance can be explained by cell wall impermeability to the antimicrobial agents, or metabolic pathways that antimicrobial agents cannot influence. Normally, natural resistance is chromosomal-mediated and is thus predictable (Fling and Richards, 1983).

2.2.4.2 Acquired antimicrobial resistance

Acquired resistance refers to the emergence of resistant strains within a bacterial population (Kallings, 1982). This form of resistance is the most important. Acquired resistance can either be indigenous (i.e. occurs through mutations) or exogenous where the bacteria acquires exogenous DNA from related or unrelated bacteria via transformation, transduction or conjugation (Courvalin, 1996).

2.2.5 Genetic basis of acquired antimicrobial resistance

Genes that code for resistance to antimicrobial agents may emerge from spontaneous mutation or may be acquired from other bacteria (Courvalin, 1996). These genes are located on the chromosome or plasmid or on both (Forster, 1983). Acquired resistance in bacteria results from R-plasmid transfer by conjugation, transduction or transformation. The distinction between plasmid mediated and chromosomally encoded resistance can be blurred due to the dynamic movements of transposons between plasmids and the chromosome (Forster, 1983).

2.2.5.1 Mutations

Changes in only a few base pairs causing substitution of one or a few amino acids in a crucial target (enzyme or cell wall) can affect chromosomal structure or control genes leading to new resistant strains. Spontaeous mutations occur in a bacterial population at a frequency of 10^{-5} to 10^{-9} per generation (Kallings, 1982). Statistically, the probability of this type of resistance emerging in bacteria is minor but becomes

significant when bacteria are exposed to selective antibiotic pressure (Doss, 1994), and the presence of other bacteria that facilitate the dissemination of resistance (Courvalin, 1996). Mutations are stably inherited, confer resistance to all members of the family and can be deleterious to the host bacterium (Courvalin, 1996).

Mutations in chromosomal DNA, without affecting the ability of bacteria to survive in the natural environment, would be expected to occur over a long period of time (Doss, 1994). The accumulation of chromosomal mutations would seem to be an unsatisfactory explanation to the rapid emergence of multi-resistant bacteria (Forster, 1983).

2.2.5.2 Transferable resistance

Plasmid-mediated resistance may spread by three main routes, transformation, transduction and conjugation

2.2.5.2.1 Transformation

Transformation is the transfer of naked DNA from one lysed bacterium to another. Certain recipient bacteria can acquire high molecular weight DNA from the surrounding medium thus transforming their genetic constitution (Stewart, 1989). The resistant strain can spread from person to person carrying its plasmid with it. This route was exemplified by an outbreak in which a multi-resistant *Pseudomonas aeroginosa* strain spread to infect several patients in a leukemiac ward (Green *et al.*, 1973). The isolates from the patients and some from the environment were shown to be similar by several epidemiological methods, and all carried a similar plasmid that

encoded resistance to carbenicillin and aminoglycosides. The plasmid itself was non-transmissible and thus could only spread in the bacterial host population. Transformation has been reported among *Pneumococci*, *Haemophilus spp* and *Neisseria spp*. These bacteria are capable of excreting short transforming DNA located on resistance plasmids or transposons in the slime capsules of certain recipient bacteria. If the bacteria are closely related, they may transfer DNA by homologous recombination into chromosomal locations of the recipient bacteria (Courvalin, 1996). Among *staphylococci*, enterobacteria and *pseudomonads* transformation is a less important mode of transfer of resistance.

2.2.5.2.2 Transduction

Transduction involves transfer of antibiotic resistance by a bacterial virus. The genetic material is carried from the donor cell by bacteriophages that infect the recipient cells (Kokjohn, 1989). For transduction to occur, transducing phage particles need to be produced from donor strains through infection with a lytic bacterial phage or by induction of a prophage. Calcium ions are required for the attachment of phage particles to cell surfaces, and so transduction can be inhibited by the presence of chelating agents such as citrate ions (Levin, 1985). The best examples include the transfer of plasmid-mediated penicillinase production in staphylococci and resistance to tetracycline and chloramphenicol in some enterobacteria (Murray, 1991).

2.2.5.2.3 Conjugation

Conjugation refers to the mating between a donor cell and a recipient cell. Conjugation is a phenomenon that occurs mainly among gram-negative bacteria but certain gram-positive bacteria such as streptococci may also acquire resistance via conjugation (Murray, 1991). Among the three genetic mechanisms of acquiring antimicrobial resistance, conjugation is by far the most important in bacteria. Conjugation occurs *in vivo* and can cause epidemic spread of plasmid-mediated resistance on a worldwide scale, crossing borders between species and genera of bacteria (Davies, 1994).

Resistance genes can be spread by means of conjugal transfer to another bacterial host of the same or different species (O'Brien *et al.* (1982). The transferred genes, located together with resistance genes on the plasmid, create a highly efficient mechanism for dissemination of antimicrobial resistance. Nearly 60-90 % of resistance determinants reside on plasmids. The transfer of R-plasmids is therefore the most important phenomenon in the spread of antimcrobial resistance (Kayser, 1983).

For conjugation to occur pilli or fimbriae projecting from the donor cell must be formed and this occurs on F+ (fertility factor) cells. The F-factor is principally carried on plasmid DNA which may be transferable or non-transferable. During mating the sex pilus on the donor cell extends to form contact with a receptor site on the surface of the recipient cell. Duplicated genetic material from the donor cell, usually extrachromosomal DNA, then passes along the pilus into the recipient (Courvalin,

1994). This method is used particularly by Gram-negative bacteria, transferring not only antibiotic resistance genes but also pathogenicity determinants and metabolic functions.

2.2.6 Pharmacodyamics of acquired antimicrobial resistance

Bacteria become resistant to antimicrobial agents by one or more biomechanisms.

2.2.6.1 Alteration of the target site

Resistance due to alteration of target sites has been encountered for beta-lactams, streptomycin and erythromycin. Alteration of penicillin binding proteins (PBP's) may lead to resistance to beta-lactams while resistance to streptomycin occurs due to mutation leading to altered ribosomes. The methylation of ribosomal RNA inhibits binding of erythromycin (Murray, 1989; Spratt, 1994).

2.2.6.2 Modification of antibiotics

Drug modification causing inactivation of the drug is probably the most common mechanism of resistance to antimicrobial agents (Murray, 1991). These include resistances caused by beta-lactamase, chloramphenicol acetyl transferases and aminoglycoside adenyl transferases (Amyes and Gemmel, 1992). It has also been reported in resistance associated with macrolides, lincosamides, streptogramins and tetracyclines (Salyers *et al.*, 1990). Resistance determinants involved in drug inactivation are usually plasmid or transposon mediated (Faragasan *et al.*, 1997).

2.2.6.3 Prevention of antibiotic entry

This mechanism is mostly expressed in gram-negative bacteria resistant to vancomycin and nafcillin and that of enterococci to low levels of aminoglycosides (Murray, 1991). Decreased permeability of the cell wall may be explained by mutation of the porin gene (e.g. Omp C and Omp F in *E. coli*) that leads to altered non-specific aqueous diffusion channels normally used for entry of antimicrobial agents into the bacterial cell (Neu, 1992). This mechanism of resistance has also been reported to be the basis of cross-resistance to chemically unrelated drugs such as resistance to tetracycline and chloramphenicol by *E. coli* (Chopra, 1990).

2.2.6.4 Production of novel enzyme substitutes

In this mechanism of resistance, the bacteria produce a novel enzyme substitute that is not susceptible to conventional antimicrobial agents (Amyes and Gemmel, 1992). An example is the transferable resistance of gram-negative bacteria to sulfonamides and trimethoprim. Resistance to trimethoprim is caused by overproduction of the target enzyme, dihydrofolate reductase, thus requiring a high inhibitor concentration (Spratt, 1994).

2.2.7 Tetracycline resistance

Tetracyclines are antimicrobial agents that interact with bacterial ribosomes and block protein synthesis. Prior to the mid- 1950s, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines (Levy, 1984). The first multiresistant

Shigella dysenteriae was isolated in 1955 and was resistant to tetracycline, streptomycin and chloramphenicol (Watanabe, 1963). Tetracycline resistance has also been reported in increasing frequencies in gram positive and cell-wall-free species (Roberts, 1994; Roberts and Kenny, 1986); and are often found in multi-drug resistant bacteria (Levy *et al.*, 1999). Resistance is often due to acquisition of new genes associated with either conjugative plasmids or transposons (Roberts, 1994).

Resistance to tetracycline occurs by three mechanisms: the use of an energy-dependent efflux of tetracycline, alteration of the ribosome to prevent effective binding of the tetracycline, and producing tetracycline-inactivating enzymes (Roberts, 1994). There have been 30 different tetracycline resistance (tet) genes characterized to date (Levy *et al.*, 1999; Ng *et al.*, 2001). Seventeen of the *tet* genes code for efflux pumps. Seven of the *tet* genes code for ribosomal protection proteins. Both the efflux and ribosomal-protection encoding genes are found in members of gram positive, gram negative and anaerobic species. The *tet(B)* gene confers resistance to tetracycline, doxycycline, and minocycline, although all the other efflux genes confer resistance to tetracycline and doxycycline (Roberts,1994; Mendez *et al.*, 1980). The *tet(B)* gene has the widest host range of the gram-negative tet genes and has been reported in 20 gram-negative genera. The ribosomal protection proteins confer resistance to tetracycline, doxycycline and minocycline. The *tet* (M) has the broadest host range and has been found naturally in 26 genera including both gram-positive and gram-negative species (Roberts, 1994; Chopra and Roberts, 2001). In contrast, other *tet* genes appear to have a more limited host range. In other cases, the *tet* genes in some bacteria, such as *tet* (M) in *E. coli*, confer only low levels of tetracycline

resistance. The *tet* (X) gene codes for an enzyme that alters the tetracycline molecule in the presence of oxygen (Roberts, 1994) while the *tet* (U) gene sequence is distantly to other mechanism of resistance (Ridenhour *et al.*, 1996). Recently, point mutations in tetracycline resistant bacteria were identified in gram-positive bacteria (Ross et al., 1998). Mutations that alter the permeability of porins and /or polysaccharides in the outer membrane can also affect tetracycline susceptibility (Chopra and Roberts, 2001). Whether these changes account for increased levels of tetracyclines is not clear at the moment.

2.2.8 Sulfonamide resistance

Sulfonamides are synthetic drugs that interact with dihydropteroate synthase (DHPS). This enzyme is part of the folic acid biosynthetic pathway that is required for thymine production and bacterial cell growth (Huovinen *et al.*, 1995). Sulfonamides were used early for prophylaxis and treatment of meningococcal disease. Unfortunately, resistance developed quickly in *Neisseria meningitidis* and was replaced by penicillin therapy. Different types of mechanisms have been found to confer sulfonamide resistance, with most common resulting in changes in DHPS genes. Maskell *et al.* (1997) examined six sulfonamide-resistant clinical *S. pneumoniae* and found three to six base pair duplication which was sufficient to confer high levels of resistance. More recently Haasum *et al.* (2001) examined 11 sulfonamide-resistant clinical *S. pneumoniae* and found a number of different amino acid duplications within the DHPS gene. In contrast, resistant DHPS genes in *Neisseria meningitides* are more

likely due to development of mosaic genes by DNA transformation (Haasum *et al.*, 2001).

2.2.9 Beta-lactam resistance

Beta-lactam antimicrobial agents comprise of natural and semi-synthetic penicillins and cephalosporins which possess a fused thiazolidine (beta-lactam) ring in their chemical structure. This group of antimicrobial agents owes its antimicrobial activity to its ability to bind to and inhibit the action of certain bacterial cell wall synthetic enzymes, namely, the penicillin-binding- proteins (PBP) (Hsieh *et al.*, 2006; Sakata *et al.*, 2006). Bacterial resistance to the beta-lactam antibiotics is usually based on any of three mechanisms, acting either independently or in concert: Low affinity between the target PBP(s) and the antibiotic (Franciolli *et al.*, 1991; Hsieh *et al.*, 2006), destruction of the antibiotic by extracellular or periplasmic hydrolytic enzymes (beta-lactamases) produced by the bacteria (Bush, 1989) and inaccessibility of the PBPs to the antibiotic (permeability barrier) (Lugtenberg and Van Alpen, 1983, Bradford *et al.*, 1997). Access of most beta-lactam antibiotics to their target enzymes is usually moderated by the presence and character of the bacterial outer membrane. This outer membrane contains porin proteins, which form water filled channels through the membrane and serve as conduits for passive diffusion of hydrophilic molecules such as beta-lactam antibiotics (Lugtenberg and Van Alpen, 1983). The porin structure of the outer membranes of certain gram-negative bacteria, such as *Pseudomonas aeruginosa*, provides only limited permeability to beta-lactam antibiotics and can contribute to the efficiency of periplasmic beta-lactamases by regulating the influx of

the antibiotic (Bradford *et al.*, 1997). However, this mechanism of resistance through diminution of outer-membrane permeability by mutation or loss of porin proteins is relatively rare and not transferable by plasmids.

The affinity of a beta-lactam antibiotic for one or more of the PBPs in a particular bacterial species is a major determinant of the potency of the antibiotic. Although there are clinical examples of acquired resistance due to alteration of the target PBP (Parr and Bryan, 1984, Franciolli *et al.*, 1991, Dowson *et al.*, 1994) this form of resistance is less frequently encountered and is not acquired via conjugation and plasmid transfer (Sahm *et al.*, 1985). Bacterial proteins collectively known as beta-lactamases encompass a large number of enzymes which have widely varying kinetics for their potential substrates. Bacterial expression of beta-lactamase-mediated resistance to a beta-lactam antibiotic depends on several factors. These include: the specific activity of the enzyme against the compound, the amount and rate of production of the enzyme by the organism, the locus of the enzyme (excreted or cell-bound) and whether it is produced constitutively or inducibly (Medeiros and Jacoby, 1986).

Some gram-negative bacteria produce beta-lactamases under direction of a gene or genes located on extrachromosomal DNA (plasmid) acquired from other bacteria via conjugation. The most commonly encountered plasmid mediated enzymes in clinically isolated gram-negative bacteria are those of the Richmond-Sykes Type IIIa, referred to as TEM 1. The production of this type of beta-lactamase is mediated by the bla_{TEM} genes and is always associated with resistance to aminopenicillins, but not the cephalosporins (Bush, 1989). Resistance to extended-spectrum betalactams

(ESBL's) in the family *Enterobacteriaceae* has commonly been associated with the expression of extended-spectrum TEM and SHV beta-lactamases (Dubois *et al.*, 1995, Heritage *et al.*, 1999) and plasmid mediated AmpC betalactamases (Bradford *et al.*, 1997, Pitout *et al.*, 1998). However, since 1992, newer plasmid mediated ESBLs-the cefotaximases have been described (Barthelemy *et a*l., 1992; Bernard *et al.*, 1992). To date more than 10 variants of the CTX-M– type beta-lactamases have been reported in various enterobacterial species, including *E. coli, salmonella entericar serovar* Typhimurium, *Klebsiella pneumonia* and *Proteus mirabillis* isolated from different parts of the world including south Africa and Kenya (Bonnet *et al.*, 2000, Kariuki *et al.*, 2001, Pitout *et al.*, 1998; Rodriguez *et al.*, 2005; Soge ***et al.***, 2006).

2.3 Epidemiolgy of antimicrobial resistance

There is convincing evidence that both subtherapeutic and therapeutic doses of antibiotics cause increased antibiotic resistance in the intestinal flora of animals (Linton, 1977a,b). Resistance may be engendered not only to the antibiotic used but also to other antibiotics (Julia and Chan, 1987) because resistance genes are often linked.

Antimicrobial resistant *E. coli* isolated from clinical sources continues to pose therapeutic problems worldwide. Consequently, commonly available and inexpensive orally administered antimicrobials have become ineffective in the treatment of serious infections caused by *E. coli* and other micro-organisms (London *et al.*, 1994). In the United States, several outbreaks of *E. coli* 0157:H7 infections have been associated with a high prevalence of resistance to commonly used oral antibiotics such as co-

trimoxazole, streptomycin and tetracycline (Kim *et al.*, 1994). In many developing countries strains of *E. coli* isolated from children with diarrhoea and domestic animals were found to show multiple resistance to almost all the commonly available antibiotics (Shears *et al.*, 1988; Faruque *et al.*, 1993; Bebora *et al.*, 1994; Kariuki *et al.*, 1997; Al-Ghamdi *et al.*, 1999). In Kenya, Senerwa *et al.* (1991) reported high prevalence of hospital EPEC strains from children with diarrhoea which were resistant to ampicillin, co-trimoxazole, tetracycline and chloramphenicol. Studies in Hong Kong (Ling *et al.*, 1994) have reported 100 % resistance to ampicillin and 41 % resistance to co-amoxyclav (amoxycillin + clavulanic acid) among clinical *E. coli* isolates. In Greece, a high prevalence of *E. coli* resistance to trimethoprim was found among patients with urinary tract infections (Tsakris *et al.*, 1993). Johnson *et al.* (1994) observed a high prevalence gentamycin resistance among *E. coli* clinical isolates in a London hospital, while fluoroquinolone-resistant *E. coli* strains have been reported in Germany (Lehn, *et al.*, 1996).

2.4 Antimicrobial use, surveillance and Risk analysis

2.4.1 Introduction

Antibiotics—naturally-occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be administered orally, parenterally or topically—are used in human and veterinary medicine to treat and prevent disease, and for other purposes including growth promotion in food animals. Antibiotic resistance is as ancient as antibiotics, protecting antibiotic-producing organisms from their own products, and other originally susceptible organisms from their competitive attack in

nature. All antibiotics can select spontaneous resistant mutants and bacteria that have acquired resistance by transfer from other bacteria (O'Brien *et al.*, 1982). These resistant variants, as well as species that are inherently resistant, can become dominant and spread in host-animal populations (Kallings, 1982). The more an antibiotic is used, the more likely are resistant populations to develop among pathogens and among commensal bacteria of an increasing number of animals in an exposed population (Davies, 1994). However, there is great diversity: whereas some bacteria very r apidly develop resistance in the individual treated, others r emain susceptible (Wray, 1997).

Antibiotic resistance defined in this way is a microbiological phenomenon, which may or may not have clinical implications depending on pharmacokinetic and pharmacodynamic parameters as they apply to specific antibiotics. Nevertheless, even intermediate resistance (diminished antibiotic potency within the clinically susceptible range) is noteworthy since it may be a first step towards clinical resistance. These considerations have always been important in definitions of rational antimicrobial therapy and have been re-emphasized by recent calls for prudent therapy in human and veterinary medicine (WHO, 1998,2001).

The campaign against what has been considered excessive clinical use has been generally evenly directed at human and animal medicine, but there has been a concerted attack on the agricultural use of antibiotics, based on the assumption that all such usage is imprudent since it might act as an important source of resistance in bacteria affecting humans (Levy, 1984; Levy, 2001; WHO, 2001). In Europe, this has led to the banning of several antibiotic growth promoters as a precaution, despite the

advice of the European Union's own Scientific Committee on Animal Nutrition (SCAN) that there were insufficient data to support a ban (SCAN, 1996, 1998), and it is proposed to withdraw the rest in 2006. There are calls for a wider application of the ban. Pieterman & Hanekamp have drawn attention to the logical, legal and moral flaws inherent in the 'precautionary principle', taking as an example the banning of growth-promoting antibiotics in Europe (Pieterman & Hanekamp, 2001).

2.4.2 Use of antibiotics in food animals

2.4.2.1 Definitions of use

The Clinical laboratory standard Institute (CLSI) has defined terms to describe herd or flock antibiotic use.

Therapy is the administration of an antimicrobial agent to an animal, or group of animals, which exhibit frank clinical disease. Control is the administration of an antimicrobial agent to animals, usually as a herd or flock, in which morbidity and/or mortality has exceeded baseline norms. Prevention/prophylaxis is the administration of an antimicrobial to exposed healthy animals considered to be at risk, but before expected onset of disease and for which no aetiological agent has yet been cultured. (Metaphylaxis is a term sometimes used when there is clinical disease in some animals, but all are treated.) Growth promotion is the administration of an antimicrobial, usually as a feed additive, over a period of time, to growing animals that results in improved physiological and production performance.

2.4.3 Therapy, control and prevention

When antibiotic treatment is necessary, it often has to be administered to food animals either parenterally or orally in feed or water. Individual animal treatment is almost never practical for poultry, but may be practical for cattle and swine.

In livestock production, the objective is to limit progression of disease in the population, since illness decreases animal per formance. Herd or flock treatment is often indicated when illness is first recognized in a small proportion of the animals. For example, one of the indications for the use of antibiotics in animals is physical stress involved, for example, in the movement of animals in large numbers. Whereas mass regimens can improve animal performance and the general welfare of the treated animals, such regimens do result in increased antimicrobial usage (van den Bogaard and Stobberingh, 1999). Mass treatment programmes generally leads to administration of treatment to individuals that do not need it, whereas limitation of therapy to recognized clinical cases errs on the side of withholding treatment from some individuals that would benefit. Attempts to limit mass metaphylaxis to those individual animals most likely to benefit, using rectal temperature as a clinical indicator for treatment, have usually been unsuccessful (Guthrie *et al.*, 1997). More sophisticated measures of disease status are being investigated as one means to improve treatment selection criteria.

2.4.4 Growth promotion

The growth promoting effects of antibiotics were first discovered in the 1940s when chickens fed by-products of tetracycline fermentation were found to grow faster than those that were not fed those by-products (Stokestad, 1949). Since then, many antimicrobials have been found to improve average daily weight gain and feed efficiency in livestock in a variety of applications (Preston, 1987; Nagaraja and Chengappa, 1998; Gaskins *et al.*, 2002), and this is known as 'growth promotion'. Whereas the precise mechanisms of growth-promoting effects were, and are still, often unknown, knowledge is improving (Andersons *et al.*, 1999). The net benefit of antibiotic feeding to food-producing livestock was, and still is, measurable (Pieterman and Hanekamp, 2001). Such measurable benefit coupled with demonstrable target animal safety, edible tissue clearance and residue avoidance, and environmental safety remains the basis for regulatory approval of growth promoting applications of antibiotics in livestock production (Preston, 1987). Whereas some growth-promoting effects are mediated through alterations of the normal intestinal microbiota resulting in more efficient digestion of feed and metabolism of nutrients (Dennis *et al.*, 1981; Nagaraja *et al.*, 1987), others are mediated through pathogen and disease suppression and immune system modulation. For example, rates of post-weaning scours increased following antimicrobial growth promoter restrictions in Sweden (Wierup *et al.*, 2001; Inborr, 1996). Similar problems have been experienced in many parts of Europe following the growth-promoter ban, requiring the increased use of therapeutic

antibiotics (DANMAP, 2003; Casewell *et al., 2003*), making it clear that infectious disease suppression is an important effect of growth promoters.

2.4.5 Antibiotic use

In 2001, 23 products with antibacterial activity, excluding coccidiostats, had United States Food and Drug Administration (FDA) regulatory approval and were marketed for feed additive applications (Anon, 2001). Fifteen of those 23 antibacterial compounds had growth promotion label claims. Of those 15, only two (bambermycins and laidlomycin) did not have additional claims for therapeutic feed additive uses. Thus, distinctions between growth promotion and prophylactic applications are sometimes misleading. For example, whereas control and treatment dosages of lincomycin and tylosin are higher than those for growth promotion, it is clear from the Danish experience after the banning of growth promoters that the compounds at the lower growth promotion doses appear to help swine ward off the pathogenic effects of *Lawsonia intracellularis* and decrease the incidence and severity of ileitis and diarrhoea (Bager and Emborg, 2000). A recent publication reviews the current usage of antibiotics in livestock in the US, explaining the complex interaction of antimicrobials with dietary factors (AVCARE, 2003). Whereas many products used for growth promotion and prophylaxis such as bacitracin, bambermycins and carbadox have little or no application in human medicine, products used for prophylaxis and therapy are often closely related to antibiotics used in human medicine. The classes used include: ß-lactams (penicillins and cephalosporins); sulphonamides with and without trimethoprim; tetracyclines; macrolides,

lincosamides and streptogramins; and quinolones (including fluoroquinolones) (Bager and Emborg, 2000). These have a variety of therapeutic and preventive applications in food animals (Prescott *et al.*, 2000).

2.4.6 Antibiotic use in humans and the problem of resistance

Antibiotics are widely used to treat and to prevent infection in humans. There are many guidelines for their rational use, and these have always considered the likelihood of the emergence of resistance as a parameter (Phillips, 1979). Such guidelines have been further developed as policies for antibiotic use within given communities, ranging from individual hospitals to whole nations. Most antibiotic prescription in developed nations is in the hands of community medical practitioners, of whom there is less control than is possible in hospitals. In some countries, it is still possible for a patient to buy potent antibiotics directly from the pharmacist without a medical prescription (Kariuki, 1996; Tollefson, 2001; WHO, 2001).

The antibiotics used in human medicine belong to the same general classes as those used in animals. In most parts of the world, ß-lactam agents (ranging from penicillin G to fourth-generation cephalosporins and carbapenems) play a major role, but sulphonamides (with or without trimethoprim), macrolides, lincosamides and streptogramins, fluoroquinolones, tetracyclines, aminoglycosides and glycopeptides are widely used, some mainly in the community and some mainly in hospitals.

With the range of antibiotics available, it is possible to treat infection with a high expectation of success. The benefits of use are clear both in the community and in hospitals, and failures of therapy are likely to be because of such factors as

misdiagnosis (for example of viral respiratory infections, or exacerbations of chronic bronchitis not caused by bacteria) or serious underlying disease (as in the treatment of sepsis) or use when clinical experience shows it to be inappropriate (as in most gastrointestinal infections caused by *salmonellae* and *campylobacters*). There has been considerable emphasis on the avoidance of such pitfalls in the pursuit of rational and prudent antibiotic therapy (WHO, 2000).

2.4.7 Antimicrobial resistance surveillance

There have been, until recently, few adequate international antibiotic resistance surveillance systems and those which exist have been driven by the interests of the pharmaceutical industry and are limited in scope (Bax *et al.*, 2001). Nonetheless, such systems as SENTRY, SMART, The Alexander Project and several others listed by Bax *et al.* (2001) have yielded valuable information on antibiotic resistance patterns in clinical isolates of resistant pathogens in different parts of the world.

The Danish National System, DANMAP, has now been reporting for seven years, and has been unique in trying (with varying success) to bring together in coordinated reports (DANMAP 97; DANMAP 98; DANMAP 99; DANMAP 2000; DANMAP 2001; DANMAP 2002 and DANMAP 2003) reliable data on the usage of antibiotics and on antibiotic resistance from human and veterinary medicine and food hygiene (Bager, 1997; 1999; 2000; Bager *et al.*, 2001; Emborg and Heuer, 2003). Denmark leads the way as the country with the most valuable data on antimicrobial resistance in bacteria isolated from animals. The Danish Veterinary Laboratory has had, for a number of years, a consistent program of surveillance of antimicrobial resistance in

normal intestinal bacteria obtained from animals as well as in selected animal pathogens, some significant for human health (Bager *et al.*, 2001; Emborg and Heuer, 2003). This work is of exceptional quality, and includes detailed molecular analysis of genes involved in resistance in animal pathogens (Aestrup, 2000a; Aestrup, 2000b; Jensen *et al.*, 2000). Their assessments of the contribution of antimicrobial growth promoters to resistance in important human pathogens are of particular value.

The Danish studies found that feeding the antimicrobial growth promoter, avoparcin, to chickens, pigs, and calves led to widespread resistance to vancomycin by species of fecal Enterococcus isolated from these animals. The finding led to the withdrawal of avoparcin as a growth promoter from use in Danish animals and, subsequently, in the entire E.U. The same laboratory also documented the relationship between use of virginiamycin as a growth promoter and resistance of enterococci to streptogramin antimicrobials, including quinupristin-dalfopristin. The latter drug was recently introduced into human medicine specifically for the treatment of Vancomycin Resistant Enterococci (VRE). These data have been used also in the E.U. to support the removal, in late 1999, of virginiamycin as a growth promoter (together with other antimicrobials: bacitracin, spiramycin, and tylosin). Also, they have been used to document the decline in vancomycin resistance in faecal enterococci in chickens and pigs following withdrawal of avoparcin as a growth promoter (Bager *et al.*, 1999). In summary, the availability of very high quality Danish data, based on resistance surveillance, with subsequent detailed investigation of specific areas once apparent problems are identified, illustrates the value of well-designed resistance surveillance in support of important policy decisions on antimicrobial drug use in food animals.

In the USA, the National Antimicrobial Resistance Monitoring System (NARMS) is an attempt to do much the same kind of study as DANMAP, and is already yielding valuable data (NARMS, 2000; Marano *et al.*, 2000; Terrence, 2001). The Centre for Disease Control (CDC)- FoodNet is another source of information on the prevalence and resistance of food-borne pathogens (CDC, 2001), as are a variety of national systems that concentrate on surveillance. NARMS was established in 1996 as a collaborative effort among the Food and Drug Administrations' Center for Veterinary Medicine (FDA, CVM), the U.S. Department of Agriculture (USDA), and the Centers for Disease Control and Prevention (CDC). The NARMS program monitors changes in susceptibilities of human and animal enteric bacteria to 17 antimicrobial drugs. Bacterial isolates are collected from human and animal clinical specimens, healthy farm animals, and food animal carcasses. The objectives of the system include provision of descriptive data on the extent and temporal trends of antimicrobial susceptibility in *Salmonella* and other enteric organisms from human and animal populations; facilitation of the identification of resistance in humans and animals as it arises; and provision of timely information to veterinarians and physicians. The ultimate goal of these activities is to prolong the lifespan of approved drugs by promoting prudent and judicious use of antimicrobial drugs and to identify areas for more detailed investigation (FDA, 1999). The NARMS program is designed as two nearly identical parts: an animal arm and a human arm. Human-origin isolates are submitted by 17 state and local Departments of Health for testing that is conducted at the National Center for Infectious Disease (NCID), CDC, in Atlanta, Georgia. Animal-origin enteric isolate susceptibility testing is conducted at the USDA

Agricultural Research Service's (ARS) Russell Research Center in Athens, Georgia. Animal and human isolates currently monitored in NARMS are non-typhoid *Salmonella, Campylobacter, E. coli, and Enterococci.* The CDC/NCID and USDA/ARS provide the NARMS results annually in comprehensive summary reports. Data acquired through this well-established surveillance system, with other data, were used to document the marked rise in fluoroquinolone resistance of *Campylobacter jejuni*, an important cause of human diarrhoeal and other illness, isolated from broiler chickens. This resistance has been attributed to the use of enrofloxacin and sarafloxacin in the control of septicemic *Escherichia coli* infections in chickens for at least the last five years. These data were used in the "Risk assessment on the human health impact of fluoroquinolone resistant *Campylobacter* associated with the consumption of chicken," conducted for the U.S. FDA CVM in October, 2000 (FDA, 2001), which led to the proposal to withdraw approval for the use of fluoroquinolones in poultry in the U.S. These surveillance studies demonstrate the value of antimicrobial resistance surveillance in supporting policy changes based on scientific data.

Much of the evidence relating to the potential for transfer of antimicrobial resistance from animals to man comes from a consideration of the epidemiology of zoonoses, mainly salmonella and campylobacter infection, and of what have become known as 'indicator organisms'—enterococci and *Escherichia coli*, which cause no disease in animals but can cause disease in man and which might be zoonotic. The epidemiology of these diseases is far from simple since there are many possible sources other than

food animals and many routes of transmission other than food of animal origin (Kariuki *et al.*, 2002).

The important antibiotic-resistant strains in this context are the multiply antibiotic-resistant salmonellae, macrolide- or fluoroquinolone-resistant campylobacters, glycopeptide- or streptogramin-resistant enterococci and multiple antibiotic-resistant *E. coli.* In all cases, the hypothesis is that the food chain is the main means of transmission. The hypothesis is intuitively attractive, and there can be no doubt of the existence of a hazard, but neither of these considerations means that the hypothesis is correct or of universal significance (Pieterman and Hanekamp, 2001).

2.4.8 Emergence of resistance in bacteria from food animals

When antibiotics are used in animals, resistance is likely to be selected in the normal and pathogenic intestinal flora (and in other colonized or infected body sites) and to increase in prevalence (Emberg and Heuer, 2003). For example, in the USA, where virginiamycin is widely used as a growth promoter, resistance to streptogramins is common in animal *Enterococcus faecium* (Welton *et al.*, 1998), whereas avoparcin has not been used and appropriately mediated acquired resistance to glycopeptides is virtually non-existent in animal enterococci (Coque *et al.*, 1996; McDonald *et al.*, 1997; Harwood *et al.*, 2001). Resistance is equally likely to diminish in prevalence when antibiotic use is decreased or discontinued, since although individual strains may retain resistance genes (Shrag and Perrot, 1996; Morrell, 1997), they are often replaced by susceptible strains when the selective pressure is removed. There is now evidence that both of these phenomena have occurred in enterococci in Europe in

relation to the use and discontinuation of use of growth-promoting antibiotics (Bager *et al.*, 2002; Van den Bogaard *et al.*, 2000; Aarestrup *et al.*, 2002). Recent studies indicate that 75% of *E. faecium* isolates from broiler chickens in Denmark were resistant to avoparcin (and thus also to vancomycin) and some 65% resistant to virginiamycin (and thus to quinupristin–dalfopristin). In addition, some 75% were resistant to avilamycin which has no current counterpart used in human medicine.

In 2000, after the growth-promoter ban, the resistance rates were less than 5% for avoparcin and avilamycin, but remained at around 30% for virginiamycin (Bager *et al.*, 2002). There is evidence from the USA and from Norway that some resistance may persist long after the use of an antibiotic has been discontinued (Langlois *et al.*, 1986; Kruse and Simonsen, 2000). The persistence of virginiamycin resistance after its ban has been attributed to the use of penicillin selecting for associated resistance to virginiamycin (Bager *et al.*, 2002), but it has recently been suggested that the use of copper as a feed supplement might also co-select antibiotic resistance in *E. faecium* (Hasman and Aarestrup, 2002). Such associated resistance is of general importance since the use of one antibacterial a gent can select for resistance to another that is unrelated because the two resistance determinants are genetically linked on the same plasmid or transposon (co-resistance) (Enne *et al.*, 2001).

2.4.9 Transfer of resistance from animals to man

It is well known that antibiotic-resistant bacteria that have been selected in animals may contaminate meat derived from those animals and that such contamination also declines when the selecting antibiotics are not used. However, most of the studies of

the food chain ignore the fact, already noted, that there are potential sources of resistant *enterococci* and *Enterobacteriaceae* other than farm animals given antibiotics. Humans themselves as well as other animals may be a source of resistant bacteria subsequently isolated from food animals, since commensals and pathogens (including resistant strains) can reach the general environment via sewage (Harwood *et al.*, 2001).

Wild animals, especially rodents, and birds, especially gulls, can acquire these environmental contaminants and pass them on via their excreta to grazing land or to the foodstuffs of food animals. Multi-drug resistant enterobacteriaceae have been found in wild rodents (Mallon *et al.*, 2002; Devriese *et al.*, 1996, Gakuya *et al.*, 2001), and in pet animals (Devriese *et al.*, 1996). Fish farming involves the use of antibiotics (although this is diminishing in Europe), and fish as food may be contaminated with resistant bacteria (Bager *et al.*, 2001).

Vegetables may also be contaminated from sewage, especially in countries in which human faeces is used as a fertilizer. Multiple antibiotic-resistant *E. coli* strains were found to be widespread contaminants of market vegetables in London during the investigation of a community outbreak of *E. coli* O157 infections (Phillips *et al.*, 1998; Riley *et al.*, 1993). Furthermore, antibiotics are widely used to prevent bacterial diseases in plants: tetracyclines and aminoglycosides are used to protect fruit trees from fire blight (Vidaver, 2002). Streptogramin-resistant *E. faecium* have been isolated from bean sprouts from sources yet to be identified (Bager, 1997; 1999). Genetic engineering in plants involves the use of a variety of antibiotics including vancomycin (Teixeira da Silva, 2002). Currently there are no rigorous

epidemiological studies on such potential reservoirs, and the assumption that they make negligible contributions to human enteric pathogen resistance has yet to be determined.

Animals that carry, or in certain cases are infected by, resistant organisms are a hazard to those who work with them since the organisms can be transferred by direct contact. This is the probable explanation of the rare but well publicized finding of indistinguishable glycopeptide-resistant enterococci—for example, in the faeces of a Dutch turkey farmer and his flock (van den Bogaard *et al.*, 1997), and of streptogramin-resistant *E. faecium* in the faeces of a Dutch chicken farmer and his chickens (Jensen *et al.*, 1998a). Even in these cases, there is a possibility that both animals and humans acquired the strains from a common source, or even that the organisms were transferred from man to his animals. The recent description of an outbreak in China of virulent but not antibiotic-resistant *E. faecium* infection in pigs and those in close contact with them seems too unusual for us to learn much about the epidemiology of 'normal' enterococci (Lu *et al.*, 2002). Isolates of enterococci from human and animal faeces that have no evidence of close conventional epidemiological links are often different on molecular testing, depending on the sensitivity of the method used, although in these studies, indistinguishable strains have sometimes been found among human and animal faecal enterococci (Descheemaeker *et al.*, 1999; Hammerum *et al.*, 2000; Simonsen *et al.*, 1998; Robredo *et al.*, 2000; Manson *et al.*, 2003). Recent work from Bruinsma *et al.* (2002) suggests that whereas human and pig faecal isolates of *E. faecium* have genetic similarities, those from poultry faeces are different.

There is experimental evidence for host-species specificity among enterococci: ingestion of heavy inocula of strains from humans by animals (Qaiyumi *et al.*, 2000) or of animal strains by humans (Sorensen *et al.*, 2001) does not result in their permanent establishment. In the experiment of Sørensen *et al.* (2001) ingestion of pig or chicken strains resulted in their excretion for a very limited period of time: in only one experimental subject out of 12 was the same organism detected at 15 days after ingestion but in none thereafter. As already noted, enterococci from chickens do not closely resemble those in human faeces, although those from pigs may have similar molecular characteristics to those from humans (Bruinsma *et al.*, 2002), but this does not mean that humans acquire their faecal enterococci from pigs. However, on the basis of analyses of *vanX* variants on Tn*1546* in *E. faecium* from chickens and pigs and humans, Jensen *et al.* (1998b) argue that spread is indeed from animals to man and not vice versa. The frequency of inter-host-species spread of faecal enterococci remains unknown.

The same host–animal specificity appears to apply to *E. coli*. van den Bogaard *et al.* (2001) give a good account of the history of the disagreement as to whether or not resistant *E. coli* from animals colonize and infect humans. In a study carried out by Parsonnet and Kass (1987), women working in a chicken abattoir, when they developed urinary tract infections (UTI), rarely yielded isolates that resembled (in terms of antibiotic resistance patterns) those from the chicken carcasses unless the woman developing UTI had been treated with antibiotics. A recent study from the Netherlands reported that among three poultry and five farmer/slaughterer populations, the Pulsed-Field Gel Electrophoretic (PFGE) patterns of ciprofloxacin-

resistant *E. coli* in the faecal flora were 'quite heterogeneous', but three farmers each had a faecal isolate of *E. coli* with PFGE patterns that were indistinguishable from those of some of the poultry isolates (van den Bogaard *et al.*, 2001). As with enterococci in farmers and their animals, it seems likely that transmission was not via animal-derived food.

Zoonoses such as *Salmonella* and *Campylobacter* infections, undoubtedly can reach humans via the food chain, but their immediate source may not be the animal fecal flora. In each case, reports of infection traced from a farm to a human non-epidemic infection are uncommon. Furthermore, *Salmonella* strains from food animals, their commonly assumed source for humans, are often genetically different from strains isolated from humans (Kariuki *et al.*, 2002; Winokur *et al.*, 2000).

The evidence that 'indicator' bacteria reach and persist in the human faecal flora via the food chain is increasingly contradictory. Although it may seem highly plausible that the VRE or streptogramin-resistant *E. faecium* found in animal faeces, or meat derived from them and in human faeces in non-hospitalized patients are the same (van ben Bogaard *et al.*, 1997a), the fact is that isolates from human faeces are usually genotypically different from those in animals (except occasionally in the case of the farmers mentioned above) and on food (van den Bogaard *et al.*, 1997b; Jensen, 1998). In deed, a recent study shows that chicken enterococci do indeed belong to a different pool from those of humans and pigs (Bruinsma *et al.*, 2002). Thus, in the absence of adequate conventional and molecular epidemiological studies, there is no sufficient evidence of the extent to which resistant enterococci or *E. coli* from food animals are able to colonize the human intestinal tract.

2.4.10 Antimic robial resistance gene transfer

The ultimate defence of those who support the farm-to-clinic hypothesis is that provided animal organisms reach the human f aeces, they need to survive only for brief periods to pass on their antibiotic-resistance genes to resident organisms. There is absolutely no doubt that transfer of resistance genes can occur, and countless *in vitro* experiments have characterized the event in endless variety, including among selected but by no means all strains of enterococci (Hammerum *et al.*, 1998), a phenomenon that may also be demonstrated experimentally in the germ-free animal gut (Jacobsen *et al.*, 1999). However, there have been no observations to determine its frequency under natural conditions—or even if it occurs at all in the normal human gut with the 'indicator organisms' from animal sources. The clearest cases of *in vivo* natural transfer have involved gut pathogens such as *salmonella, shigella, E. coli* and other enterobacteriaceae. The transfer of vancomycin resistance from VRE to *Staphylococcus aureus* under experimental conditions a decade ago (Noble *et al.*, 1992) has to date been reported to occur onl y twice in nature. The first case was recently reported in the USA, related to intensive vancomycin use in humans—the single case of *S. aureus* with *VanA* that was presumably acquired from a vancomycin-resistant *E. faecalis* strain from the same patient (CDC, 2002a), and a second case of a similar nature (CDC, 2002b). However, it is without doubt true that although some genetic elements, such as the transposon Tn*1546*, are heterogeneous both in animal and human faecal enterococci, indistinguishable variants may be found. For example, Jensen (1998) found two variants of the *vanX* gene, T and G, in human faecal

vancomycin-resistant *E. faecium*, but only T in pigs and G in poultry. On this basis, they concluded that spread from animals to humans was the likely explanation. Jensen *et al.* (1999) later reported that six human isolates (one of them from an infected patient) carried Tn*1546* variants that were indistinguishable from those common in pig isolates. In the UK, Woodford *et al.* (1998) found 10 variants of Tn*1546* in human isolates, eight only in animals but six in both and concluded that 'non-human sources cannot be excluded as a reservoir'. However, animal strains are not the only potential source of resistance since other species with the genes responsible for the *VanA* phenotype have been found, including some in the normal intestinal flora (Sundsfjord *et al.*, 2001) but it cannot be assumed that the genes have passed from these organisms to enterococci rather than vice versa.

The truth about gene transfer from animal isolates of indicator organisms to human isolates in the human intestine (or even in other relevant sites) still remains beyond our grasp. The results of the Danish ingestion experiment in which no human faecal isolates were used other than the animal strains swallowed by the experimental subjects, and in which no permanent carriage was demonstrated, suggest that it is not a common event *in vivo* (Sorensen *et al.*, 2001).

2.5 Monitoring of antimicrobial use

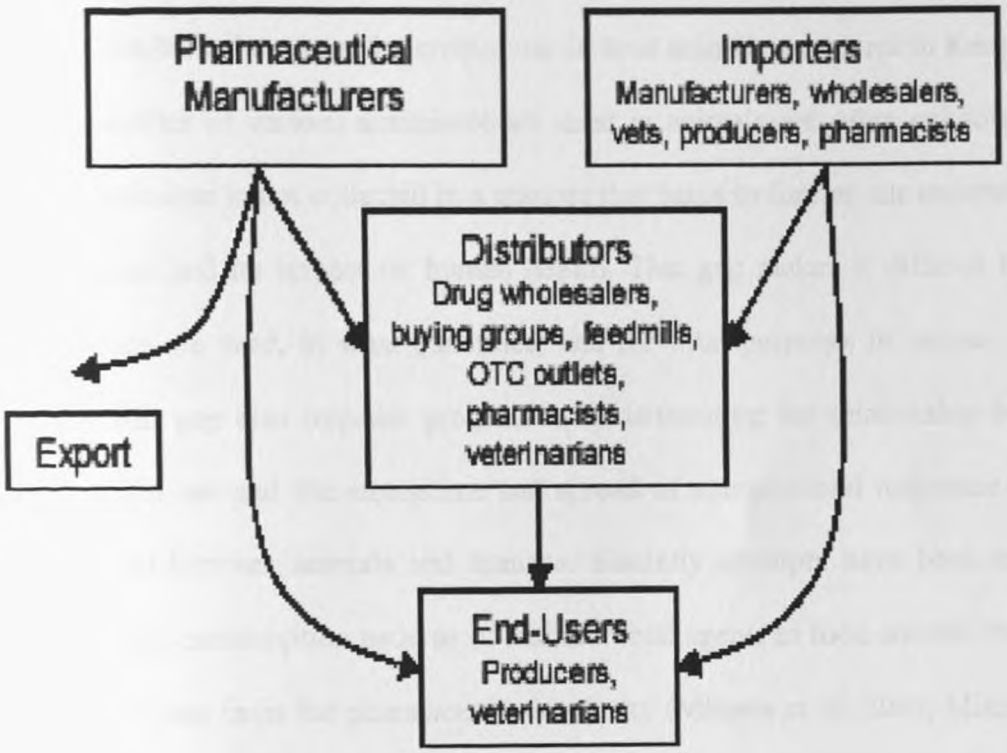
Because of the complexity of the drug distribution system (Figure 2.1) for antimicrobial drugs, an integrated approach combining data from several sources will probably be necessary. For example, the monitoring baseline could be provided by annual antimicrobial sales (including export) data from pharmaceutical manufacturers

and importation data, including "own-use importation" and the importation of bulk chemicals. A model could be developed using information from end-users and the baseline manufacturer/import data to develop annual use estimates reported by drug class and species/livestock class. End-user data could be verified by periodic monitoring of antimicrobial use by producers and veterinarians. This could be done through a rotating sentinel site system, possibly making use of quality assurance program records. Additional information from other points in the distribution system (e.g., Agrovets, pharmacies, OTC outlets, and wholesalers) could be used to validate the model and/or adjust the model estimates.

The following information is essential for a functional, meaningful and comprehensive monitoring system on antimicrobial use:

- volume produced (kilograms of active ingredient);
- volume imported.
- volume exported;
- quantitative data at end-use and use patterns (by species, use, drug, region); and
- quantitative data collected at various points in the antimicrobial distribution system (e.g., agrovets, drug wholesalers, pharmacies and NGO's).

Figure 2.1: Monitoring of the patterns of use of antimicrobial drugs



2.5.1 Monitoring of antimicrobial drugs used in food animals

2.5.1.1 Kenya

Publicly available data on antimicrobial use in food animals are scarce in Kenya. The exact quantities of various antimicrobials used in animals are often unknown, and consumption data is not collected in a manner that helps to further our understanding of resistance and its impact on human health. This gap makes it difficult to state which drugs are used, in what quantities, and for what purposes in various animal species. This gap also impedes progress in understanding the relationship between antimicrobial use and the emergence and spread of antimicrobial resistance among animals and between animals and humans. Recently attempts have been made to determine the consumption patterns of antimicrobial agents in food animals based on importation data from the pharmaceutical industry (Mitema *et al*, 2001; Mitema and Kikuvi, 2005). These studies provided some preliminary data on antimicrobial use in Kenya. To date, there is no official antimicrobial use monitoring program and Kenya has not clearly defined the rules, roles and responsibilities of stakeholders for implementing such a program.

Currently, the Pharmacy and Poisons Board of the Ministry of Health is solely responsible for collection of data on antimicrobial use and statutory regulation of antimicrobial drug use.

2.5.1.2 Monitoring practices in other countries

In some countries, however, a number of organizations, including the World Health Organization, Health Canada and the United States Department of Health and Human

Services have stated that monitoring the use of antimicrobials in animals is an essential component in controlling the development of antimicrobial resistance in bacteria affecting the health of humans and animals (WHO, 2001; Health Canada, 1997). Some of the following information is derived from the WHO Consultation on the Monitoring of Antimicrobial Usage in Food Animals for the Protection of Human Health, in Oslo, Norway.

2.5.1.2.1 Canada

In Canada, there is, for the most part, no existing mechanism by which data on the consumption of antimicrobial drugs by food-producing animals is collected, analyzed, and reported (an exception is the monitoring of antimicrobial use in aquaculture feed by the B.C. Ministry of Agriculture Food and Fisheries (Stephen and Iwama, 1997; Sheppard, 2000). Canada differs very little from most countries in this regard. As a result, there are no comprehensive estimates of antimicrobial consumption in livestock production for Canada, although some data are available from targeted research studies (Dunlop *et al.*, 1998; McEwen *et al.*, 1992).

A number of projects investigating methodologies for collecting quantitative data on antimicrobial use, as well as the behaviour patterns of veterinarians and food-animal producers relative to antimicrobial use, have been undertaken by Health Canada (Laboratory for Foodborne Zoonoses) and various research partners, including the University of Guelph, the Centre for Coastal Health, several provincial ministries of agriculture, and food and livestock commodity group (Rajic *et al.*, 2002; Bair *et al.*, 2002; Reid-Smith *et al.*, 2002; Leger *et al.*, 2002 and Reid-Smith *et al.*, 2001). These

studies provided some preliminary information on antimicrobial use in Canadian livestock production and will contribute to the development of a system for monitoring antimicrobial use in food animals.

2.5.1.2.2 Sweden

Sweden was the first country to develop a system for monitoring antimicrobial consumption in animals. All veterinary use of antimicrobials in Sweden requires a prescription. The 1986 *Feedstuffs* Act restricted the use of antimicrobials to veterinary use only. Prescriptions can be filled only by pharmacies or feed mills, which are supplied by two drug wholesalers. Sales data have been available from the drug wholesalers and compiled by the Swedish National Veterinary Institute (SVA) since 1980, although the data do not report consumption by species. Species-specific information has been accessible since 1996 in a centralized database maintained by the National Corporation of Swedish Pharmacies (Apoteket AB), which contains information on all veterinary prescriptions. These two sources are used to determine the use of antimicrobials in animals. Currently, only antimicrobial use in birds is reported by species/class. In 1999, an additional system that records data on all visits by veterinarians to food animals was developed. Although this system does not provide information on antimicrobial use, it has the potential to do so. Despite its early progress in recording antimicrobial use data, Sweden has not clearly defined the roles and responsibilities of stakeholders for implementing an antimicrobial use monitoring program (Greko and Odenvik, 2001; National Veterinary Institute, 2001). Sweden has developed the Anatomical Therapeutic Chemical veterinary classification

(ATCvet) system, which includes classification and codes for antimicrobial drugs. This system has greatly facilitated standardization in recording drug use, which is key to providing credible, accurate data and to facilitating comparisons of data from different jurisdictions and/or countries. The ATCvet system has been adopted by the European Union and is being considered by the WHO as a possible international standard. It is currently administered by the WHO Collaborating Centre for Drug Statistics Methodology, in Oslo (Greko and Odenvik, 2001; Harr, 2001).

2.5.1.2.3 Denmark

In Denmark, veterinarians can only prescribe antimicrobials for use in practice or for re-sale to food-animal producers through a pharmacy. Denmark has developed a monitoring system of antimicrobial use similar to Sweden's, but with more resources dedicated to the task. The system has two components: 1) collection, since 1995, of antimicrobial sales data from pharmaceutical companies and importers, reflecting sales to veterinary drug wholesalers, and 2) collection of antimicrobial prescription data from veterinarians through the newly developed VETSTAT system. Also, Denmark is recording on-farm antimicrobial use, beginning with dairy producers. Antimicrobial use data are reported annually, along with human consumption data and animal, food, and human antimicrobial resistance data in the DANMAP report. The data are broken down by ATC code and route of administration by species (Bager et al., 2001; DANMAP, 2001, 2002, 2003).

2.5.1.2.4 Norway

In Norway, use of antimicrobials in animals requires a prescription. These are filled by pharmacies, which are supplied by drug wholesalers or feed mills authorized by the Norwegian Medicines Agency. Sales data collected from Norwegian drug wholesalers and registered feed mills represent all antimicrobial use in agriculture. In July 2001, reporting of sales data from these two sources was made mandatory. Additionally, since 1989, a program monitoring antimicrobial use in aquaculture has collated data from prescribing veterinarians and the dispensing pharmacy or feed mill. In order to augment and validate the data collected from the wholesalers and feed mills, a program requiring veterinarians to register all prescriptions begun in 2002. Furthermore, Norway has plans to institute on-farm recording of antimicrobial use. In 2000, the Norwegian Zoonoses Centre, in collaboration with the Norwegian School of Veterinary Science, launched NORM-VET. This official monitoring program reports antimicrobial use data and antimicrobial resistance surveillance data from animals and humans on an annual basis (NORM/NORN-VET, 2001; Grave and Ronning, 2001).

2.5.1.2.5 The Rest of the European Union

In 1997, Fedesa (European Federation for Animal Health) provide information on antimicrobial use in Europe. Reported total sales volume was 10,494 MT of active ingredients. Of this, 5,400 MT(52%) was for human use, 3,494 MT(33%) for animal health, and 1,599 MT(15%) for growth promotion). They estimated that 90% of antimicrobials for animal use were administered in feed; 60% were used in pigs, 20%

in poultry and rabbits, 18% in ruminants, and 1% each in fish and pets. Within the animal health category (therapy, prevention and control), 66% were tetracycline, 12% macrolide, 9% penicillin, and 12% other drugs (Schwarz and Chaslus-Dancla, 2001).

An attempt was made to compare use figures between European countries based on the size of animal populations (antimicrobials used by tonne of live weight of slaughter animals). Based on animal census and production data, countries could be classified into three groups: in the highest use group were U.K., Greece, Spain, and the Netherlands; the lowest group comprised Sweden, Denmark, and Finland; with remaining countries in the middle group. These differences were attributed to varying husbandry conditions, but antimicrobial regulatory and distribution policies within countries were probably also contributing factors. Much has happened in Europe to change the situation since these data were assembled, including the removal of several growth promoters from the market.

The European Union has proposed that all member states and the broader European Community should monitor consumption of antimicrobials within veterinary medicine. Several member states, including the U.K., France and the Netherlands, have initiated programs and pilot projects to this end (Veterinary Medicines Directorate, 2002; Orand and Sanders, 2001; Pelican, 2001). A community system to collect data on the supply and consumption of antimicrobial feed additives was initiated in January 2000 (Makela, 2001).

2.5.1.6 Australia

All antimicrobials are imported either in end-product or bulk form. Since 1992, importers have been required to identify the intended end use (human, stock feed, veterinary therapeutic). Data have been compiled since 1992 by the Therapeutic Goods Administration (TGA) (JETACAR, 1999). There are several data quality issues related to completeness and accuracy of the importation records, especially situations in which the importer is unaware of the intended end use of imported antimicrobials. However, the data are considered reasonably representative of overall consumption. At present there is neither mechanism for separating the stock-feed category into growth promoter and prophylactic uses, nor for reporting use by species. No formal collection of enduse data has been undertaken or planned (JETACAR, 1999; Dyke, 2001).

2.5.1.7 United States of America

As is the case for Canada, there is no existing mechanism for the routine collection of quantitative data on the use of antimicrobials in agriculture. Some estimates have been made by various organizations. The most widely quoted estimate of total use is found in the 1989 Institute of Medicine (IOM) report (Comm. Health Risk Analysis (IOM), 1989), which estimated that approximately 50 million lb. of antimicrobials are produced annually in the U.S., and that approximately 50% is used in animals. This estimate was made over 10 years ago and was based on extrapolations from uncertain sources. Recently, the Union of Concerned Scientists (UCS), a non-profit organization representing consumer issues, estimated that approximately 35 million

lb. of antimicrobials are used annually in the U.S.; 4.5 million lb. (9%) in humans and 30.6 million lb. (87%) in animals (Mellon *et al.*, 2001). The vast majority (24.5 million lb.) of this estimate was classified as non-therapeutic (e.g., growth promotion, prophylaxis) in three types of food animals: cattle, swine, and poultry. To estimate human use, UCS cited outpatient prescription data from the National Center for Health Statistics and inpatient data from the U.S. Hospital Anti-Infective Market Guide. For animal estimates, UCS used an indirect method based on animal population estimates from agricultural census data, coupled with expert opinion and the results of USDA surveys of on-farm treatment practices and lists of FDA-approved antimicrobials.

The FDA requires pharmaceutical manufacturers to report quantities of drugs marketed as part of the annual Drug Experience Report. However, this reporting program was not designed to be the basis of a monitoring system of antimicrobial use. The reports are issued for each drug based on the drug's approval date, not the calendar year, so compilation of use data is virtually impossible. Furthermore, domestic sales are not distinguished from export sales, and there is no information on animal species, actual use conditions, commodity distribution, or geographic region (Tollefson, 2001).

Since 1999, the FDA and the Centers for Disease, Control and Prevention (CDC) have requested antimicrobial sales data from the Animal Health Institute (AHI), an organization that represents manufacturers of animal health products in the United States. A third-party research company collects the data provided by AHI. The data

are categorized in three ways: kilograms (kg) of active ingredient; use - therapeutic/preventive (14.7 million lb., or 83% of the total in the 1999 survey), or growth promotion (3.1 million lb., or 17% of the total); and antimicrobial drug class (aminoglycosides, fluoroquinolones, ionophores/arsenicals, penicillins, sulfonamides, tetracyclines). AHI has been collecting this type of data for its own use since 1980 (Carnevalle, 2001). There are several issues that complicate the usefulness and interpretability of the AHI data. Not all manufacturers of antimicrobials for agricultural use belong to the AHI. Also, members of the AHI are not required to give actual sales figures, and in some cases estimates are provided. The way in which the estimates are derived has not been presented. In cases where a given product is labelled for both growth promotion and therapeutic/preventive use it is classed as therapeutic/preventive (Carnevalle, 2001; AHI, 2001).

Antimicrobial use data are available also from the USDA's National Animal Health Monitoring System (NAHMS). NAHMS administers surveys to food-animal producers covering various aspects of animal health, including the use of antimicrobials (USDA, 2002). These surveys are conducted annually on a rotational basis. The data are primarily qualitative/descriptive but the mechanism could be used to collect quantitative data. These data cannot be used to develop total-use data, but could be used to interpret antimicrobial sales data.

The FDA plans to develop an official monitoring program on antimicrobial use. The nature of this has not been finalized. In the initial proposal, the program will require manufacturers of antimicrobials in the U.S. to provide sales data on an annual basis.

The sales data will be recorded on report forms and returned to the FDA for analysis. The report forms will include the following elements: market pack container sizes and number of marketable units sold within the calendar year (by month), estimates of drug use within each labelled species or target animal, estimates for the actual dose regimen use and active drug units sold within the calendar year (by month). The possibility of breaking this information down by geographic region is being considered. The resulting data will be reported annually, while maintaining manufacturer product confidentiality as stated under U.S. law (Tollefson, 2001).

2.5.2 Managing antimicrobial resistance risks

2.5.2.1 General principles

2.5.2.1.1 Risk

Risk is the probability that an adverse event will occur, along with its impact or consequences (Vose, 2000). An important role of government is to decide which risks should be publicly managed and how best to accomplish this using legislation and resources. These decisions are often difficult to make and sometimes very controversial. This is especially true in situations involving new, potentially serious risks, and where a simple, widely accepted remedy is unavailable. Under these conditions, there are advantages to a regulatory decision-making process that is open, clearly communicated, based on scientific evidence, and consistent with societal values. The Society of Risk Analysis (SRA) describes risk analysis as "a

fundamentally science based process that strives to reflect the realities of nature in order to provide useful information for decisions about managing risks" (SRA, 2001). SRA guiding principles include the view that risk analysis "seeks to integrate knowledge about the fundamental physical, biological, social, cultural, and economic processes that determine human, environmental, and technological responses to a diverse set of circumstances (SRA, 2001). Because decisions about risks are usually needed when knowledge is incomplete, risk analysts rely on informed judgment and on models reflecting plausible interpretations of the realities of Nature."

2.5.2.1.2 Risk management

In the context of human health, risk management is the process of choosing, implementing, and evaluating the optimal set of actions for the alleviation or mitigation of health risk from among the range of options available. Consideration should be given to societal benefits and costs of the available management options, relevant laws, public values, and results of consultation with interested parties in industry, government, academia, and the general public. Thus, in the case of regulatory matters, risk management necessarily and properly involves "political" considerations.

Risk management and analysis are thoroughly discussed in literature (NRC, 1994; Vose, 2000). The risk analysis structure that is accepted internationally as the model for setting food safety standards consist of three components; risk assessment, risk management and risk communication (FAO, 1997).

2.5.2.1.3 Risk assessment

Risk assessment is the process of estimating the probability and impact of adverse health effects attributable to resistance arising from using antimicrobials, for example, on farms. These estimates may be expressed in qualitative terms (e.g., low, medium, or high); however, quantitative expression of risk is preferred whenever possible [e.g., expected number of human infections, illnesses, or fatalities per year] (May, 2000).

2.5.2.1.4 Risk communication

Risk communication is the process of consultation, discussion and review that seeks to enhance the validity, effectiveness, and general acceptance of risk assessment and risk management. Good risk management decisions emerge when the views of those affected by the decision are elicited and when incentives for research, innovation and risk prevention are included. Risk communication with its participatory approach can potentially play a role in the process of making complex, value-added food safety decisions by all stakeholders (Smith and Halliwell, 1999).

2.5.2.2 Human health risks from residues and resistance

Assessment of human health risk from antimicrobial residues in food is the current focus of safety evaluations of veterinary antimicrobials in Kenya and most other countries. Assessments of risk from residues in food and from resistance in bacteria of animal origin differ in at least two important ways:

(a). Drug residues are chemicals, and their post-harvest concentrations in edible animal products do not change very much with processing and temperature changes. Bacteria, however, are very dynamic; they can die, grow, and interact with other organisms between harvest and eventual consumption. This has important implications for exposure assessment; and

(b). Drugs are approved for intentional administration to animals and treatments can be scheduled to minimize exposure to residues. Conversely, microbial contaminants are naturally occurring, and exposure cannot be so readily manipulated.

2.5.2.3 Socioeconomic impact

Few formal analyses of the economic impacts of antimicrobial use and their withdrawal from animal production have been conducted. The ban on growth promoters in Europe and some early data on the effects on animal production provide some insight into the impacts. The potential economic effects of restrictions on subtherapeutic antimicrobial use in the United States (U.S.) were recently assessed (Committee on Drug Use in Food Animals, 1999). One report by the National Academy of Sciences (NAS) stated that producers using good management practices would be affected less than producers using poor management practices. The report suggested this was because antimicrobial drugs are most effective in animals living in poor conditions, e.g., stress due to crowding and sub-optimal sanitation. Based on assumed 4-5 % feed efficiency/growth promotion, estimated average annual per capita costs of a hypothetical ban on subtherapeutic antimicrobial use were U.S.$

4.84 to $9.72 (U.S.$ 1.2 to 2.5 billion over the U.S. population). Estimated increases in cost per pound were lowest for chicken (U.S.$ 0.013 to 0.026) and highest for beef and pork (U.S.$ 0.03 to 0.06).

2.5.2.4 Consumer perspectives

On the one hand, antimicrobials have been important for the control of animal infections that could be spread to humans. They have allowed the consumer a safer, more abundant and more affordable food supply than in previous decades, which ought to contribute to a healthier population. However, it is argued that the misuse/overuse of antimicrobials in food animals is compromising our ability to fight certain human diseases because of the development of antimicrobial resistant pathogens in animals that are transferred to humans. From the consumer's perspective, which of the current options poses the greatest risk to one's health: eating food that may carry drug-resistant pathogens; eating food that is "drug free" but may be diseased; or eating no food animals? What level of risk are consumers willing to tolerate? Can regulatory policy-makers give the consumer improved options by, for example, banning the use of antimicrobials as growth promoters?

Antimicrobial growth promoters are not used in certified "organic" animal production. The National Standard of Canada for Organic Agriculture specifies that under no circumstances should feed medications, including all hormones and antibiotics used to promote growth, be added to livestock diets (Canadian General Bureau of Standards, 1999). Organic foods currently represent a small, but growing, segment of Eurpean and Canadian food production. In Sweden, for example,

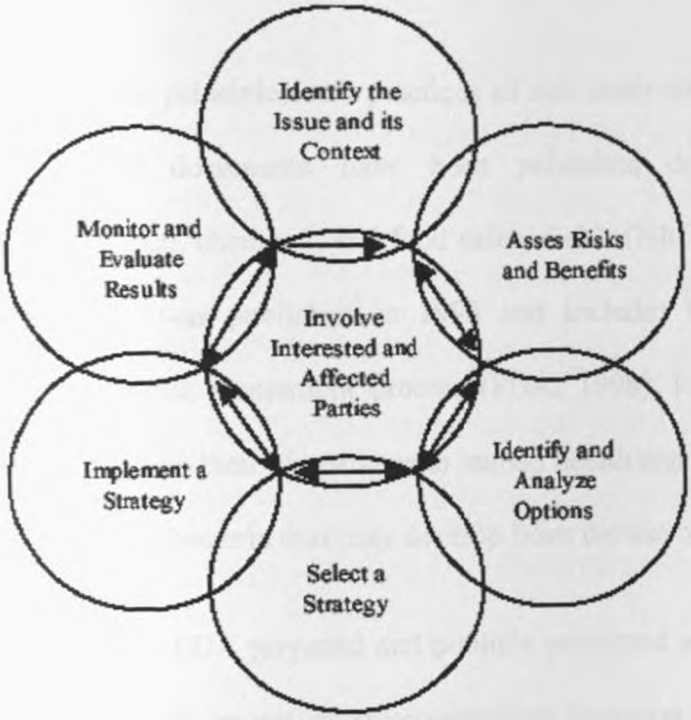
consumers are making "increasing demands for more openness, transparency, and accountability in foodstuff production. The consumer co-operatives believe that the use of antibiotics as growth promoters, together with intensive and industrialized production systems, does not address consumer expectations on food safety." (Council of European Parliamentary Association, 1999).

2.5.2.5 Risk analysis practices

To the best of our Knowledge, risk assessment on antimicrobial resistance in Kenya has not been done. In some developed countries scientists and health authorities have conducted assessments of a variety of human health risks related to food and water safety (Lammerding and Ffazil, 2000; Todd and Harwig, 1996). Canada first published a framework for risk assessment and risk management in 1993 and revised it in 2000 (Health Canada, 2000). The "Health Canada Decision-Making Framework for identifying, Assessing, and Managing Health Risks," articulates several major underlying principles, (Health Canada, 2000). The framework lays out the necessary steps in the decision-making process, including issue or hazard identification, risk/benefit assessment, identification and analysis of management options, strategy adoptions, implementation and follow-up. The approach outlined is similar, conceptually, with approaches used in other countries, including that described in the "United States Presidential Commission/Congressional Commission on Risk Assessment and Risk Management," (PC/CCRARM, 1997). The recent "Report of the Committee on the Drug Review Process of the Science Advisory Board to Health Canada" also contains information and recommendations relevant to effective risk

analysis of veterinary drugs (Committee on Drug Review, Health Canada, 2001). Although focused on human drugs, the reports emphasize the need for transparency throughout the approval process and the desirability of harmonization with other countries. Figure 2.2 illustrates the essential components of the decision-making framework and emphasizes the interconnectedness of all stages of the risk analysis process.

Figure 2.2: Decision-making framework



Source: FAO, 1997.

2.5.2.6 Risk analysis practices in other countries

2.5.2.6.1 United States

Many of the principles and practices of risk analysis were developed in the U.S. A number of documents have been published describing applications to the environmental, chemical, and food safety fields (NRC, 1994). The FDA "Framework Document" was published in 1998 and includes the essential components of a qualitative risk assessment process (FDA, 1998). It provides for categorization of drugs based on their importance to human health and potential for human exposure to any resistant bacteria that may develop from the use of antimicrobials in animals.

In 1999, the FDA prepared and publicly presented a "Draft Risk Assessment on the Human Health Impact of Fluoroquinolone Resistant *Campylobacter* Associated with the Consumption of Chicken" (FDA, 1999a). It is an attempt to estimate, in quantitative terms, the public health risk in one year from resistant foodborne pathogens due the use of antimicrobials in food-producing animals. Within the assessment, a mathematical model was developed that related the prevalence of fluoroquinolone-resistant *Campylobacter jejuni* infections in humans to the prevalence of fluoroquinolone-resistant *C. jejuni* in chickens, which is a major source of *C. jejuni* infection in the U.S. Using data from epidemiological studies and the FOODNET surveillance system in the U.S., the model estimated the most likely number of people sick with resistant *Campylobacter* infections, and estimated the possible range of fluoroquinolone-resistant *C. jejuni* infections that occur in one year in the U.S., as well as which are treated with fluoroquinolones by physicians.

FDA outlined in a 1999 discussion document, *Proposed Framework for Evaluating and Assuring the Human Safety of the Microbial Effects of Antimicrobial New Animal Drugs Intended For Use in Food-Producing Animals,* possible strategies for managing the potential risks associated with the use of antimicrobial drugs in food-producing animals (FDA, 1999b). The Framework Document describes both pre-approval and post-approval approaches. The strategies include (1) categorization of antimicrobial drugs based on the importance of the drug for human medicine; (2) revision of the pre-approval safety assessment for antimicrobial resistance for new animal drug applications to include an evaluation of all uses for microbial safety; (3) post-approval monitoring for the development of antimicrobial drug resistance; (4) collection of food animal antimicrobial drug use data; and (5) the establishment of regulatory thresholds. The Framework Document, as well as the individual strategies outlined, has been the subject of a number of public meetings.

A key component of the Framework Document is the concept of categorizing antimicrobial drugs according to their importance for treating disease in humans. The Framework Document discusses 3 categories, with the most important drugs being considered Category I. The categorization process is an integral part of assessing safety in that it provides a mechanism for characterizing the potential human health impact resulting from treatment failure due to resistance. The categorization process also serves to focus the greatest level of attention on those antimicrobial drugs of greatest importance to human medical therapy.

Consistent with the risk-based approach outlined in the Framework Document, applications for antimicrobial drugs for food-producing animals will initially undergo an antimicrobial resistance risk assessment. This assessment will characterize the proposed drug product as to the potential for resistance to emerge in animals, the potential that such resistance would be transmitted from animals to humans, and the potential consequence of that resistance to human health. These factors taken together will be used to characterize the overall risk that the proposed drug use in animals would cause antimicrobial resistance to emerge and to impact human health.

In addition to the risk assessment, applications for antimicrobial drugs for food-producing animals will undergo a risk management assessment. The recognition that the use of antimicrobial drugs exerts microbial selection pressure focuses attention on the need for appropriate or judicious use of antimicrobial drugs. A principle consistent with the judicious use concept is the idea that certain drugs, or certain drug-use conditions, are more (or less) likely than others to exert pressures favorable to the emergence of resistance.

This risk-based approach to characterizing antimicrobial drugs is consistent with the basic concepts outlined in the Framework Document (FDA, 1999b). That is, risk management steps would be scaled to account for differing levels of risk associated with certain drugs and certain drug use conditions. Based on the conclusions of the antimicrobial resistance risk assessment completed prior to drug approval, the defined risk may be most appropriately managed, for example, by approving only limited use conditions for a particular drug entity. Use restrictions may include limitations

regarding product marketing status, extra-label use provisions, or dosage and administration instructions.

In 2000, the FDA extended its risk assessment to risk management with publication of "An Approach for Establishing Thresholds in Association with the Use of Antimicrobial Drugs in Food-Producing Animals" (FDA, 2000). It identifies the concept of a resistance threshold in humans beyond which the risk of illness in people is no longer acceptable, and describes in detail a proposed methology for determining such thresholds. In 1989, the National Research Council (NRC) Institute of Medicine published a risk assessment entitled "Human Health Risks with the Subtherapeutic Use of Penicillin and Tetracyclines in Animal Feed" (NRC, 1989). This assessment used methods similar, conceptually, to the more recent FDA assessment. The former assessment focused on the annual number of human fatalities attributable to resistance in *Salmonella* infections from in-feed medications.

2.5.2.6.2 Europe

In July 1999, the European Medicines Evaluation Agency (EMEA) Committee for Veterinary Medicinal Products published a qualitative risk assessment of *Salmonella* Typhimurium and the quinolone / fluoroquinolone class of antimicrobials in the E.U. (EMEA, 1999). Specifically, the assessment addressed the following question: "What is the risk of adverse human health effects consequent upon the development of antibiotic resistance to fluoroquinolones in *S.* Typhimurium which is due specifically to the use of fluoroquinolones as veterinary medicines in farm livestock?" A number of potential risk pathways were examined, with the result that the probability of

adverse health effects was considered low, but with a high degree of uncertainty overall.

2.5.2.6.3 United Kingdom

The U.K. has had more than its share of food safety crises. It has recently reviewed its risk procedures and use of expert advisory groups (May, 2000). In essence, these reviews highlight the varied approaches that exist in risk practices associated with food safety and the need to closely link the essential stages of risk analysis (communication, management, and assessment). The reviews noted improvements in the openness and accessibility of U.K. risk procedures, but stated that communications could be better. It was emphasized that distinctions between voluntary and involuntary risks and the needs of vulnerable groups required greater recognition. A number of best practices for committees advising the government on risk were also laid out.

2.5.2.6.4 Office International des Epizooties

The Office International des Epizooties (OIE) ad hoc group on antimicrobial resistance published a draft set of guidelines entitled "Risk Analysis Methodology For The Potential Impact On Public Health Of Antimicrobial Resistant Bacteria Of Animal Origin" (OIE, 2000). It contains detailed descriptions of the principles of risk analysis, and a general description of good risk analysis practices related to antimicrobial resistance.

2.5.2.6.5 The "precautionary principle"

The precautionary principle stipulates that risk reduction actions should not await scientific certainty. The E.U. interpretation of the precationary principle presupposes there could be negative effects from a process or practice. If, after scientific assessment, there remains sufficient uncertainty of the risk, it warrants precautionary action (EC, 2002). The decision to act or not i.e., take risk management action, often weighs the political consequences of each option. In theory, the precautionary principle is consistent with qualitative risk analysis, however other countries outside of the E.U. are suspicious that the precautionary principle could be used in ways that are inconsistent with existing trade agreements.

2.5.2.7 Risk assessment - classification of human health risk of antimicrobials used in food animals

A variety of methods may be used to assess resistance risk, including description and enumeration of documented cases of human illness, analysis of disease data from resistance surveillance programs, extrapolation from animal experiments, or use of models of human exposure and disease (Baillers and Travers, 2002). Careful study of naturally occurring illness in humans is the traditional, and perhaps most reliable method; however, it is severely constrained in many situations by the limits of our technical ability to correctly correlate illness with exposure to hazards, e.g., resistant bacteria arising from antimicrobial treatment of food animals. Scientific data for risk assessments may be assembled from a variety of sources, including published

scientific literature, government reports, or from industry. Unfortunately, unlike the clearly defined mechanism for ascertaining the toxicological safety of drug residues in food, no such predictive models currently exist to precisely estimate the rate and extent of bacterial resistance that may emerge from the use of antimicrobial drugs in food animals. Despite the current lack of such models, certain information can be generated to support a pre-approval antimicrobial resistance safety assessment.

CHAPTER THREE

3.0 DETERMINATION OF ANTIMICROBIAL CONSUMPTION IN FOOD ANIMALS

3.1 INTRODUCTION

Antimicrobial agents are commonly used in the food animal industry for the treatment of bacterial and protozoal infections, prophylactic use in intensive animal rearing or for growth promotion purposes. Increasing levels in bacterial antibiotic resistance have renewed interest in obtaining relevant antibiotic consumption data from humans and food animal industry. Standard methodology for calculating drug consumption has been developed during the past 20 years by the WHO through its collaborating center for DrugStatistics Methodology (Oslo, Norway). These data are essential for risk analysis and planning and can be timely in interpreting AMR surveillance data.

Resistance to the effects of antimicrobial drugs is a serious problem in the world today. The problem, often referred to as antimicrobial resistance or AMR threatens the ability to treat infections in humans and animals. The World Health Organization (2001) reports indicate that there are serious problems with microbial resistance to frontline drugs used to combat disease causing pathogens. The resistance problem is most acute in the case of bacterial infections.

Although our traditional response to the development of antimicrobial resistance is to use different, often new, drugs to treat the disease, this approach is no longer tenable because the supply of new, effective, safe, and affordable antimicrobial agents is expected to diminish in the future. Thus, the protection of the antimicrobial drugs

now available is inevitable in order to minimize resistance impacts on our health and economies. Although emergence of AMR occurs whenever antibiotics are used, evidence indicates it can be slowed by prudent use of antimicrobials and better infection control (Bager *et al.*, 2002).

Prudent antimicrobial use maximizes therapeutic effect while minimizing resistance. With respect to clinically important infections in food animals and humans, most resistance problems probably arise from direct use of antimicrobials. Serious questions have been raised about the inappropriate use of antimicrobials for treatment of viral infections, non-prescription use in some countries, and incomplete treatment courses (WHO, 2001; Dept. of Health, UK, 1998; Harrison and Lederberg, 1998). Clearly, improvements can be made in how antimicrobials are used in human and veterinary medicine (Bager, 1997).

Inevitably, however, when considering the use of antimicrobials in Kenya and the rest of the world, attention turns to the use of antimicrobials in agriculture. In countries where reliable data are available, upto 50% or greater of the total volume of antimicrobials produced or imported in these countries are administered to animals (DANMAP, 2003). Previously, a significant proportion of antimicrobials were used in food animals for growth promotion and disease prophylaxis (Bager, 1997).

AMR is an international problem; resistant bacteria are carried easily between countries by travellers, animals, food, and other carriers. Most solutions to the problem, however, are necessarily national or local in scope because they involve government regulation or changes in prevailing farming practices. The European Union (EU), for example, banned four drugs (avorpacin, virginiamycin, tylosin and

olaquindox) for use as growth promoters because they are also used for therapy in humans and animals and recently announced plans to eliminate remaining growth promoters by 2006. However, antimicrobials of critical importance to human medicine (e.g., fluoroquinolones, cephalosporins) are still used in the E.U. for the treatment of food animals (DANMAP, 1999; McEwen, 2001). The United States is taking a somewhat different approach by focusing its regulatory efforts on reshaping the approval process for new drug applications. Recently, the U.S. used quantitative risk assessment to guide its decision to seek revocation of approval of a fluoroquinolone for therapy in poultry (FDA, 1999). Australia also recently examined its antimicrobial programs and policies and made recommendations aimed at improving regulatory controls, surveillance, infection prevention, education and research (JETACAR, 1999) while Canada established a national surveillance system to monitor antimicrobial resistance and use in the agri-food and aquaculture sectors (Health Canada, 1997). In the year 2000, British Columbia and Ontario introduced antimicrobial resistance monitoring programs (Provincial Healthcare Office, BC, 2000; Healthcare Ontario, 2001).

Denmark is seen as the leading country in the world in reducing antimicrobial use in animal production (Bager, 1997; Bager *et al.*, 2001). It banned the use of avoparcin in May 1995 and issued the regulations restricting veterinarians' sale of antimicrobials to farmers and the use of antimicrobials for production enhancing purposes. In 1998, Danish farming organizations determined to voluntarily withdraw animal growth promoters (AGPs). By 1999, AGPs were also phased out in weaner pigs. Today, all the AGPs have been banned from use in food animals except four of them,

avilamycin, flavomycin, salinomycin and monensin in Denmark and other EU countries. The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) has been publishing an annual report on consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food, and humans in Denmark since 1997. The purpose of the DANMAP is to monitor the changes in antimicrobial use and antimicrobial resistance before and after the ban of AMGPs.

In Kenya, data on antimicrobial usage in food animals is scanty. To date, there are only two reports on antimicrobial consumption in humans and food animals (Mitema *et al.*, 2001; Mitema and Kikuvi, 2004). These studies document antimicrobial consumption among food animals in Kenya for the period 1995 to 1999. Also, a number of studies have reported antimicrobial resistance in humans (Chunge *et al.*, 1992; Kariuki *et al.*, 1997; Graham *et al.*, 2000; Kariuki *et al.*, 2002) as well in food animals (Bebora *et al.*, 1994; Ombui *et al*, 1995). In spite of these reports, the impact of antimicrobial use in food animals on the emergence of AMR has not been studied. Since antimicrobial resistance eventually develops in bacteria hosted by animals when antimicrobials are administered to animals, bacteria including those resistant to antimicrobial drugs, spread from animals to humans. However, the overall magnitude of the impacts of antimicrobial resistance on human health has yet to be determined. Moreover, the contribution of antimicrobial usage in food animals to the development of AMR remains unknown.

Therefore, the aim of this study was to generate data on antimicrobial consumption in food animals in Kenya for the period January, 2000 to December, 2001 as a follow-up

of previous studies. These data was correlated with antimicrobial resistance data from indicator bacteria isolated during the same period. This information is necessary;

a) for the interpretation of antimicrobial resistance surveillance data from human, animal, food, and environmental sources;

b) for the development and evaluation of programs designed to contain antimicrobial resistance and to maintain and promote a wholesome and nutritious food supply for human consumption.

c) for use in risk analyses relating to the use of antimicrobials in food-animal production and the protection of human health; and

d) for use in identification of agricultural antimicrobial use practices that are likely to result in the development of antimicrobial resistance of veterinary or human medical significance.

3.2 MATERIALS AND METHODS

3.2.1 Sources of data

Most of the data on antimicrobial usage in animals was based on drug imports by the pharmaceutical industry. These data, representing the volume of drugs imported from overseas as well as those locally manufactured, was collected from the official records of the Pharmacy and Poison's Board (PPB) registry of the Ministry of Health. The PPB has the legal responsibility for monitoring the importation and consumption of all human and veterinary medicinal products in Kenya.

3.2.2 Data collection

Data was collected as previously described by Mitema *et al.* (2001). The name (brand and generic), quantities (number of vials or packs) including potency of pharmaceutical preparation imported for sale or manufactured for local use were recorded for each antibiotic, antimicrobial class or combination.

Data collected for each antimicrobial product included the following information: name of importing or manufacturing company, date of importation or manufacture, brand name of the product, generic name (non-proprietary name), dosage form (solution/ suspension/ powder/ bolus) and route of drug administration (oral/ parenteral/ intramammary/ topical), amount or quantities (kilogrammes) of active ingredients of each antimicrobial class, intended use of antimicrobial agent (specific therapy/ prophylactic therapy or growth promotion) and target species for instance large animals (cattle, sheep, goats, pigs) or poultry. Antimicrobials were classified in accordance with the international classification system for drug consumption studies

(ATC) code maintained by the WHO Collaborating Centre for Drug Statistics and Methodology. Antimicrobial classess were categorised as either: Beta-lactams (penicillins and cephalosporins), tetracyclines, sulfonamides and trimethoprim (including combinations), aminoglycosides, macrolides and lincosamides, quinolones and others (e.g. tiamulin and nitrofurans).

4.9 Data analysis

The results from data collected for each antimicrobial product were stored in a resistance database (Microsoft Excel). Amounts of antimicrobial agents were calculated in kilograms of active ingredient for each of the specific variables like antimicrobial class, intended use of antimicrobial agent, route of administration and target species. All manipulation of data and evaluation of results were carried out using Microsoft Excel.

3.3 RESULTS

3.3.1 Trends in consumption of animal antimicrobial agents

3.3.1.1 Overall consumption

In Kenya, all antimicrobials used in food production from January, 2000 – December, 2001were imported from overseas countries. Table 3.1 shows the trend from 2000 and 2001 in the consumption of antimicrobial used in production animals. During the study period the annual increase of total antibacterials for systemic use (ATC group JO1, 2004 definition) averaged 5,400 kg. The quantity of antimicrobial used in food animals increased by 54.7 % to an estimated 15,234 kg active compound. The increase was mainly in quinolones and to a lesser extent in tetracyclines. The veterinary consumption of fluoroquinolones increased by 2500 %, from 200 kg in 2000 to 5204 kg in 2001. The consumption of tetracyclines increased by 6.4 %, from 8,481 kg in 2,000 to 9,020 kg in 2001. The consumption of sulfonamides decreased by 21 %, from 148 kg to 117 kg in 2001, while aminoglycosides consumption declined by 60 %, from 318 kg to 127 kg in 2001. The consumption of penicillins (including Extended Spectrum Beta-lactams) marginally increased by 4 %, from 520 kg in 2000 to 540 kg in 2001.

The mean antimicrobial consumption and percentage mean antimicrobial usage for the two-year period (2000 - 2001) is shown in Fig.3.1 and Fig. 3.2, respectively. Tetracycline was the most prescribed antibiotic class during the study period. A mean total of 8,751 kg (69.8 %) was consumed per year. In this class, oxytetracycline was the only tetracycline administered during the study period. Fig. 3.3 shows the

percentage mean oxytetracycline consumption per year of various food animals. The mean total oxytetracycline consumption per year was 8,499 kg (97.1 %) for large animals, 193 kg (2.2 %) for poultry and 59 kg (0.7 %)for both poultry and large animals. Approximately 98 % (8,505 kg) of mean oxytetracycline consumption per year in food animals was used for specific therapeutic purposes while 2.4 % (211 kg) was used for prophylactic purposes. Similarly, 97 % (8,499 kg) was administered parenterally and only 3 % (252 kg) was given orally.

The mean consumption per year for the fluoroquinolones (2,702 kg) represented 21.6 % of total antimicrobial consumption. Quinolones consumption comprised of 2,575 kg (95.3 %) of enorfloxacin, 70 kg (2.6 %) of flumequin and 57.2 kg (2.1 %) of norfloxacin.

The mean annual consumption for the Beta-lactams was 530 kg, representing 4.2 % of antimicrobial consumption during the study period. The mean penicillin consumption was 487.5 kg (92 %), while mean consumption for ampicillin and cloxacillin was 25 kg (4.7%) and 17.5 kg (3.3 %), respectively.

The mean consumption per year for the aminoglycosides was 222 kg, this amount represents 1.8 % of mean total antibiotic consumption per year during the study period. In this class, streptomycin consumption was 103 kg (88.7 %) while neomycin consumption amounted to 13 kg (11.3 %). With regard to sulfonamides, the percentage mean total consumption per year during the study period was approximately 1 %. The mean annual consumption (117 kg) consisted of 94 kg (80.3 %) of sulfadiazine and 23 kg (19.7 %) of trimethoprim.

The percentage mean antimicrobial consumption per year among various food animals is shown in Table 3.2. Approximately 9,232 kg was intended for consumption by large animals (cattle, shoats and goats), 2,886 kg and 422 kg was meant for large animals and poultry (LAP) and poultry alone, respectively. These quantities represented 74 % in large animals, 3 % in poultry alone and 23 % in both large animal and poultry.

The percentage mean antimicrobial consumption per year in food animals according to intended use was about 98 % (12,300 kg) for specific therapy while approximately 2 % (208 kg) was used for prophylactic purposes. With respect to administration route, 76 % (10,371 kg) of the total antimicrobial consumption for systemic use was intended for parenteral use while 24 % (3,355 kg) was intended for oral administration.

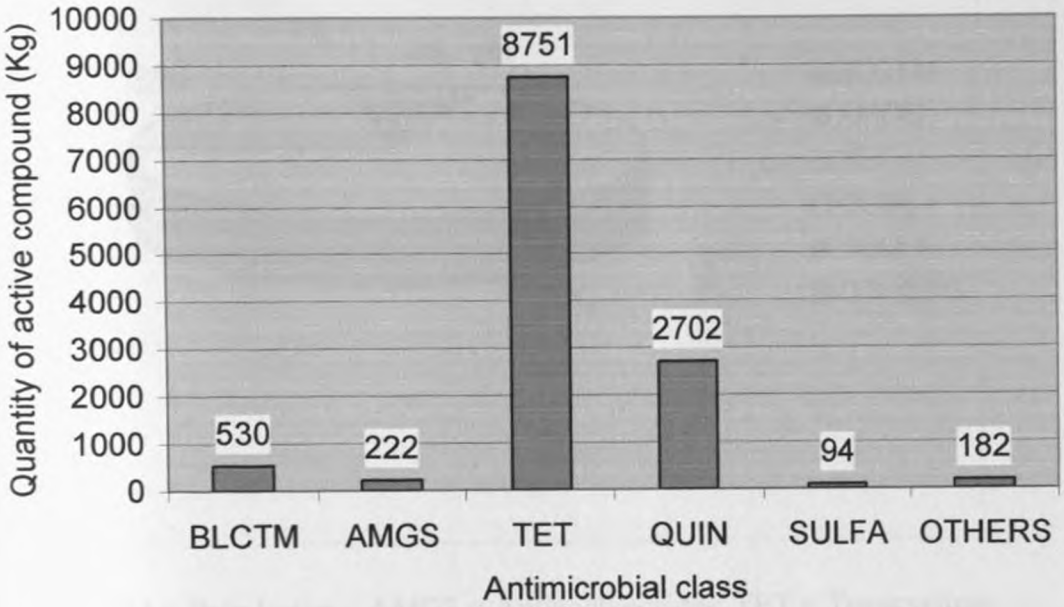
3.4.2 Antimicrobial consumption by oral administration

The various data for oral antimicrobial use in food animals is shown in Table 3.3 and Figure. 3.4. Approximately 82 % (2,754 kg) of mean oral antimicrobial prescription was administered in both large animals and poultry while 18 % (601 kg) was administered to poultry alone. Moreover, about 75 % (2,337 kg) of mean total oral consumption was administered for specific treatment while 25 % (762 kg) was administered for prophylactic purposes.

With respect to various antimicrobial classes five antimicrobials were administered using the oral route to the various food animals during the 2-year period. The percentage mean consumption per year was as follows: 2,702 kg (80.5 %) of

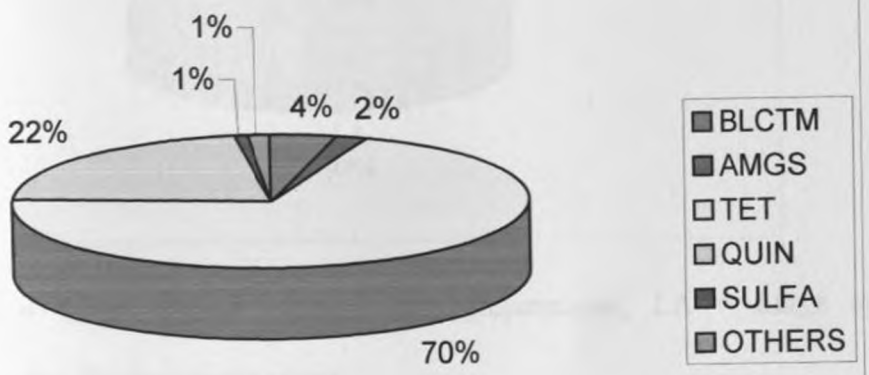
fluoroquinolones, 459 kg (10.6 %) of oxytetracycline, 180 kg (5.4 %) of nitrofurans and 117 kg (3.5 %) of sulfonamide + trimethoprim (Fig. 3.4). Tiamulin consumption was less than 1 % while the beta-lactams, aminoglycosides and macrolide antibiotics were not administered orally.

Fig 3.1. Mean antimicrobial consumption (Kg) per year in food animals in Kenya during 2000-2001.



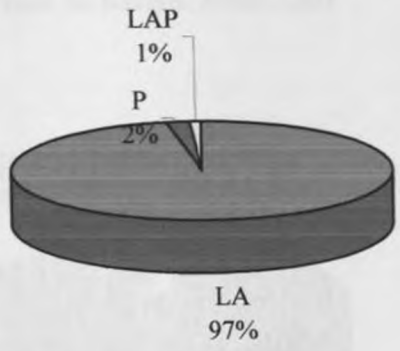
Key: BLCTM = Beta lactams; AMGS = Aminoglycosides; TET = Tetracyclines QUIN = Quinolones; SULFA = Sulfonamides.

Fig. 3.2. Percentage mean antimicrobial consumption per class per year in food animals in Kenya.



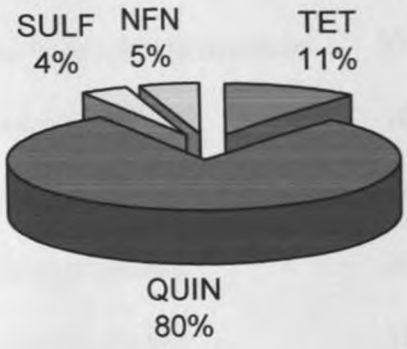
Key: BLCTM = Beta lactams; AMGS = Aminoglycosides; TET = Tetracyclines QUIN = Quinolones; SULFA = Sulfonamides.

Fig. 3.3. Percentage mean oxytetracycline consumption per year among the various food animals in Kenya.



Key: LAP = Large animals and poultry preparations; LA = Large animals preparations; P = Poultry preparations.

Fig. 3.4. Percentage mean oral antimicrobial consumption per year administered in food animals in Kenya, 2000-2001.



Key: SULF = Sulfonamides; QUIN = Quinolones; TET = Tetracyclines; NFN = Nitrofurans.

Table 3.1: Total consumption of antimicrobial agents (kg) for treatment of food animals during 2000-2001, Kenya

ATC group	compound	2000	2001	Mean
QJ01AA	Tetracycline	8481	9020	8751
QJ01CE	Penicillins, B-lactamase sensitive	504	521	513
QJ01C	Other penicillins	18	18	18
QJ01	Sulfonamides + trimethoprim	148	86	117
QJ01MA	Fluoroquinolones	200	5204	2702
QJ01G	Aminoglycosides	318	127	222
QJ01XX	Tiamulin	2	1	2
	Others (nitrofurans)	174	185	180
Total		9845	15234	12540

Table 3.2: Amount (kg) and percentage of antimicrobial agent consumption in food animals in Kenya, 2000-2001

Category	Mean (Kg)	Percentage (%)
Species		
Large animals	9232	74
Large animals & Poultry	2886	23
Poultry	422	3
Intended use		
Prophylaxis	208	2
Specific treatment	12300	98
Route of administration		
Parenteral	10371	76
Oral	3355	24

Table 3.3: Total consumption of antimicrobial agents (kg) administered orally for treatment of food animals in Kenya, 2000-2001.

ATC group	compound	Period (Year)		
		2000	2001	Mean
QJ01AA	Tetracycline	459	251	355
QJ01CE	Penicillins, B-lactamase sensitive	0	0	0
QJ01C	Other penicillins	0	0	10
QJ01	Sulfonamides + trimethoprim	148	86	117
QJ01MA	Fluoroquinolones	200	5204	2702
QJ01G	Aminoglycosides	0	0	0
QJ01XX	Tiamulin	2	1	2
	Others (nitrofurans)	174	185	180
Total		983	1528	3355

Table 3.3: Total consumption of antimicrobial agents (kg) administered orally for treatment of food animals in Kenya, 2000-2001.

ATC group	compound	Period (Year)		
		2000	2001	Mean
QJ01AA	Tetracycline	459	251	355
QJ01CE	Penicillins, B-lactamase sensitive	0	0	0
QJ01C	Other penicillins	0	0	10
QJ01	Sulfonamides + trimethoprim	148	86	117
QJ01MA	Fluoroquinolones	200	5204	2702
QJ01G	Aminoglycosides	0	0	0
QJ01XX	Tiamulin	2	1	2
	Others (nitrofurans)	174	185	180
Total		983	1528	3355

3.4. DISCUSSION

Total antimicrobial consumption in food animals increased by 55 % in 2001 compared to 2000. However, mean consumption during this period declined by almost 16 % when compared to mean consumption between 1995-1999 (Mitema *et al.*, 2001). Tetracyclines constituted 70 % of total antimicrobial consumption, fluoroquinolones 22 %, penicillins 4 %, aminoglycosides 2 % and sulfonamides 1 %. When compared to the report (Mitema *et al.*, 2001) of a previous study on antimicrobial consumption in food animals this study indicates increasing trends in oxytetracycline (55 % to 70 %) and fluoroquinolone (0.6 % to 22 %) usage and declining trends in sulfonamides (21 % to 1 %), penicillins (6 % to 4 %), aminoglycosides (7 % to 2 %) and macrolides (0.24 % to 0 %) consumption in food animals.

In Kenya veterinary consumption of fluoroquinolone appears to be on the increase despite reduction levels of consumption in Europe and USA due to legal restrictions. Excessive administration of antimicrobial agents through the oral route can lead to selection of resistant bacteria of the gut resulting in development of AMR strains. In this study 24 % of total antimicrobial consumption was administered orally; quinolones and tetracycline accounted for nearly 81 % and 11 %, respectively of total oral antimicrobial consumption. The increase in fluoroquinolone consumption occurred mainly in large animal and poultry and was associated with specific treatment. On the other hand, the increase in oxytetracycline usage occurred only in large animals, parenteral administration and for specific treatment purposes.

The dramatic increase in fluoroquinolone consumption in 2001 was unlikely to be due to either change in treatment regimens (i.e. increased doses or longer duration of treatment or increased incidence of infections) or an increase in animal production. It is more likely explained by a markedly reduced price per defined animal daily dose (ADD) due to the influx of generic fluoroquinolones and the increasing levels of sulfonamide resistance. The significant increase in fluoroquinolone consumption in food animals in Kenya in 2001 calls for a closer surveillance of consumption and its effect on possible emergence of fluoroquinolone resistance. The Center for Veterinary Medicine of the Food and Drug Administration (FDA) has banned the use of fluoroquinolones in poultry following evidence of development of fluoroquinolone-resistant Campylobacter bacteria in poultry; and that these fluoroquinolone-resistant Campylobacter bacteria are transferred to humans and causes the development of fluoroquinolone-resistant Campylobacter in humans (FDA, 1999).

Data on antimicrobial consumption from various countries is rather scarce. However, surveillance data from Nordic countries has been available since 1994 and indicate that total antimicrobial usage (including those used for both growth promotion and treatment purposes) has declined markedly. DANMAP reports indicate that in 1994, a total of 206,000 kg of active compound of Animal Growth Promoters (AGPs) and therapeutic antimicrobials were used in Denmark (DANMAP, 1997). By the year 2000, the total antimicrobial usage fell to 80,600 kg of active compound, which represents a 61% reduction. In 1999, the therapeutic antimicrobial use in Denmark was 61,900 kg of active compound but this amount went up to 80,600 kg in 2000,

which represents a 30% increase. This increase in therapeutic antimicrobial use was associated with the increase of oral compounds, mainly tetracycline and macrolides/lincosamides (DANMAP, 2000; DANMAP, 2001).

The use of fluoroquinolones in food animals in the Danish animal production slightly increased from 1999 to 2000 and remains at a much lower level than in 1998 (DANMAP, 1988). The total use of active compound of fluoroquinolones was slightly over 400 kg in 1998, 150 kg in 1999, about 165 kg in 2000 decreasing to 14 kg in 2002 (DANMAP, 2002; 2003). It was reported that the reduction of fluoroquinolones use was accompanied by a decline in quinolone resistance among *E. coli* O149 isolated from young pigs with diarrhoea.

In 1997, Fedesa (European Federation for Animal Health) provided information on antimicrobial use in Europe. Reported total sales volume for the European Union was 10.5 million kg of active ingredients. Of this, 5.4 million kg (52%) was for human use, 3.5 million kg (33%) for animal health, and 1.6 million kg (15%) for growth promotion). They estimated that 90% of antimicrobials for animal use were administered in feed; 60% were used in pigs, 20% in poultry and rabbits, 18% in ruminants, and 1% each in fish and pets. Within the animal health category (therapy, prevention and control), 66% were tetracycline, 12% macrolide, 9% penicillin, and 12% other drugs (Schwarz and Chaslus-Dancla, 2001). In the Netherlands Veterinary consumption of antimicrobial agents was estimated at 300 000 kg (van den Bogaard, 1997)

In the United states the most widely quoted estimate of total use is found in the 1989 Institute of Medicine (IOM) report (Committee on Human Health Risk Assessment,

IOM, 1989), which estimated that approximately 11.4 million kg of antimicrobials are produced annually in the U.S., and that approximately 50% is used in animals. Recently, the Union of Concerned Scientists (UCS), a non-profit organization representing consumer issues, estimated that approximately 16 million kg. of antimicrobials are used annually in the U.S.; 1.5 million kg (9%) in humans and 14 million kg (87%) in animals (Mellon *et al*, 2001). Approximately, 11 million kg (70 %) of this estimate was classified as non-therapeutic (e.g., growth promotion, and /or prophylaxis) in three types of food animals: cattle, swine, and poultry.

These consumption patterns are distinctly different from Kenya where, according to the present study, 98 % of antimicrobial agents were used for specific therapy.

This study indicates that conventional AGP's were hardly used in food animals during the study period. These results are in agreement with previous observations (Mitema et al., 2001). Hence, the use of antimicrobial agents for growth promotion purposes in food animals if it hardly occurs, perhaps utilizes water-soluble oral antibiotics for example oxytetracyclines and sulfonamides.

Currently, actual levels of antimicrobial consumption in food animals in Kenya relative to production figures remain relatively unknown because available antimicrobial consumption data is currently based on importation figures alone. Yet, these data are needed to interpret trends in resistance over time, to assess the impact of resistance on human health, and for development and evaluation of programs designed to contain antimicrobial resistance. Given the way that antimicrobial agents are distributed and used in the Kenyan food production industry, an integrated approach combining antimicrobial consumption data from several sources (end-users)

will be necessary. These should include annual antimicrobial sales data from pharmaceutical manufacturers, importation data, periodic monitoring of antimicrobial use by farmers and veterinarians, and information from other points in the distribution system (e.g., agrovets, pharmacies, wholesalers and NGO's).

In conclusion, this study indicates increasing levels in tetracycline and fluoroquinolone usage and declining trends in sulfonamides in food animals in Kenya.

CHAPTER FOUR

4.0 PHENOTYPIC AND GENOTYPIC CHARACTERIZATIONS OF ANTIMICROBIAL RESISTANCE IN *E. COLI* ISOLATES FROM FOOD ANIMALS

4.1 INTRODUCTION

The emergence and dissemination of antimicrobial resistance in bacteria has been well documented as a serious problem worldwide (WHO, 2000). Problems associated with the presence of antibiotic-resistant bacteria have reached epidemic proportions in recent years, with cost estimates exceeding $4 billion in the United States alone (Boyce *et al.*, 2001; Jones *et al.*, 2003). As a consequence, antimicrobial-resistant bacteria are selected for, thereby posing a critical public health threat in that antimicrobial treatment efficacy may be reduced. The spread of antibiotic-resistant bacteria in the environment is dependent on the presence and transfer of resistance genes among microorganisms, mutations, and selection pressure to keep these genes in a population. The causes and effects of antibiotic overuse are varied. One of the most controversial applications of antibiotics, however, is for growth promotion in livestock, and this application has raised concerns about its contribution to the presence of resistant bacteria in humans (Aarestrup *et al.*, 2001; Wegener *et al.*, 1999).

To date there has never been a program of systematic monitoring of antimicrobial resistance of bacteria originating from food animals in Kenya and there are no preliminary attempts to develop a systematic monitoring program. Data on resistance

in bacteria derived from food animals, when available, tends to be highly fragmented and opportunistic. Moreover, in most studies the bacteria are from diagnostic and research submissions; therefore, they are not systematically collected and the findings may be biased due to lack of geographical representation.

The reports of Kenya Medical Research Institute (Kariuki *et al.*, 1997, 1999) provide a possible exception to the above. The center conducts ongoing monitoring of serovars of *Salmonella* isolated from animals, including the highly virulent *Salmonella* Typhimurium definitive phage type DT 104 (Kariuki, *et al.*, 2002; Kariuki, *et al.*, 2005). Typically, data on antimicrobial resistance in Kenya for animal pathogens has addressed resistance only in the context of efficacy (or lack of it) on treatment of infections in animals. Therefore, these data may not be representative of exposure to antimicrobial resistance in the food chain.

With the worldwide progressive increase in AMR, treatment of bacterial infections has become increasingly difficult. In Kenya aminopenicillins, oxytetracycline, trimethoprim-sulfonamides, and, to a lesser extent, the aminoglycosides streptomycin and dihydrostreptomycin are the usual antimicrobials used in food animals (Mitema *et al.*, 2001); however, with the appearance of increased multi-drug resistance, flouroquinolone usage is on the increase.

The dissemination of antibiotic resistance genes among bacterial strains is an increasing problem in infectious diseases. Many antibiotic resistance genes are located on plasmids and/or transposons, enabling their transfer among a variety of bacterial species (Roberts, 1996). In recent years, another mechanism of resistance

gene dissemination has been discovered. That mechanism involves a DNA element that mediates the integration of resistance genes by a site-specific recombinational mechanism. This newly recognized DNA element, called an integron, either is found as part of a transposon within the Tn*21* family or is found independently on several groups of broad-host-range plasmids. Class 1 integrons possess two conserved segments separated by a variable region (VR) which includes integrated antibiotic resistance genes or cassettes of unknown function (Recchia and Hall, 1997). The 3' conserved segment contains the *qacE* and *sulI* genes and an open reading frame (ORF) called *orf5*. The *qacE* and *sulI* genes determine resistance to ethidium bromide and quaternary ammonium compounds and resistance to sulfonamide, respectively (Recchia and Hall, 1997).

In Kenya a relative paucity of information exists regarding antimicrobial resistance in *E. coli* from food animal sources. The distribution of antimicrobial resistance genes and presence of integrons remains unknown. Therefore, in this study, antimicrobial susceptibility profiles were determined for *E. coli* isolates of food animal origin. The primary objective was to characterize the extent of antimicrobial resistance in these *E. coli* isolates from cattle, swine and poultry. Moreover, *E. coli* strains from these food animals were also characterized for their genotypic resistance gene profiles.

MATERIALS AND METHODS

4.2.1 Animal sampling

A total of 55 (12 %) out of 472 cattle slaughtered at Dagoreti slaughter houses (Nairobi) during the study period and 58 swine slaughtered at Ndumbuini slaughterhouse (Kiambu district) were sampled. Fecal and carcass swabs were obtained from cattle and swine after evisceration from the slaughter line. In addition, sixty-nine (69) live chickens were sampled at various markets around Nairobi and 59 cloacal swabs and 10 pharyngeal swabs were obtained. All collected samples were inoculated into Stuart's transport medium and transported to the laboratory in cool boxes.

4.2.2 Isolation of *E. coli*

Upon arrival at the laboratory, approximately two milliliters of Stuart's transport medium containing the specimen were inoculated into peptone water (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours for enrichment. A loopful of each specimen was then subcultured onto Eosin methylene blue (EMB) agar (Oxoid, Hampshire, England), a selective medium for *E. coli*, and then incubated at 37°C for 24 hours. Typical *E.coli* colonies appear dark centred with or without a characteristic green metallic sheen.

4.2.3 Identification of *E. coli*

Identification of *E. coli* was done using both cultural and microscopic characteristics and confirmed using the following biochemical tests in accordance to Food and Drug Administration -Bacteriological Manual, (1998).

4.2.3.1 Indole production

E. coli test organisms were inoculated into 5 ml of sterile 1.0 % w/v tryptone (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours. After incubation, 2 ml of Ehrlich's reagent (containing 1g, p-dimethylaminobenzaldehyde dissolved in 95 ml absolute ethanol and 20 ml concentrated HCl) was added and mixed. Indole production (positive result) was indicated by a pink/ red colouration in the upper layer of the solution.

4.2.3.2 Methyl red reaction

E. coli test organisms were inoculated into 5 ml of sterile 1.0 % w/v tryptone water (Oxoid, Hampshire, England) and incubated at37°C for 24 hours. After incubation 2 drops of test solution (containing 0.04 g Methyl red in 40 ml ethanol and 100 ml distilled water) was added and mixed vigorously. A positive result was indicated by a red colour while yellow colour indicated a negative reaction.

4.2.3.3 Vogues-Proskauer reaction

Tubes containing 5 ml of Voges-Proskauer broth were inoculated with test bacteria and inoculated at 37°C for 24 hours. One ml of 40 % (w/v) potassium hydroxide was

mixed with the broth culture and one gram of creatine was added. The tubes were shaken and kept in a slanting position for one hour. Production of acetyl methyl carbinol was indicated by development of a red (eosin pink) colour.

4.2.3.4 Citrate utilization

E. coli test organisms were inoculated into slants of citrate medium and incubated for 24 hours at 37°C. Utilization of citrate was indicated by growth and a blue colour due to the production of an alkaline reaction.

4.2.3.5 Interpretation of results

All bacterial cultures that appeared as gram-negative rods or cocci and gave IMVic pattern of + + - - (Biotype 1) or - + - - (Biotype 2) were considered to be *E. coli*.

4.2.4 Storage of stock culture

Each *E coli* isolate was purified and maintained as stock culture in duplicate. One batch was stored in 5 % glycerol and the second batch was maintained in cooked meat media and stored at -20°C until analysed.

4.2.5 Antimicrobial susceptibility tests

E. coli isolates were tested for their susceptibility to eight different antimicrobial agents by use of disc diffusion technique as recommended by The CLSI (2004). The bacteria stored in cooked meat media at –20 °C were allowed to thaw for 15 minutes. They were inoculated on blood agar (BA) plates and incubated at 37°C for 24 hours

so as to get actively growing bacterial cells. Three to five discrete colonies from each of the BA plates were suspended in 5 ml sterile distilled water. The concentration of the suspension was standardized by adjusting the turbidity of the bacterial suspension to match that of Barium sulphate (0.5 Marcfarland turbidity standard). Mueller Hinton agar plates (Oxoid, Hampshire, England) were then inoculated with this bacterial suspension by dipping a sterile swab into the inoculum and pressing and rotating the swab firmly against the side of the bottle above the level of the liquid to remove excess inoculum. The swab was then streaked all over the surface of the media three times while rotating the plate through an angle of approximately 60^{o} after each application in order to spread the bacteria as evenly as possible. The plates were left to dry for five minutes at room temperature with the lid closed. Multidiscs containing eight different antimicrobial agents including ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulfamethoxazole (25 µg), co-trimoxazole (25 µg), streptomycin (10 µg), kanamycin (30 µg) and gentamycin (10 µg) were placed on each of the inoculated agar plates using a pair of sterile forceps. The multidiscs were gently pressed to ensure even contact with the medium. Plates were then incubated at $37^{o}C$ for 24 hours. After incubation the diameter of the zone of inhibition for each antimicrobial agent was measured with a ruler to the nearest millimeter. *E. coli* ATCC 25922 was used as a control. The disc susceptibility tests were interpreted in accordance to CLSI criteria (Table 4.1).

4.2.5.1 Multiple antibiotic resistance (MAR) index

The multiple antibiotic resistance index was defined as **a/ b** where **a** represents the number of antibiotics to which the particular isolate was resistant and **b** (n = 8) the number of antibiotics to which the isolate was exposed (Krumperman, 1983). The number and the proportion (%) of *E. coli* isolates with an MAR index $\geq$ 0.20 was noted (MAR index > 0.2 indicates high risk).

Table 4.1: Zone diameter (mm) interpretive criteria for enterobacteriaceae

Antimicrobial agent	Disk content	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin	10 µg	≤ 13	14-16	≥ 17
Tetracycline	30 µg	≤ 14	15-18	≥ 19
Chloramphenicol	30 µg	≤ 12	13-17	≥ 18
Sulfamethoxazole	25 µg	≤ 10	11-15	≥ 16
Co-trimoxazole	25 µg	≤ 10	11-15	≥ 16
Streptomycin	10 µg	≤ 11	12-14	≥ 15
Kanamycin	30 µg	≤ 13	14-17	≥ 18
Gentamycin	10 µg	≤12	13-14	≥ 15

Source: CLSI, 2004

4.2.6 Genotypic characterization of AMR genes using PCR

Ampicillin resistant *E. coli* isolates were screened for β-lactamase encoding genes (bla_{TEM}). Tetracycline resistant *E. coli* isolates were screened for the presence of *Tet(A)* and *Tet(B)* resistance genes that are associated with drug efflux mechanism in gram-negative bacteria. Similarly, sulfonamide resistant *E. coli* isolates were examined for the presence of sulfonamide resistance genes, *SulI* and *SulII*.

4.2.6.1 Oligonucleotide primers

Five sets of oligonucleotide primers (amplimers) were used for PCR amplification of five antimicrobial resistance genes that confer resistance to β-lactams, tetracycline and sulfonamides. The oligonucleotide sequences used to amplify resistance genes were synthesized using a DNA synthesizer (3900 ABiosystems, CA USA.) at International Laboratory Research Institute (ILRI) laboratories Oligosynthesis Unit, Kabete, Kenya. The sequences of the primers and expected sizes for PCR-amplified products (amplicons) are shown in Table 4.2. To derive the working concentrations, the primers were dried under vacuum (speed vac), dissolved in 500µl triple distilled water (TDW) and spun for two minutes. Their DNA concentrations were then determined by spectrophotometry, using gene Quent, and the primers diluted to 100 ng/µl in TDW.

Table 4.2: Oligonucleotide sequences of primers used in PCR for amplification of AMR genes

Name	Primer sequence	Amplicon (bp)	Reference
$Bla_{(TEM)}$	5'GCACGAGTGGGTTACATCGA3'-F 5'GGTCCTCCGATCGTTGTCAG3'-R	290	Carlson *et al.*, 1999
Tet(A)	5'GCTACATCCTGCTTGCCTTC3'F 5'CATAGATCGCCGTGAAGAGG3'-R	210	Levy, *et al.*, 1999
Tet(B)	5'TTGGTTAGGGGCAAGTTTTG3'-F 5'GTAATGGGCCAATAACACCG3'-R	659	Levy, *et al.*, 1999
SulI	5'TCACCGAGGACTCCTTCTTC3'F 5'CAGTCCGCCTCAGCAATATC3'-R	822	Sandvang *et al.*, 1998
SulII	5'CCTGTTTCGGACACAGA3'-F 5'GAAGCGCAGCCGCAATTCAT3'R	722	Sandvang *et al.*, 1998
Class1 Integron	5'GGCATCCAAGCACAAGC3'-5'-CS 5'AAGGCAGACTTGACTGAT3'-3'CS	Variable	Sandvang *et al.*, 1998

Key:

R = Reverse primer

F = Forward primer

CS = Conserved segment

4.2.6.1.1 Polymerase Chain Reaction

4.2.6.2.1 DNA template preparation

Two colonies of *E. coli* isolates grown on nutrient agar plates were picked and suspended in 500 µl of distilled water and used as a source of DNA template for PCR.

4.2.6.2.2 PCR conditions and amplification

The PCR was performed using an MJ minicycler (MJ Research Inc.MA USA). The reaction mixture consisted of 12.5µl of 10 x PCR buffer (containing 1 mM each of deoxynucleoside triphosphate mixture, 2.5 mM Mg Cl_2), 0.25 µM each of primers, 3 µl of bacterial suspension, 1 U of Taq DNA polymerase and 8.5 µl of sterile water. The reagents were then thoroughly mixed and 25 µl of mineral oil overlaid to reduce evaporation of the mixture during the amplification process. Preincubation was at 95°C for 5 minutes. Thirty five PCR cycles were run under the following conditions: denaturation at 95°C for 1 minute, primer annealing at 60°C for 30 seconds, and DNA extension at 75°C for 30 seconds in each cycle. After the last cycle, the PCR tubes were incubated for 3 minutes at 72°C and then 4°C till product analysis.

4.2.6.3 Identification of integrons

Since resistance to sulfonamides is characteristic of class 1 integrons, sulfamethoxazole-resistant *E. coli* isolates were screened for the presence of such integrons. The class 1 integron is characterized by the *qacE*1 and *sulI* genes at its 3' conserved segment (CS). Primers located at the 3' conserved segment were used as described by Sandvang and Aarestrup (2000) to characterize the class 1 integrons.

Amplifications were performed with 5 µl of supernatant from bacterial preparations that had been boiled for 5 minutes. The PCR mixture (total volume, 50 µl) included 29.6 µl of H_2O, 5.0 µl of 10x PCR buffer, 5.0 µl of 2 mM deoxynucleoside triphosphates, 1 U of *Taq* polymerase and 25 pmol of each primer. DNA amplification was carried out using the following conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. A sample of 3 µl of the PCR product was verified for size and purity by gel electrophoresis.

4.2.6.4 Detection of amplified DNA products

Ten microlitres of the PCR reaction products were electrophoresed in 1 % agarose gel on horizontal tanks containing 0.5X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 M EDTA) buffer stained with 0.05 % ethidium bromide solution. In preparing the gel, 1 gm of agarose was dissolved in 100 ml of distilled water. The mixture was heated to dissolve the agarose gel completely and then cooled to 55°C before pouring into the horizontal gel tanks. Wells were made in the agarose gel using a teeth comb placed in molten agar and the gel was allowed to set for 30 minutes. The teeth comb was removed and the gel was placed on an electrophoresis tank containing TBE buffer. Ten microlitres of the PCR products were mixed with 3 µl of 6x loading dye and the mixture was loaded onto the wells and allowed to run for 45 minutes at 100 V. PCR products were then visualized on a UV transilluminator in a dark room. The sizes of the PCR products were determined by comparing them with migration of a 100 bp ladder (Gibco, BRL). Photography was done using a digital camera.

4.2.7 Mating studies and conjugation gene transfer

In vitro conjugation tests on transferable antimicrobial resistance was performed according to Walia *et al.* (1987) with modification. Single discrete colonies of each donor isolate and recipient *E. coli* K12F- (nalidixic resistant) were separately subcultured onto tryptic soy broth (5 ml) and incubated at 37°C overnight. The donor and recipient broth cultures were diluted 1:10 in fresh tryptic soy broth and allowed to multiply to the logarithmic phase for 4 hours. The donor and recipient broth cultures were mixed at the ratio of 2:1 respectively and incubated at 37°C for 24 hours. To select transconjugants (progeny of donors and recipient), 3 µl samples were drawn from the overnight cultures and plated onto macConkey agar plates containing 16 µg/ml nalidixic acid + 8 µg/ml tetracycline, 16 µg/ml nalidixic acid + 16 µg/ml streptomycin and 16 µg/ml nalidixic acid + 16 µg/ ml amoxycillin. The plates were incubated at 37°C overnight and thereafter growth on agar plates confirmed presence of transconjugants. The antimicrobial susceptibility testing and genotypic characterization of the transconjugants were carried out as previously described in sections 4.2.5 and 4.2.6, respectively.

4.2.8 Data analysis

The observations from primary examination of animal samples for the isolation of *E. coli* were stored in a database (Microsoft Excel). The AMR were categorized as susceptible, intermediate or resistant as defined by the relevant CLSI breakpoints. Each isolate was identified by the date and place of sampling, species of animal, source (carcass or fecal) and for poultry isolates breed (Exotic or indigenous). All

manipulation of data and evaluation of results were carried out using Microsoft Excel. Multi-drug resistance was defined as resistance to at least two or more antimicrobial agents.

The chi square (χ^2) test was used to assess significant differences in the overall prevalence of antibiotic resistance between the different species of food animals, locations and management regimes. Group means were compared using t-tests. Comparisons of the associations between resistance phenotypes were made using Pearson's chi-square test. The statistical significance was set at a P value of ≤ 0.05.

4.3 RESULTS

4.3.1 Bacterial isolates

Overall, 42 *E.coli* isolates from cattle, 48 from poultry and 114 from pigs were studied. Of these bacterial isolates, 57 were obtained from animals that originated from the Rift Valley (Kajiado, Kericho, Bomet, Uasin-Gishu, Nakuru, West Pokot and Narok), 121 originated from Central province (Kiambu), 16 were obtained from animals brought from Eastern province (Makueni, Kitui and Mwingi) while 10 isolates were from other areas including Nairobi province. Among the poultry isolates 28 were derived from exotic birds while 20 were from indigenous birds.

4.3.2 Susceptibility testing

Of the 204 *E. coli* isolates in this study, about 38 % showed antibiotic resistance of which 26 % were multi-drug resistant. The overall prevalence of resistance is shown in Table 4.3. The highest prevalence of antibiotic-resistant phenotypes was observed for *E. coli* from swine and poultry. Among swine isolates resistance was observed in seven out of the eight antimicrobial agents tested, in six antimicrobial agents for poultry isolates while cattle isolates were resistant to four antimicrobial agents in the test panel.

Approximately 26 % of the isolates from swine were resistant to sulfonamides, 22 % to ampicillin, 22 % to tetracycline, 13 % to streptomycin and 7 % to chloramphenicol. Whilst, 31 % of the isolates from poultry were resistant to sulfonamides, 16 % were resistant to ampicillin, 10 % were resistant to tetracycline, 8 % were resistant to

streptomycin and 2 % were resistant to chloramphenicol. The prevalence of resistance was lowest for cattle isolates. However, 17 % were resistant to ampicillin, 14 % to tetracycline and 2.4 % to sulfonamides. All the isolates regardless of species of origin were sensitive to gentamycin (Table 4.4).

Sixteen (38.1 %) of the 42 *E. coli* isolates from cattle, fifty-four (50.9 %) of the 106 from pigs and twenty one (43.8 %) of the 48 from poultry were fully sensitive to all eight (8) antimicrobials tested. Only 9.4 % of the isolates from pigs, 9.5 % from cattle and 14.6 % from poultry were resistant to a single antimicrobial agent; mainly ampicillin or tetracycline for swine, ampicillin for bovine and sulphonamides for poultry. Figures 4.1 and 4.2 show the proportion (%) of antimicrobial resistance among *E. coli* isolates from food animals.

Overall, 58.6 % of all isolates resistant to a single antimicrobial agent were resistant to ampicillin while 20.7 % and 17.2 % were resistant to tetracycline and sulfonamides respectively.

Some 27.1 % of the *E. coli* isolates from pigs and 29.2 % from poultry were multi-drug resistant (resistant to two or more antimicrobials). In contrast, only 2.4 % of the bovine isolates were multi-drug resistant. Of these multi-drug resistant isolates, 16 different resistance patterns were observed (Table 4.5). The commonest resistance phenotypes were ampicillin, sulphonamides, tetracyclines in swine; sulphonamides and tetracycline in poultry. Moreover, five isolates from pigs and one isolate from poultry showed penta-drug resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline while three isolates were resistant to at least six antibiotics in the test panel.

4.3. 2.1 AMR profiles of carcass and faecal isolates

Among cattle and swine isolates a higher proportion of *E. coli* isolates from carcass (68.4 %) were multi-drug resistant compared to 31.6 % of faecal isolates ($P > 0.05$). However, a significantly higher proportion of isolates from carcass were resistant to ampicillin (65.3 % vs 34.7 %; $P = 0.04$), but not significant for tetracycline (55.6 % vs 44.4 %; $p = 0.25$), streptomycin (62.5 % vs 37.5 %; $P = 0.24$) and sulfonamides (55.2 % vs 44.8 %; $P = 0.41$) compared to fecal isolates. Conversely, a higher proportion of fecal isolates were resistant to chloramphenicol (75 % vs 25 %; $P = 0.19$) and kanamycin (67% vs 33 %; $P = 0.18$) compared to carcass isolates although these differences were not significant.

4.3.2.2. Prevalence of AMR based on sampling locations

The overall prevalence of AMR *E. coli* from food animals is shown in Figure 4.3, while the individual prevalence of AMR in three different provinces of Kenya is shown in Figure 4.4. The prevalence of AMR was lowest in Rift Valley where resistance to tetracycline, ampicillin and sulfonamides was noted; and highest in Eastern province where resistance to ampicillin, streptomycin and sulfonamides was reported. In Central province resistance to all antimicrobial agents except gentamycin was observed. However, the prevalence of chloramphenicol and kanamycin resistance noted was low (< 5 %) and was confined to multi-drug resistant isolates from swine and poultry species.

4.3.2.3 Association between AMR phenotypes

In order to determine whether possible associations exist between the resistance phenotypes found among the *E. coli* isolates and whether the co-appearance of some resistance phenotypes could be confirmed statistically, an analysis of association was done using Pearson's chi-square test. Significant associations ($P < 0.05$) with respect to the occurrence of individual resistance phenotypes among the *E. coli* isolates were detected between tetracycline and chloramphenicol ($r = 0.991$; $p < 0.05$) and tetracycline and kanamycin ($r = 0.806$; $p < 0.05$).

4.3.2.4 AMR in poultry: Intensive versus indigenous management systems

The prevalence of antibiotic resistant *E. coli* isolates from two different groups of poultry representing two different management systems and antibiotic use profiles is presented in Table 4.6. In Intensively reared group (high antibiotic consumption) a higher degree of resistance was found for oxytetracycline (14.2 % versus 5 %) and streptomycin (10.7 % versus 5 %) and chloramphenicol (3.6 % versus 0 %) compared to indigenous chickens ($P > 0.05$). Conversely, higher prevalences of resistance were found in indigenous chickens (low antibiotic consumption group) compared to intensively reared poultry for sulfonamides/ trimethoprim (44 % versus 21.4 %), ampicillin (20 % versus 14 %). Moreover, no resistance was found for kanamycin and gentamycin.

4.3.3. Multiple antibiotic resistance (MAR) index

A total of 194 *E. coli* isolates obtained from food animals from the Rift Valley, Central and Eastern provinces were included in the study. All the isolates were found to have MAR index ranging between 0 and 0.88. The mean MAR index of isolates from Rift Valley, Eastern and Central province were 0.10, 0.18 and 0.16, respectively. Moreover, 17.5 %, 28 % and 43.8 % of the isolates from Rift Valley, Central and Eastern province, respectively had MAR index > 0.2. The mean MAR index for each region is shown in Table 4.7.

The mean MAR index for the various food animals is shown in Table 4.8. The mean MAR index obtained from *E. coli* isolates from bovine was 0.1. Six (6) out of 42 (14 %) isolates had MAR index > 0.2. The mean MAR index for poultry and swine isolates was 0.14 and 0.17 respectively. Morever, 33% and 31 % of the *E. coli* isolates from poultry and swine, respectively had MAR index > 0.2. Among swine, fecal isolates had a higher MAR index (0.21, n = 40) compared to carcass isolates (0.17, n = 57). Conversely, bovine carcass isolates had a slightly higher MAR index (0.1, n = 25) compared to fecal isolates (0.07, n = 17).

4.3.4 Genotypic characterization of AMR *E. coli* using PCR

Selected *E. coli* isolates previously identified phenotypically as resistant to ampicillin, tetracycline and sulfonamides were confirmed by PCR amplification using specific primers. The specifics and proportions of antimicrobial resistance genotypes examined are shown in Tables 4.9 and 4.10.

4.3.4.1 β-lactam resistance genes

Of the 29 ampicillin resistant *E. coli* isolates tested, PCR assay results revealed that 27 (93.1 %) expressed the TEM- β-lactamase genes. Eleven (100 %) out of 11 bovine isolates, nine (100 %) out of nine poultry and seven (78 %) of the nine swine isolates that were resistant to ampicillin expressed the bla_{TEM} gene.

Nine out 25 (36 %) ampicillin resistant *E. coli* isolates that possessed β-lactamase genes came from faecal sources, whereas 16 (64 %) of these isolates originated from carcass samples.

4.3.4.2 Tetracycline resistance genes

Twenty-five tetracycline resistant (Tc^R) *E. coli* were investigated for the presence of tetracycline resistance genes using PCR. Tetracycline resistance was encoded by the presence of a single gene, either *Tet(A)* or *Tet(B)*. Eleven (44 %) isolates carried the *Tet (A)* gene while two (8 %) isolates carried the *Tet(B)* gene. Of the 17 swine isolates, 7 (41.2 %) possessed the *Tet(A)* gene and two (11.8 %) possessed the *Tet(B)* gene. Among the eight poultry isolates tested, four (50 %) expressed the *Tet(A)* gene only. Seven out 13 (54 %) *E. coli* isolates identified with tetracycline resistance genes came from faecal sources while five (46 %) were obtained from carcass samples (Table 4.10).

4.3.4.3 Sulfonamide resistance genes

Nineteen sulfamethoxazole and co-trimoxazole resistant *E. coli* were examined for the presence of sulfonamide resistance genes. Overall, the *SulII* gene was found in 67 % of the isolates, *SulI* in 32 % while 22 % possessed both *SulI* and *SulII* genes. Of the swine isolates, 20 % possessed the *SulI* gene while 80 % possessed the *SulII* gene. Among the poultry isolates 33 % possessed the *SulI* gene while 67 % possessed the *SulII* gene.

Eight out of 12 (67 %) sulfamethoxazole-resistant *E. coli* isolates that possessed either *SulI* or *sulII* genes came from faecal sources, whereas four (33 %) of these isolates originated from carcass samples (Table 4.10).

4.3.4.4 Identification of antibiotic resistance integrons

Of the 20 *E. coli* isolates, a fragment from the class 1 integron 3' conserved region was amplified from 3 (15 %) isolates by PCR (Table 4.9). All the positive isolates were phenotypically resistant to sulfonamide and trimethoprim and also positive for *sulI* gene by colony PCR. These isolates produced amplicons larger than 2 kb. One fecal isolate from swine, phenotypically resistant to ampicillin, chloramphenicol, sulfamethoxazole, trimethoprim, streptomycin and tetracycline (ACSST) possessed an integron of 2.5 kb.

4.3.5 *In-vitro* conjugation tests

Overall, 17 antibiotic-resistant isolates of *E. coli* were examined. Ten (58.8 %) isolates transferred at least one resistance marker to recipient *E. coli* K12 (Table

4.11). Nine (64.3 %) of the tetracycline resistant isolates transferred resistance to tetracycline. Five (45.5%) of the 11 sulfonamide resistant *E. coli* isolates transferred resistance to sulfamethoxazole and co-trimoxazole. Six (42.9 %) of the 14 ampicillin-resistant isolates transferred resistance to ampicillin. Whilst, seven (53.8 %) of the 13 multi-drug resistant isolates transferred resistance to two or more resistance markers to recipient *E. coli* K12.

Fig. 4.1: Multiple antimicrobial resistance (%) among 204 *E.coli* isolates from food animals in Kenya

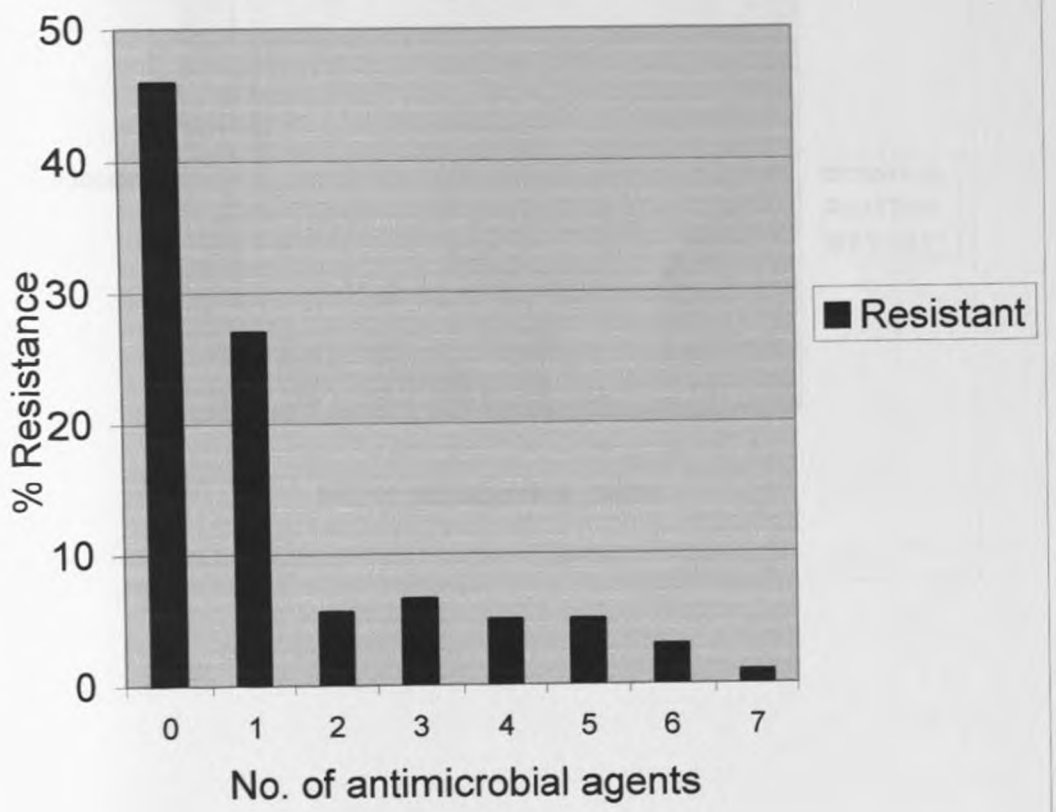


Fig. 4.2: Multiple antimicrobial resistance (%) among 204 *E. coli* isolates of food animal origin from Rift Valley, Central and Eastern province, Kenya

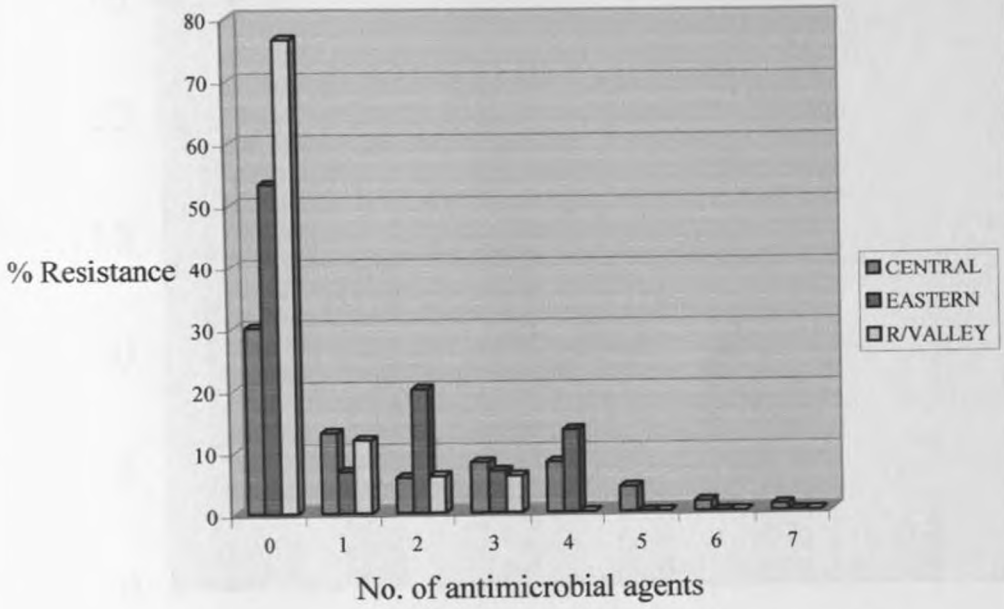


Figure 4.3: The Overall prevalence of AMR among 204 *E. coli* isolates from food animals in Kenya

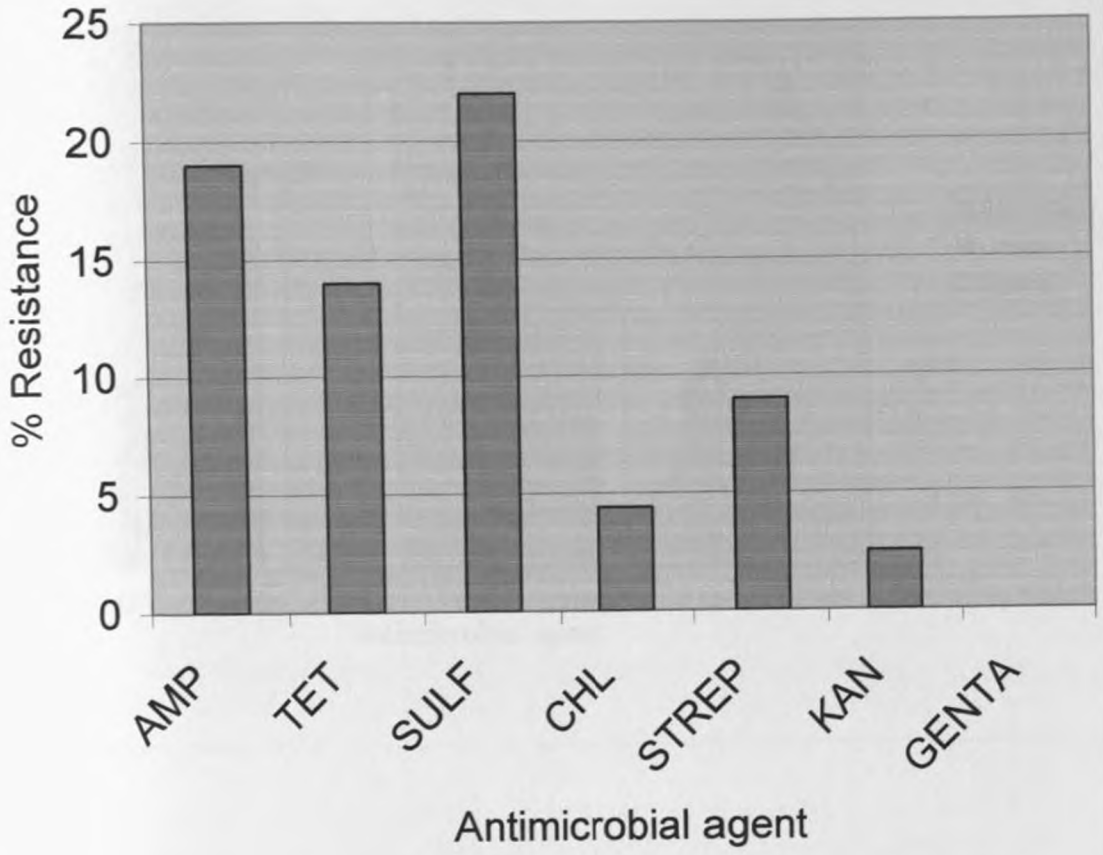
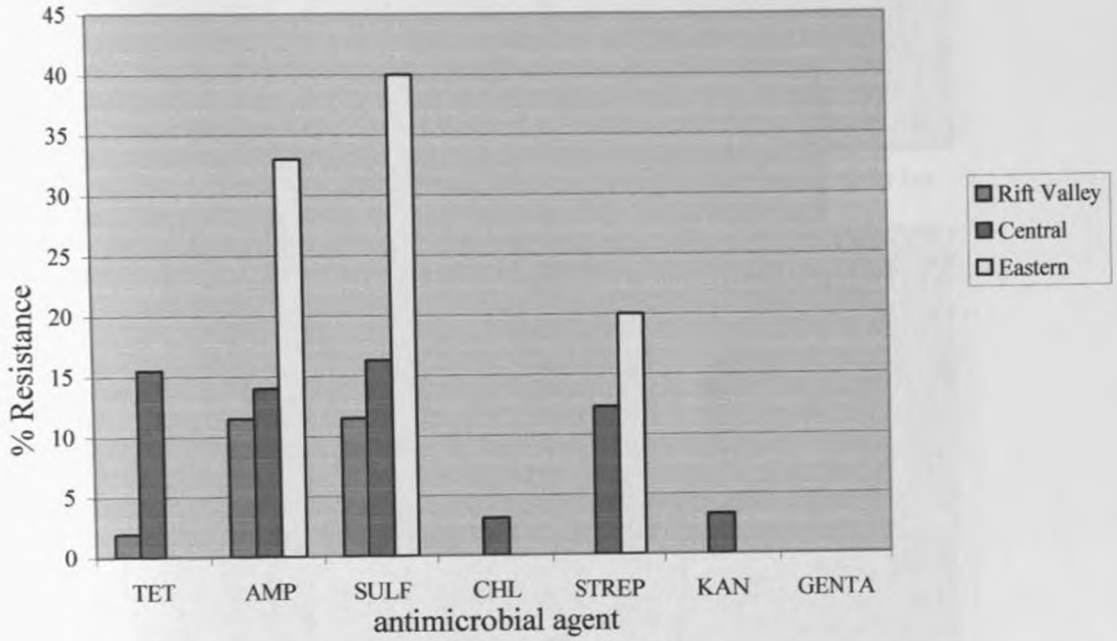


Figure 4.4: The prevalence of AMR among 204 *E.coli* isolates of food animals from Rift Valley, Central and Eastern province, Kenya



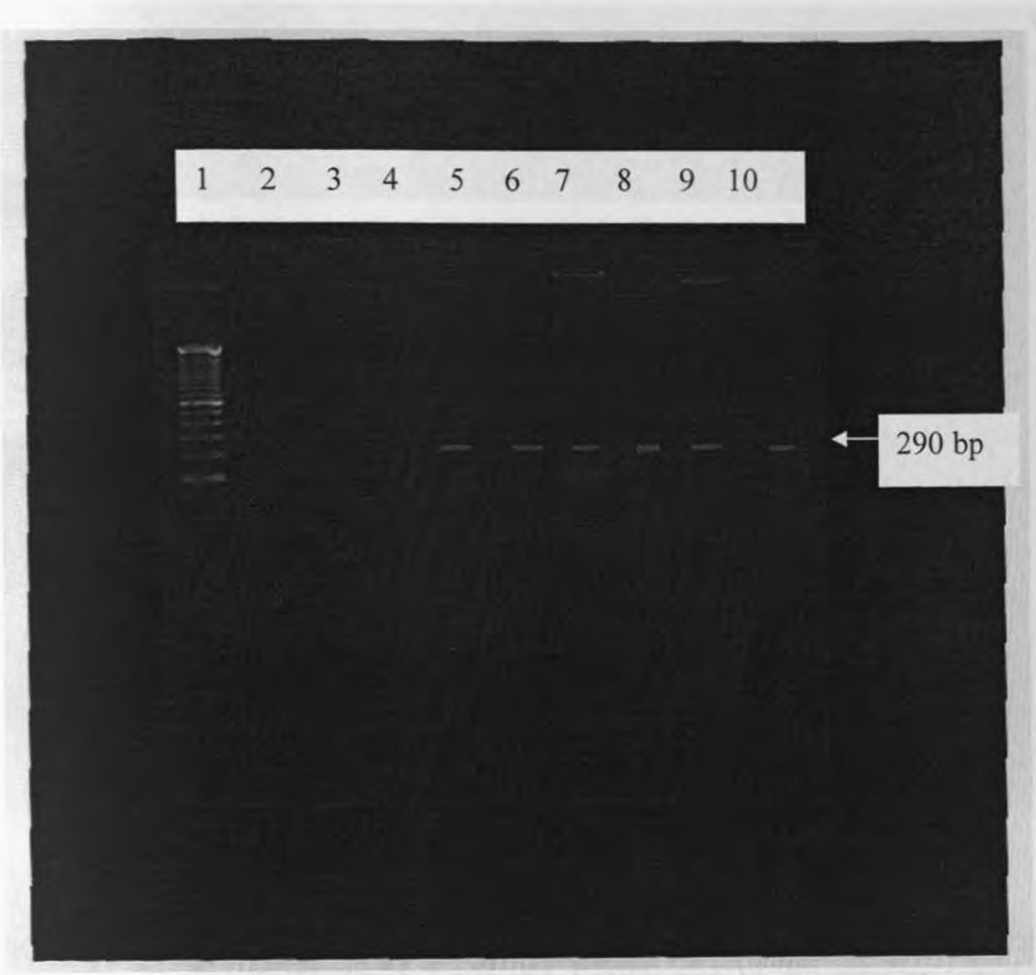


Figure 4.5: Agarose gel electrophoresis of PCR amplification products from *E. coli* isolates using $Bla_{(TEM)}$ primers specific for beta-lactamase production gene (TEM). Lane 1 represents marker (100-bp ladder), lane 2, negative control; lanes 3-10 used bacterial suspension from ampicillin resistant bacteria.

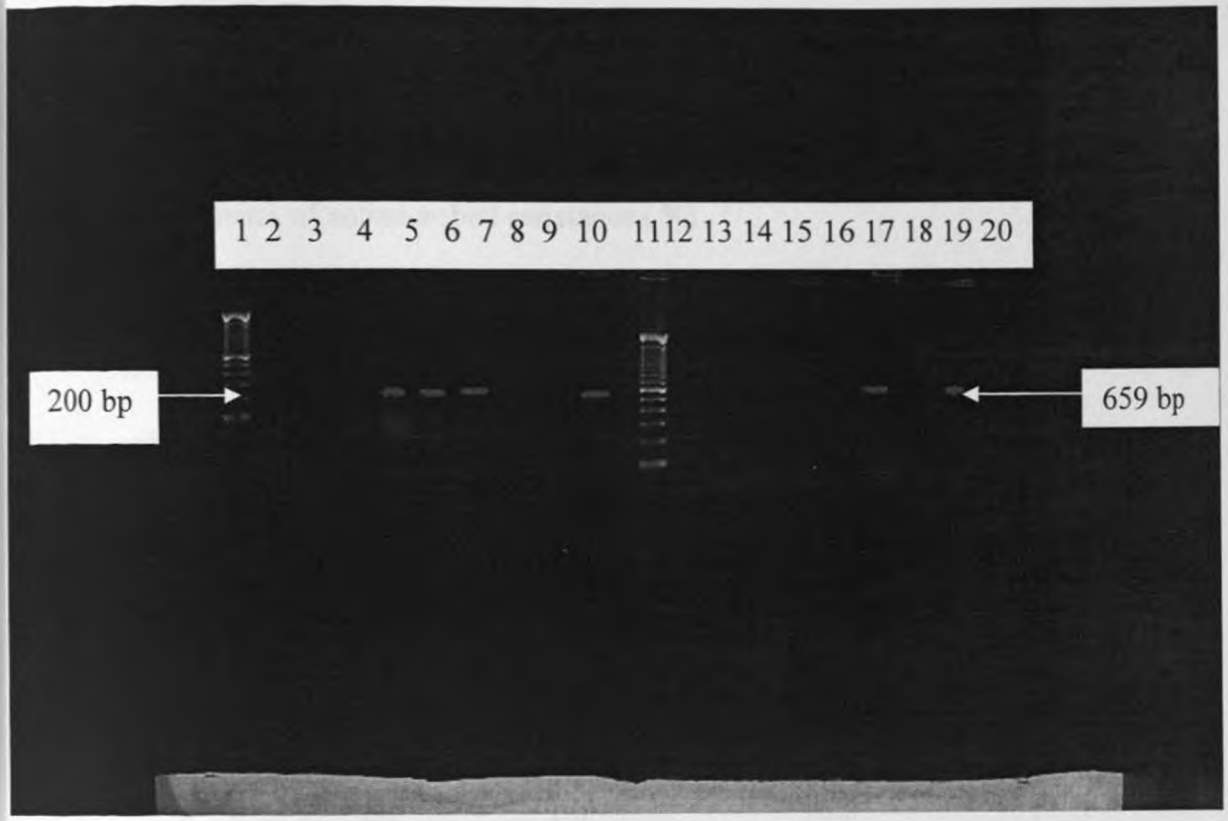


Figure 4.6: Agarose gel electrophoresis of PCR amplification products from *E. coli* isolates using *Tet(A)*[lanes 2-10] and *Tet(B)*[lanes 12-20] primers specific for tetracycline resistance genes. Lanes 1 and 11 represents marker (100-bp ladder); lane 2 and 12, negative controls (drug sensitive); lanes 3-10 and lanes 13-20 used bacterial suspension from tetracycline resistant bacteria.

Table 4.3: The prevalence of antimicrobial resistance for 204 *E. coli* isolates from healthy food animals in Kenya

The prevalence of antimicrobial resistance (%)			
Antibiotic	Resistant	Intermediate	Sensitive
Ampicillin	19.2	10.6	70.2
Tetracycline	14.1	8.8	77.1
Co-timoxazole	21.1	0	78.9
Streptomycin	9.1	16	74.1
Sulfamethoxazole	21.8	1.5	76.7
Chlorampenicol	4.4	1.0	94.6
Kanamycin	2.5	1.0	96.5
Gentamycin	0	0	100

Table 4.4: The prevalence of antimicrobial resistance among *E. coli* isolates from healthy cattle, poultry and swine in Kenya

The prevalence of antimicrobial resistance (%)			
Antimicrobial agent	Cattle (n= 42)	Poultry (n= 48)	Swine (n= 112)
Ampicillin	16.7	16.3	21.7
Tetracycline	14.1	10.0	21.8
Sulfamethoxazole	2.4	30.6	25.7
Co-trimoxazole	2.4	30.6	24.1
Streptomycin	0	8.3	13.2
Chloramphenicol	0	2.0	7.1
Kanamycin	0	0	4.7
Gentamycin	0	0	0

Table 4.5: Resistance Patterns of multi-drug resistant *E. coli* isolates from healthy food animals in Kenya

Resistance patterns	No.of multi-drug resistant *E. coli*		
	Cattle	Poultry	Swine
Am, Tet	0	1	2
Am, St	8	0	0
Am, Sm, Cot	0	1	0
Tet, Sm, Cot	1	3	3
Am, Tet, Kan	0	0	2
Am,Tet, St	0	0	1
St, Sm, Cot	0	0	2
Am, St, Sm, Cot	0	2	0
Tet,St, Sm, Cot	0	1	1
Am, Tet, Sm, Cot	0	0	5
Am, Chl, Tet, Kan	0	0	1
Am, Chl, Tet, Sm, Cot	0	1	0
Am, St, Sm, Cot, Tet	0	1	8
Am, Chl, St, Sm, Cot, Tet	0	1	5
Am, St, Sm, Cot, Tet, Kan	0	0	1
Am, Chl, St, Sm, Cot, Tet, Kan	0	0	2

Key: Am = Ampicillin, Tet = Tetracycline, St = Streptomycin, Sm = Sulfamethoxazole, Cot = Co-trimoxazole, Chl = Chloramphenicol, Kan = Kanamycin.

Table 4.6: The prevalence (%) of resistant *E. coli* among poultry isolates from intensively reared and indigenous management systems.

Antimicrobial agent	Intensively reared birds (N = 28)	Indigenous birds (N = 20)	P-value
Oxytetracycline	14.2	4	0.382
Chloramphenicol	3.6	0	0.355
Sulfamethoxazole/ Trimethoprim	21.4	44	0.208
Ampicillin	14.3	20	0.500
Streptomycin	10.7	5	0.413
Kanamycin	0	0	-
Gentamycin	0	0	-

Table 4.7: The overall mean MAR index of *E. coli* isolates of food animal origin from selected regions in Kenya

Province	Mean MAR Index	No.of isolates (MAR index >0.2)	% (MAR Index >0.2)
Rift Valley	0.10	10	17.5
Central	0.16	34	28.1
Eastern	0.18	7	43.8

Table 4.8: The overall mean MAR index of *E. coli* isolates from various food animals in Kenya

Province	Mean MAR Index	No.of isolates (MAR index >0.2)	% (MAR Index >0.2)
Bovine	0.10	6	14.3
Porcine	0.17	16	30.5
Poultry	0.14	7	33.3

Table 4.9: Genotypic analysis of AMR *E. coli* isolates from food animals in Kenya, 2001-2002

Antimicrobial agent	No.of isolates	Genotypic profiles	
		Genotype	Percentage (%)
Tetracycline	25	*Tet(A)*	44
		Tet(B)	8
Ampicillin	29	Bla_{TEM}	93
Sulfamethoxazole	20	*SulI*	24
		SulII	71
		SulI & SulII	24
Sulfamethoxazole	20	Class 1 integrons	15

Table 4.10: The distribution of some AMR genes among *E. coli* isolated from carcass and fecal samples from food animals

Genotype	Number of Isolates	Fecal	carcass	P-value
		No. (%)	No. (%)	
$Bla_{(TEM)}$	25	9 (36)	16 (64)	0.04
SulI,SulII	12	8 (67)	4 (33)	0.04
Tet(A),Tet(B)	12	7 (58.3)	5 (41.7)	0.15

Table 4.11: Resistance transfer profiles for 17 resistant *E. coli* isolates from food animals

Identification	Origin	Resistance pattern	Phenotype transferred
EC 1	Poultry	Tet Cot Sm	Tet
EC 2	Poultry	Tet	-
EC 3	Poultry	Am Tet	-
EC 4	Poultry	Am Chl Tet Cot Sm	Sm Cot
EC 5	Poultry	Am Tet	Tet
EC 6	Poultry	Am Chl Cot Sm Tet	Am Tet
EC 7	Bovine	Am Tet Sm Cot	-
EC 8	Bovine	Am	-
EC 9	Bovine	Am	-
EC 10	Bovine	Am	-
EC 11	Swine	AmTet Sm Cot	-
EC 12	Swine	Tet Sm Cot	Tet Sm Cot
EC 13	Swine	Am Chl Tet Sm Cot	Am Chl Tet Sm Cot
EC 14	Swine	Am Tet Sm Cot	Am Tet Sm Cot
EC 15	Swine	Am Sm Tet	Am Tet
EC 16	Swine	Am Tet Sm Cot	Am Tet Sm Cot
EC 17	Swine	Amp Chl Tet Sm Cot	Am Tet

Key: Am = Ampicillin, Tet = Tetracycline, Chl = Chloramphenicol, Sm = Sulfamethoxazole, Cot = Co-trimoxazole.

4.4 DISCUSSION

4.4.1 Antimicrobial resistance in indicator bacteria

4.4.1.1 Phenotypic resistance in *Escherichia coli*

Escherichia coli are the most predominant enterobacteriaceae in the gut of domestic animals (Atlas, 1984; Honda, 1992). In this study *E. coli* was isolated in 70.8 % of the animals that were sampled. However, the proportion of *E. coli* recovery differed among species i.e. 57.3 %, 76.8 % and 80.2 % in cattle, poultry and swine, respectively.

The intestinal tract of animals and humans forms a reservoir in which resistant bacteria and resistance genes can accumulate. Consequently the prevalence of resistance in the intestinal flora can therefore be used to estimate and predict resistance in pathogens (Lester, 1990).

In the present study the prevalence and degree of resistance of *E. coli* (considered an indicator bacteria) in fecal and carcass samples from food animals showed resistance to commonly prescribed antimicrobial agents. These findings demonstrated differences in animal populations, animal production systems and antimicrobial usage between the different regions in Kenya. The observations of this study are in agreement with those of Guerra *et al.* (2001) who reported antimicrobial resistance in German *E. coli* isolates fom poultry, swine and cattle. Other studies have reported higher levels of antimicrobial resistance, for instance, Al-Ghamdi *et al.* (1999) compared the prevalence of antibiotic-resistant *Escherichia coli* isolates from faecal samples from poultry industry workers, human patients and healthy chicken in Saudi

Arabia. They reported resistance rates of *E. coli* isolates from chickens to ampicillin, chloramphenicol, gentamicin, tetracycline and co-trimoxazole ranged from 57% to 99 %; significantly higher than those isolated from patients (range 21.9% - 71.4%) and workers (range 35% - 71.8%). However, for drugs not used in poultry, such as amoxicillin + cluvalanate, ceftazidime and nitrofurantoin, resistance rates (range 0% - 2.6%) of chicken isolates were significantly lower ($P < 0.05$) than those of patient isolates (range 8.7% - 30%). In Ireland, prevalence rates of 47.6 % to 68 % for sulfonamides, 55 % to 62.5 % for tetracycline and 23 % to 25 % for ampicillin were reported from *E. coli* isolates from poultry flocks (Cormican *et al.*, 2001).

Overall, 26 % of *E. coli* in this study demonstrated resistance to at least two or more of the antibiotics tested. The same level of resistance was reported earlier in poultry isolates in Kenya (Kariuki *et al.*, 1997). In the present study multi-drug resistance was much more common among isolates from poultry and swine (29.2 % and 27.1 %, respectively) than from cattle (2.4 %). In agreement with this study, Guerra *et al.* (2001) reported a multi-drug resistance rate of 32 % in German isolates and that resistance was significantly higher in isolates from poultry and swine than from cattle. Moreover, trends in the prevalence of antimicrobial resistance among isolates in this study were similar to those reported in Denmark (Emborg and Heuer, 2003). However, the prevalence rates of resistance in this study are lower for swine isolates and higher for cattle and poultry when compared with those in Denmark. Higher prevalence rates of AMR have also been reported among poultry isolates in Netherlands (van den Bogaard *et al.*, 2001). These findings may be explained by differences in animal populations, animal production systems and by extension

antimicrobial prescription patterns between Kenya, Netherlands and Denmark. For instance total antimicrobial consumption in food animals in Denmark during 2002 was 102, 000 kg and pig production accounted for 80 % of the total antibiotic consumption (Emborg and Heuer, 2003). In the Netherlands total antimicrobial consumption in food animals in 2000 was approximately 300, 000 kg and poultry production amounted up to 20 % of total antimicrobial consumption (van den Bogaard, 1997) while the mean annual antimicrobial consumption in food animals in Kenya is approximately 15, 000 kg (Mitema *et al.*, 2001). Relative to animal production figures, the amount of meat produced in Denmark in 2000 amounted to 1,840 million kg of bacon and 169 million kg of beef while in Kenya beef production amounted to 250 million kg (FAO, 2001).

In Denmark resistance to ampicillin, sulfonamides and streptomycin continue to increase in indicator *E. coli* from pigs. These observations coincide with increased consumption of penicillins, sulfonamides and tiamulin in pigs (Emborg and Heuer, 2003).

Despite a ban on the use of chloramphenicol in food animals in Kenya, an increase in the rate of chloramphenicol resistance among poultry and swine isolates was observed. Other investigators have also observed the persistence or an increase in the rate of chloramphenicol resistance among *E. coli* isolates from swine (Bischoff *et al.*, 2002; Lanz *et al.*, 2003; Maynard *et al.*, 2003; Teshager *et al.*, 2000) and other animal species (Werckenthin, *et al.*, 2002). In this study, resistance to chloramphenicol was closely associated with resistance to tetracycline. The direct use of antimicrobials can drive the co-selection of resistance genes. For example, the use of injectable

oxytetracycline in cattle receiving chlortetracycline in their feed was associated with an increase in the incidence of resistance to chloramphenicol and sulfisoxazole (O'Connor *et al.*, 2002). This suggests that the increased use of tetracycline or sulfamethoxazole-trimethoprim in pig and poultry production in the past could have co-selected for resistance to chloramphenicol, thus explaining the increase in chloramphenicol-resistant isolates, even though this antimicrobial agent has never been used in food animal production.

Interestingly, 5 % of the swine isolates were resistant to kanamycin, even though this antimicrobial agent was not used in the Kenyan food animal industry during the study period. It is likely the result of the cross-resistance caused by most of the aminoglycoside resistance genes (Werckenstein *et al.*, 2002).

4.4.1.2 Genotypic analysis of AMR in *E. coli* from food animals

Although treatment of enteric *E. coli* infection in food animals commonly includes the use of antimicrobials (Bertchinger and Fairbrother, 1999; Hampson, 1994), relatively few studies have been directed toward the characterization of the genotypic resistance profiles of *E. coli* strains isolated from animals. One study characterized the tetracycline and sulfonamide resistance gene profiles of *E. coli* strains isolated from animals and humans (Lanz *et al.*, 2003), while a second one (Sandvag and Aarestrup, 2000) characterized the aminoglycoside resistance gene profiles of porcine and bovine *E. coli* isolates. This is the first study in Kenya to characterize *E. coli* strains isolated from cattle, pigs and poultry for detection of acquired resistance to three different antimicrobial agents; namely tetracyclines, penicillins and sulfonamides.

Tetracyclines are the most widely used antimicrobial agents in Kenya as well as Danish food animal production (Mitema *et al.*, 2001; Emborg and Heuer, 2003) accounting for 56 % and 26 %, respectively of total antibiotic usage. Resistance to tetracycline is conferred by one or more of the 30 currently described *tet* genes, which encode one of three mechanisms of resistance: an efflux pump, a method of ribosomal protection, or direct enzymatic inactivation of the drug (Chopra and Roberts, 2001). Efflux mechanisms appear to be more abundant among gram-negative microorganisms, while ribosomal protection mechanisms are more common among gram-positive organisms (Chopra and Roberts, 2001). Generally speaking, the rapid spread of tetracycline resistance among bacteria is due to the localization of *tet* genes on plasmids, transposons, and integrons (Chopra and Roberts, 2001; Levy *et al.*, 1976b; Roberts, 2003).

While several studies have examined tetracycline resistance among bacteria, most have employed clinically isolated bacteria (Arzese *et al.*, 2000; Guillamume *et al.*, 2003) These studies have not documented the presence and types of *tet* genes that are present in natural (non-clinical) populations of bacteria especially from healthy food animals. Recently, Sengeløv *et al.* (2003) examined 100 *E. coli* isolates from both healthy and diseased animals for the presence of five *tet* resistance determinants while Blake *et al.* (2003) used PCR to examine 200 tetracycline-resistant commensal *E. coli* strains for seven *tet* genes.

In the current study *tet(A)* was found to be the most predominant tetracycline-resistant gene. In contrast to the observations of this study, *tet(B)* was reported to be the most

prevalent resistance gene among tetracycline-resistant *E.coli* from food animals in Germany. Similarly, Maynard *et al.* (2003) reported that among the tetracycline-resistant isolates from Canada, the *tet*(B) gene was largely predominant until 1994, when two other closely associated tetracycline resistance genes, *tet*(A) and *tet*(C), became dominant during the period of 1995 to 2000. Similar results were observed in another study in pigs from three herds with different histories of antimicrobial exposure, in which *tet*(B) was predominant in two of the herds exposed to antimicrobials, and whenever present, *tet*(A) and *tet*(C) were also found together (Lee *et al.*, 1993). In agreement with our study, Lanz *et al.* (2003) showed that the *tet*(A) gene alone is the most prevalent *tet* gene among *E. coli* isolates from pigs with diarrhea or enterotoxemia. In the United States, Bryan *et al.* (2004) reported that the most common tetracycline resistance determinants in *E. coli* from food animals are *Tet(B)* (63%) and *Tet(A)* (35%). In contrast, Sengeløv *et al.* (2003) reported that 71 % and 25 % of 100 *E. coli* isolates from the diseased and healthy animals contained *tetA* and *tetB*, respectively. The modes of action as well as the specificities of certain antimicrobial enzymes could exert positive selection pressure and contribute to the emergence of new genes over time. For example, class A, B, and C tetracycline resistance determinants are efflux pumps with different specificities. Most of the efflux proteins confer resistance to tetracycline but not to the minocycline or glycylcycline antimicrobial group. In contrast, the *tet*(B) gene encodes an efflux protein which confers resistance to tetracycline, doxycycline, and minocycline but not glycylcycline (Petersen *et al.*, 1999). These specificities correlate with the emergence of the diverse distribution of different tetracycline resistance genes over time.

In the present study, higher levels of sulfonamide resistance were reported in *E. coli* isolates from poultry (30.6 %) and swine (25.7 %). Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes *sulI* and *sulII*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (Enne *et al.*, 2001). The *sulI* gene is normally found linked to other resistance genes in class 1 integrons, while *sulII* is usually located on small nonconjugative plasmids (Skold *et al.*, 2000) or large transmissible multiresistance plasmids (Enne *et al.*, 2001). Similarly, *sulII* was found to be the most prevalent gene encoding sulfonamide resistance among food animals in Kenya. In contrast with the observations of this study, Lanz *et al.* (2003) reported the predominance of *sulI* among pig isolates. Similar observations were made in Canada by Maynard *et al.* (2003). In agreement with this study, Guerra *et al.* (2001) found *sulII* the most predominant gene among cattle, swine and poultry *E. coli* isolates from Germany.

The *sulI* and *sulII* genes encode dihydropteroate synthase enzymes with different sensitivities (K_i values) for *p*-aminobenzoic acid, which is implicated in bacterial folic acid biosynthesis. The enzyme encoded by *sulII* discerns the normal *p*-aminobenzoic acid substrate from the inhibitor, the sulfonamides (Skold, 2001). Morever, this study indicates that 24 % of sulfonamide resistant isolates possessed both *sulI* and *sulII* genes. It is possible that strong selection pressures provided by environments containing elevated levels of sulfonamide lead to the acquisition of more than one resistance gene in a given strain due to their prevalence in the environment, rather than to a selective advantage.

The dissemination of multi-drug resistant bacteria through food has important public health implications. The ability of bacteria to acquire antibiotic resistance genes and subsequently spread them to different bacterial species is well known (Hall, 1997). Integrons, mobile DNA elements have been associated with the transfer of resistance and often contain one or more linked antimicrobial resistance genes (Hall *et al.*, 1993). The integrons identified in this study were similar to those reported in pathogenic Enterobacteriaceae (Ridley and Threlfall, 1998; Sandvang *et al.*, 1998; White *et al.*, 2001). These integrons were associated with sulfonamide and streptomycin resistance, suggesting that integrons may play a crucial role in the dissemination of resistance among *E. coli* bacteria. However, class 1 integrons and their associated gene cassettes did not entirely account for the observed resistance phenotypes indicating that other resistance mechanisms were also involved.

Resistance genes are associated with mobile DNA such as plasmids, transposons, and integrons, which facilitate resistance gene distribution (Jacoby, 1994; Tenover and Rasheed, 1998). Some of the isolates (15%) in this study possessed a class 1 integron. Interestingly, these isolates were multi-drug resistant and notably resistant to sulfonamides and trimethoprim. Because integrons are characterized by their integration of many different gene cassettes between insertion sites in the VR, site-specific insertion represents another mechanism driving the evolution of the plasmids and transposons of gram-negative bacteria. Most class 1 integrons in the *E. coli* isolates in this study were larger than 2 Kb. Similar to our observation, an increase in the VR length was observed in *E. coli* strains isolated in Canada during the same period (Maynard et al., 2004), a phenomenon also observed by Schmitz *et al.* (2001).

They showed that the VRs of the class 1 integrons in human *E. coli* strains isolated in 1993 ranged from 0.65 to 1.8 kb and that those in human *E. coli* strains isolated in 1999 ranged from 0.75 to 3 kb, suggesting an accumulation of gene cassettes inserted in the class 1 integron among the ETEC isolates due to selection by antimicrobial agents.

In this study resistance to ampicillin, tetracycline, sulfonamides and chloramphenicol were co-transferred via conjugation in ten (58.8 %) of antibiotic-resistant *E. coli* to recipient *E. coli* K12. These results are in agreement with earlier reports that indicate that multi-drug resistant *E. coli* isolates transfer of resistance to *E. coli* K12 ranges from 24 % to 76 % (O'Brian *et al.*, 1993; Niljesten et. al., 1996). About 93 % of ampicillin resistant *E. coli* isolates in this study possessed β-lactamase (TEM) genes that code for β-lactamase production. This finding agrees with reports from previous studies from both Kenya (Kariuki *et al.*, 1997) and developed countries (Ling *et al.*, 1994; Maynard *et al.*, 2003; Guerra *et al.*, 2001) reporting that 90 % of resistance to β-lactam antibiotics is through production of β-lactamases-enzymes capable of hydrolyzing and inactivating the β-lactam antibiotics. Resistance to β-lactam antibiotics presents a major challenge as it is one of the most widely prescribed antibiotic in humans for the treatment of bacterial infections in Kenya (Kariuki *et al.*, 1997; Mitema and Kikuvi, 2004) and accounts for 28 % of the total amount of therapeutic antibiotics used for food animals in Denmark (Emborg and Heuer, 2003). Therefore the evolution of TEM β-lactamase genes emphasizes the importance of and

need for surveillance over time and space, in order to monitor the spread of AMR and to determine their potential impact on antimicrobial therapy.

In summary, by examining the frequency and distribution of AMR genes and integrons among diverse natural *E. coli* populations present in different animal species, a picture of the selection pressures in the various host animals can be inferred. Not only did those food animals that presumably had continuous exposure to antimicrobial agents have a higher prevalence of multi-drug resistant *E. coli* isolates, but also those isolates carried a greater diversity of resistance genes. This suggests that human activity provides environments that select for resistant strains and encourages the transfer of genetic information from unrelated bacterial species. Although this study examined only nonclinical *E. coli* isolates, the prevalence of AMR genes among these unrelated bacteria, and direct evidence of horizontal gene transfer, suggests that these same resistance determinants may also be present in animal and human pathogens. Moreover, the high prevalence of multi-drug resistant *E. coli* in food animals reflects a reservoir of resistance in animals that can be transmitted to humans. Even though the occurrence of resistant bacteria in food animals is low compared to the human reservoir, mobile resistance genes (as demonstrated by this study) may be transferred horizontally to pathogenic bacteria and ultimately lead to teatment failure.

This study emphasizes the need to reduce the prevalence of antibiotic resistant bacteria present in food animals. Therefore a national surveillance programme to monitor the emergence of AMR and to fast-track antimicrobial consumption in food

animals should be established. In addition policies and drug regulations that encourage more appropriate and rational use of antimicrobial agents in food animals should be established and adopted as long-term interventions for the containment of AMR.

In conclusion, MDR (upto six antimicrobial agents) was mainly observed among *E. coli* isolates from swine and chickens. Choramphenicol resistance driven by oxytetracycline co-selection was observed among swine and poultry isolates. *Tet(A)* and *SulII* genes are the predominant genotypes encoding for tetracycline and sulfonamide resistance, respectively in Kenya..

CHAPTER FIVE

5.0 ASSOCIATIONS BETWEEN ANTIMICROBIAL USE IN FOOD ANIMALS AND ANTIMICROBIAL RESISTANCE IN *E. COLI* FROM ANIMALS AND NON TYPHOIDAL *SALMONELLAE* FROM HUMANS

5.1 INTRODUCTION

A global trend of increasing antimicrobial resistance, but with wide variations at national levels, is well documented in the literature (Livermore, 2003). In this regard, the World Health Organization (WHO) and the European Commission have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control (WHO, 2000; CDC, 2000; EC, 2000).

Strong evidence supports an association between antibiotic use and resistance in hospitals (McGowan, 1983; Harbarth, *et al.*, 2001). For instance, an ecologic study linked penicillin resistance in *Streptococcus pneumoniae* with β-lactam and macrolide use in 12 European countries (Bronzwaer, *et al.*, 2002). Recently, McCormick *et al.* (2003) have shown that variation in *pneumococcal* resistance in the United States is best explained by geographic variation in antibiotic selection pressure, rather than by clonal dynamics.

A major risk factor for the development of resistance is thought to be inappropriate use of antimicrobial drugs especially in food animals. Most studies that have investigated the relationship of antimicrobial use and antimicrobial resistance have been undertaken in hospital, multicenter, or country settings (Fridkin *et al.*, 1999;

Goettsch *et al.*, 2000). For infections with penicillin-nonsusceptible *S. pneumoniae* (PNSP), studies have demonstrated that at the individual level, previous use of beta-lactam antibiotics such as penicillin is an important risk factor (Tan *et al.*, 1993; Nava *et al.*, 1994; Deeks *et al.*, 1999). Studies on carriage of PNSP in children have shown that sulfamethoxazole-trimethoprim (co-trimoxazole) and macrolides have also been associated with selection of PNSP (Arason *et al.*, 1996; Melander *et al.*, 2000). Therefore, consumption of beta-lactam antibiotics, co-trimoxazole, or tetracyclines in a given geographic region may be proportional to antimicrobial resistance.

Studies have been carried out to evaluate the correlation between the antimicrobial agents used to treat persons with *Salmonella* infections and antimicrobial-resistance among human *Salmonella* isolates. Information provided from surveys conducted by CDC within selected counties in the United States in 1985 (McDonald *et al.*, 1987), 1990 (Lee *et al.*, 1994) and 1995 (Herikstad *et al.*, 1997a) indicated that the proportion of persons with *Salmonella* infection receiving an antimicrobial agent who were treated with ampicillin declined from 60% in 1985 to 5% in 1995, while the proportion of isolates resistant to ampicillin steadily increased. The proportion of persons treated with sulfonamides remained constant while trimethoprim-sulfamethazone resistance increased slightly. Most significantly, the proportion of patients with salmonellosis treated with ciprofloxacin or extended-spectrum cephalosporins markedly increased without an emergence of resistance to either of these antimicrobial agents among human *Salmonella* isolates (Herikstad *et al.*, 1997a,b). Taken together, these data suggest there is little correlation between the antimicrobial agents used in persons with *Salmonella* infections and development of

antimicrobial resistance among human *Salmonella* isolates. If human antimicrobial use is not associated with the increasing antimicrobial resistance seen among *Salmonella* isolates, what is the cause of the increasing prevalence of antimicrobial-resistance observed among *Salmonella* isolates?

Several outbreak investigations of antimicrobial-resistant *Salmonella* infections in humans have combined epidemiologic fieldwork and laboratory subtyping techniques to trace back antimicrobial-resistant *Salmonella* through the food distribution system to farms, and antimicrobial use on the farms was found to be associated with the antimicrobial resistance (Holmberg *et al.*, 1984; Tacket *et al.*, 1985; Spika *et al.*, 1987; Angulo *et al.*, 2000; Busani *et al.*, 2004; Gorman and Adley, 2004). In one investigation, hamburgers contaminated with antimicrobial-resistant *Salmonella* were traced, using a unique plasmid profile, from supermarkets, through meat processing, to beef cattle which had been fed subtherapuetic antimicrobial agents (Holmberg *et al.*, 1984). In another investigation, approximately 1,000 persons were infected by hamburger contaminated with antimicrobial-resistant *Salmonella* serotype Newport with an unusual marker - chloramphenicol resistance. Chloramphenicol-resistant *S. Newport* was traced from ill persons, through processing, to dairy cattle on farms where chloramphenicol had been used (Spika *et al.*, 1987). Recent studies have documented a ceftriaxone-resistant salmonella infection in a child that was acquired through exposure to cattle in the United States of America (Fey, 2000). Although such investigations provide considerable insight into the complexity of *Salmonella* transmission, they suffer from the limitations of epidemiological studies. However, when combined with other lines of evidence, such investigations illustrate the

potential human health consequences of the use of antimicrobial agents on food animal production.

The continued emergence of *S. Typhimurium* DT104 with decreased susceptibility to fluoroquinolones in humans in the United Kingdom provides increasingly strong evidence that antimicrobial-resistance among *Salmonella* isolates from humans result from the use of antimicrobial agents in food animals. Decreased susceptibility to fluoroquinolones among human *Salmonella* isolates was rare in the United Kingdom prior to 1993, despite the widespread use of ciprofloxacin in humans since 1987. Following the 1993 approval and widespread use of enrofloxacin in veterinary medicine, human *Salmonella* isolates (and food animal isolates) with decreased susceptibility to ciprofloxacin rapidly emerged beginning in 1994 (Threfall *et al.*, 1997). The number of cases of infection with serotype typhimurium DT104 has since increased in many countries (Calvert *et al.*, 1998; Izumiya *et al.*, 1999; Baggessen *et al.*, 2000; Aarestrup *et al.*, 2003).

Non-Typhoidal *Salmonella spp* (NTS) are an important cause of infection in both human and animals, and there is an increasing prevalence of multi-drug resistance (MDR) in Kenya (Graham *et al.*, 2000; Kariuki *et al.*, 2002). However, there are no data from developing countries including Kenya providing evidence that antimicrobial-resistance among *Salmonella* isolates in humans results from the use of antimicrobial agents in food animals. Therefore, this study utilized an ecologic study design to examine the correlation between use of relevant antibiotics in the food

animal setting and the proportion of MDR among *E. coli* isolates from food animals and NTS isolates from humans.

The aim of this study was to investigate the association between antimicrobial use in food animals and reported prevalence rates of AMR in Kenya using a correlation matrix. The study sought to identify needs for specific interventions and to provide a basis for policy recommendations for animal and public health.

5.2 MATERIALS AND METHODS

5.2.1 Antimicrobial Use Data

National antimicrobial sales data for food animals from 1995 to 1999 were obtained from published information (Mitema *et al.*, 2001) while data from 2000 to 2001 was obtained from the results of the present study (extracted from Chapter Three).

5.2.2 Antimicrobial Resistance Data

An ecologic study design was used, which allows measurement of the effect of total and individual antibiotic exposure on antimicrobial resistance. *E. coli* in food animals and Non typhi *Salmonella spp* in humans were chosen as indicator organisms for those effects. Using PUBMED and local literature search systems, a search of national surveillance studies on AMR published in English from 1995 to 2001 was performed. Data from the present study (extracted from Chapter four) was included for the period from 2000 to 2001.

5.2.3 Statistical Analysis

The proportion of resistance among *E. coli* and Non-typhi *Salmonellae* isolates from food animals and humans, respectively reported during 1996-2001were included. Least-square linear regression analysis was used to determine the relationship between antimicrobial use (expressed in tonnes) and the log odds of prevalence of resistance (R/ 1-R).

The drug-use factors thought to be associated with antimicrobial resistance were modelled using multivariate regression analysis. Generalised linear models (McCullagh and Nelder, 1989) were performed on drug use-level factors, using the log odds of prevalence of multi-drug resistance (% MDR) of *E. coli* as the independent variable and antimicrobial use indices (i.e total consumption [TC], oral consumption [OC], parenteral consumption [PC] and prophylactic use [PU]) as outcome variables. The coefficient of determination (r^2) was used to determine the strength of the association. Spearman Rank correlation coefficient (r) and its corresponding p value were used to determine the degree of correlation and the level of significance ($p \leq 0.05$), respectively.

5.3 RESULTS

5.3.1 Antimicrobial usage correlations

Table 5.1 shows the consumption of antimicrobial agents used by food animals in Kenya for the period, 1996-2001. The use of a number of different antimicrobial agents was interrelated. The results of correlation analysis of total antimicrobial consumption with individual antimicrobial agents were as follows: tetracycline consumption was positively correlated with total antimicrobial consumption ($r = 0.371$; $p > 0.05$) whilst, β-lactams consumption was positively correlated with total antimicrobial consumption ($r = 0.486$; $p > 0.05$). The consumption of macrolides was also positively correlated with total antimicrobial usage ($r = 0.395$; $p > 0.05$). With respect to antimicrobial classifications, the relationship between various antimicrobial agent usage were as follows: tetracycline consumption was positively correlated with sulfonamide usage ($r = 0.314$; $p > 0.05$) and negatively correlated with beta-lactam consumption ($r = - 0.543$; $p > 0.05$), aminoglycoside consumption ($r = -0.429$; $p > 0.05$) and fluoroquinolone consumption ($r = -0.543$; $p > 0.05$). β-lactams usage was not significantly associated with aminoglycoside consumption ($r = 0.771$; $p = 0.006$) while quinolone consumption was inversely correlated with sulfonamide consumption ($r = -0.771$).

5.3.2 Antimicrobial resistance correlations (food animals)

Table 5.2 shows trends in AMR among *E. coli* isolates from food animals in Kenya during the period between 1996- 2001. From this data the relationship between the

prevalence of resistance to ampicillin, tetracycline and sulfonamide was determined. Findings of the correlation analysis were as follows: a significant association was found between both tetracycline resistance and ampicillin resistance ($r = -0.994$; $p < 0.05$) and tetracycline resistance and sulfonamide resistance ($r = -0.972$; $p < 0.05$). Moreover, there was a significant association between ampicillin resistance and sulfonamide resistance ($r = 0.992$; $p < 0.05$).

5.3.3 Antimicrobial usage and AMR correlations

5.3.3.1 Food animals

Figure 5.1 shows trends in resistance to selected antimicrobial agents for *E. coli* and total antimicrobial consumption in food animals; showing a clear association between antimicrobial resistance and antimicrobial usage. In 1998, there was a reduction in tetracycline resistance following a reduction in total antimicrobial consumption during 1996-97 while ampicillin and sulfonamide resistance increased following an increase in total antimicrobial consumption. Figure 5.2 shows trends in tetracycline resistance among *E. coli* isolates from food animals and the consumption of tetracycline. There was a direct relationship between tetracycline resistance and tetracycline consumption.

Observations of the correlation analysis of antimicrobial usage in food production with antimicrobial resistance prevalence among *E. coli* from food animals were as follows: total antimicrobial consumption in food animals was positively correlated with the prevalence of multi-drug resistance ($r = 0.203$; $p > 0.05$) and negatively correlated with both sulfonamide resistance ($r = -0.239$; $p > 0.05$) and ampicillin

resistance ($r = -0.119$; $p > 0.05$). However, total antimicrobial consumption was significantly associated with tetracycline resistance ($r = 0.878$; $p < 0.05$). With regard to individual antimicrobial agent consumption and resistance correlations, tetracycline's resistance and consumption were significantly associated ($r = 0.953$; $p < 0.05$). Although β-lactam consumption was strongly correlated with ampicillin resistance ($r = 0.717$; $p = 0.062$) while sulfonamide resistance was marginally correlated with sulfonamide consumption ($r = 0.443$), these associations were not significant. Interestingly, sulfonamide consumption was negatively correlated with sulfonamide resistance ($r = -0.478$; $p > 0.05$) but significantly associated with tetracycline resistance ($r = 0.956$; $p < 0.05$).

5.2.3.2 AMR in humans

Table 5.3 shows trends in prevalence of AMR among NTS isolates from human patients from two Kenyan Hospitals for the period 1996-2001. Figure 5.3 depicts trends in total antimicrobial consumption in food animals and prevalence of MDR among NTS isolates from human patients for the same period; showing an increase in prevalence of MDR and its relationship with total antimicrobial consumption. Figure 5.4 illustrates trends in tetracycline resistance in NTS human isolates and tetracycline consumption in food animals in Kenya; showing a direct non-linear relationship between resistance and consumption.

Observations from correlation analysis of antimicrobial usage in food animals with antimicrobial resistance prevalence among NTS isolates from humans were as follows: total antimicrobial consumption was significantly associated with

tetracycline resistance (r = 0.801) but negatively correlated with sulfonamide resistance (r = - 0.733) and ampicillin resistance (r = - 0.790); however, these associations were not significant ($p > 0.05$). Moreover, a negative correlation was found between total antimicrobial consumption and prevalence of multi-drug resistance (r = - 0.786; $p > 0.05$). β-lactam consumption was positively correlated with ampicillin resistance (r = 0.489; $p > 0.05$) while aminoglycoside consumption and streptomycin resistance showed a similar trend (r = 0.265; $p > 0.05$). Nalidixic resistance was negatively correlated with total antimicrobial consumption (r = - 0.512; $p > 0.05$) as well as total fluoroquinolone consumption in food animals (r = - 0.136; $p > 0.05$). Although ciprofloxacin resistance was not reported during the entire study period (see Table 5.3), fluoroquinolone consumption in food animals was strongly correlated with the MIC_{90} of ciprofloxacin (r = 0.660).

5.2.3.3 Regression analysis of tetracycline, Beta-lactams and sulfonamides

Observations from regression analysis of antimicrobial usage in food animals with antimicrobial resistance prevalence among *E. coli* isolates were as follows:

Linear regression of tetracycline consumption (TC) and the log odds of tetracycline resistance showed a coefficient of determination, $r^2 = 0.50$ ($p = 0.118$) and a regression coefficient (slope), $\beta = 0.023$. The equation for the linear regression was:

$$Ln[R/ 1\text{-}R] = 0.0227 \times TC + 0.04.$$

Similarly, linear regression of beta-lactams consumption (BC) and the prevalence of ampicillin resistance showed an $r^2 = 0.34$ ($p = 0.28$) and $\beta = 0.42$.

The equation for the regression was:

$$Ln[R/1-R] = 0.418 \times BC - 2.02$$

For the use of sulfonamides (SC) and the prevalence of sulfonamide resistance, the calculated $r^2 = 0.32$ ($p = 0.25$) while $\beta = 0.42$. The linear regression equation was:

$$Ln[R/1-R] = -1.497 \times SC - 0.97.$$

The observations from the linear regression analysis model predicts that the prevalence of tetracycline resistance increases approximately by 2.3 % annually per additional 1000 kg (tonne) of tetracycline usage. For sulfonamide and beta-lactams usage in food animals the prevalence of resistance could not be accurately predicted in the regressional model due to low sensitivity/ specifitivity of the regressions ($r^2 < 0.5$; $p > 0.1$).

The results of modeling of drug-use factors associated with the development of multi-drug resistance in food animals in Kenya are shown in Table 5.4. All the factors examined, (i.e total consumption, oral consumption, parenteral consumption and prophylactic use) were not significantly ($p < 0.05$) associated with the prevalence of multi-drug resistance.

Fig 5.1 Trends in resistance to selected antimicrobial agents among *E. coli* from food animals and the consumption of antimicrobials in food production, Kenya

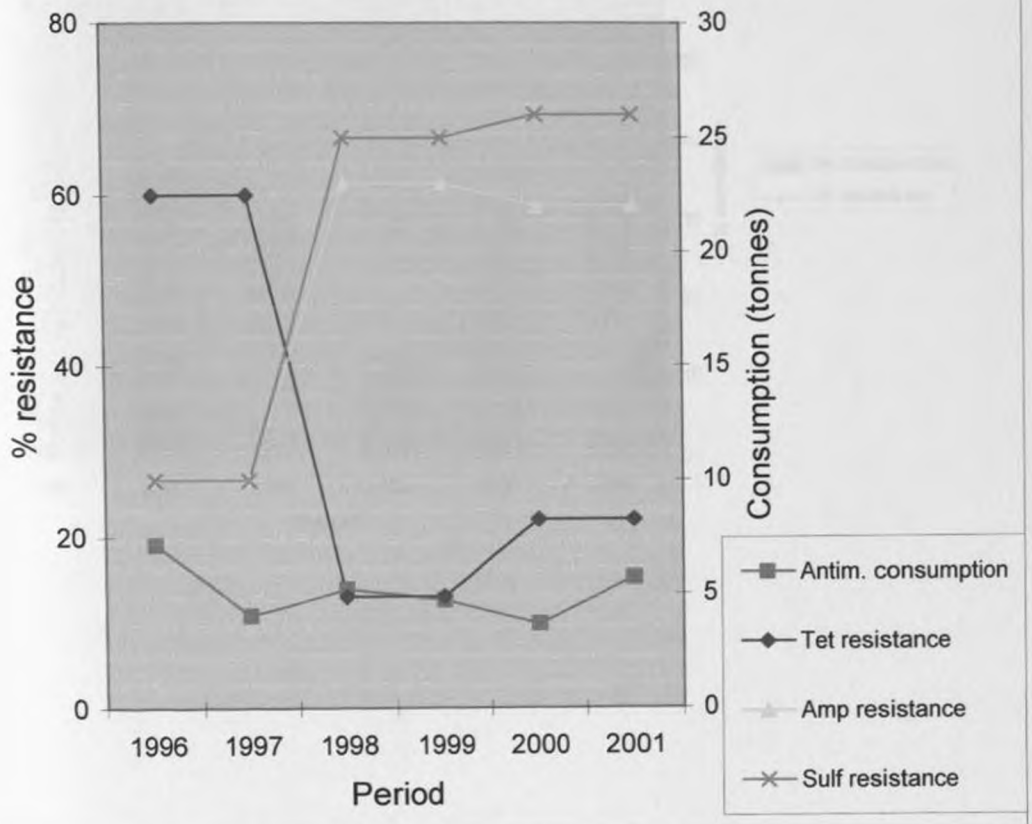


Fig. 5.2 Trends in tetracycline resistance among E. coli isolates from food animals and the consumption of tetracycline in food production, Kenya

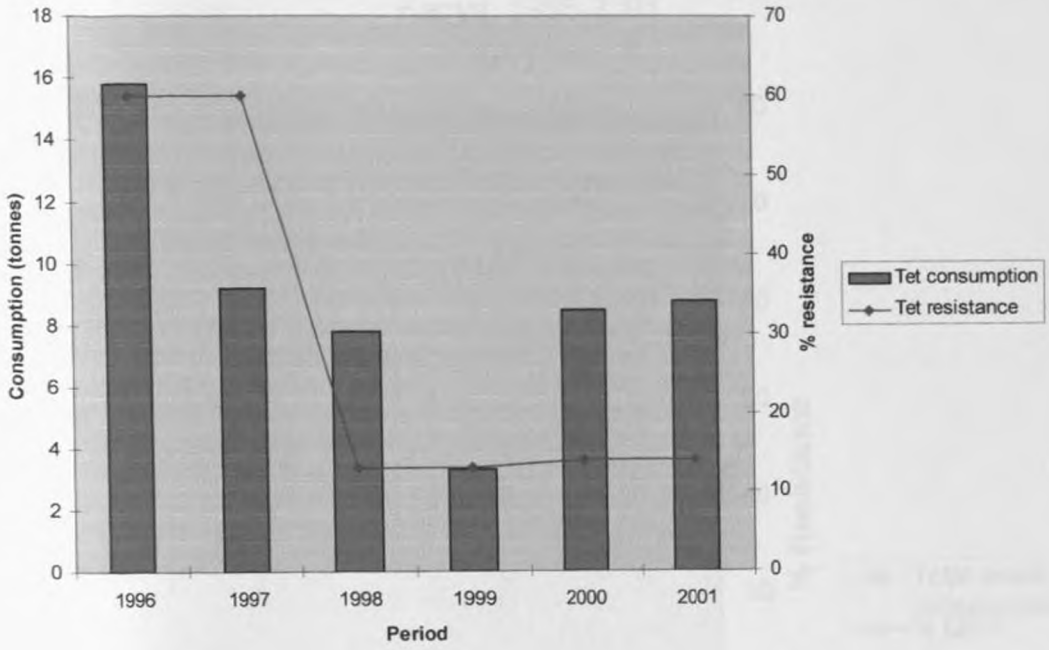


Fig. 5.3 Trends in antimicrobial consumption in food animals and development of MDR in NTS isolates from humans in Kenya, 1995-2001

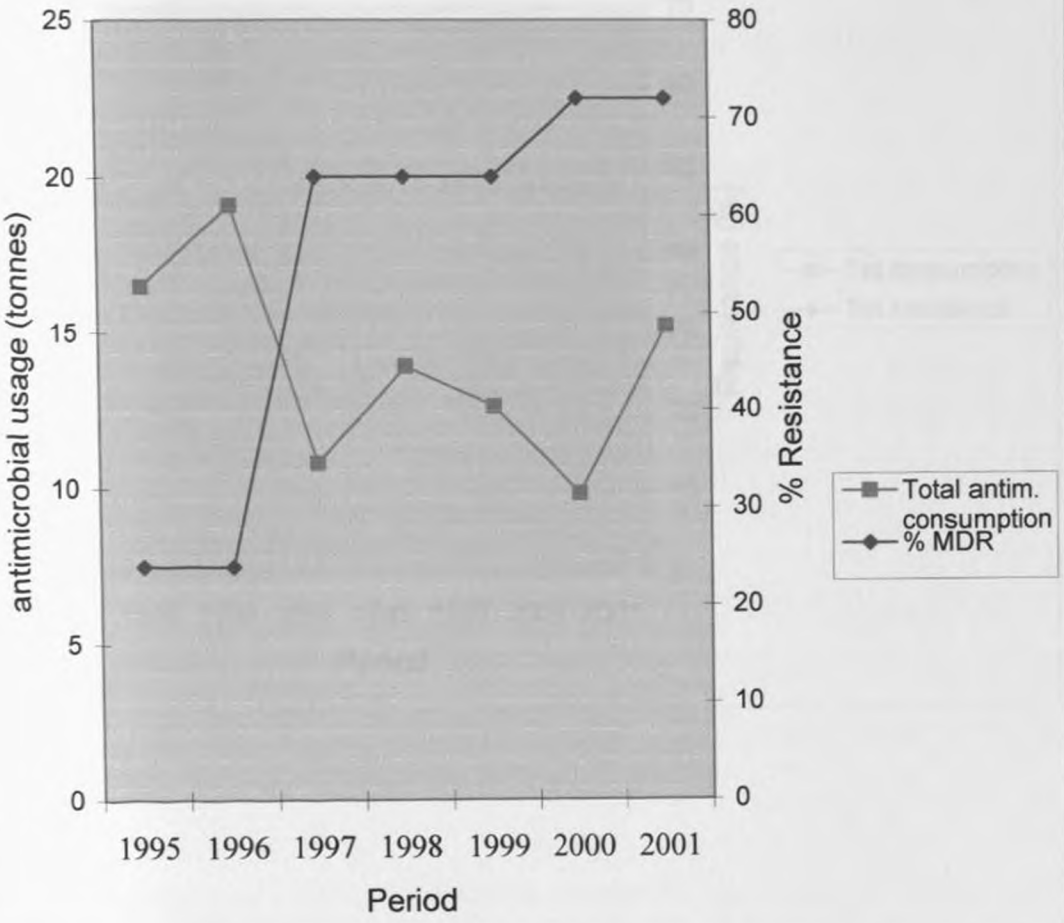


Fig. 5.4 Trends in tetracycline consumption in food animals and resistance in NTS human isolates, Kenya

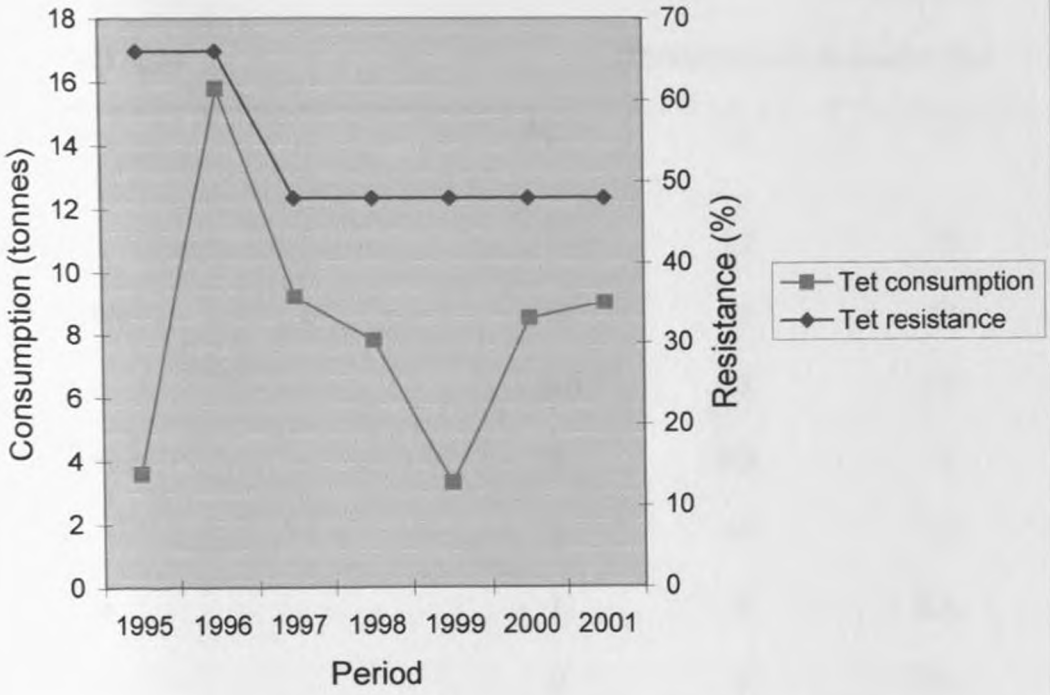


Table 5.2. Occurrence of resistance (%) in *E. coli* from food animals, Kenya*

	Period (Year)		
	1996-97	1998-99	2000-01
Antimicrobial agent	Prevalence of resistance (%)		
Tetracycline	60	13	14
Ampicillin	10	23	20
Co-trimoxazole	10	25	22
Streptomycin	NA	13	9
Chloramphenicol	8	NA	4
Gentamycin	5	0	0
Nalidixic acid	1	0	NA
Ciprofloxacin	0	0	NA

Key:
NA = data Not Available

*** Source: Kariuki *et al.*, 1997; Kariuki *et al.*, 1999; Gakuya *et al.*, 2001(resistance data for 1996-1999).**

Table 5.3. Occurrence of resistance (%) among Non-Typhoid *Salmonella* (NTS) from humans, Kenya *

	Period (Year)		
	1996-97	1996-99	2000-01
Antimicrobial agent	Prevalence of resistance (%)		
Tetracycline	66	48	48
Ampicillin	48	65	62
Co-trimoxazole	46	60	68
Streptomycin	49	70	67
Chloramphenicol	26	40	50
Gentamycin	16	9	11
Nalidixic acid	0	11	4
Ciprofloxacin	0	0	0

*** Source: Kariuki *et al.*, 2000; Kariuki *et al.*, 20002; Kariuki *et al.*, 2005.**

Table 5.4 Model of drug-use factors in food animals associated with multi-drug resistance in *E. coli* from food animals in Kenya.

Variable	Estimate	P-value
Total consumption	2.38	0.319
Oral consumption	-4.01	0.303
Parenteral consumption	-1.94	0.315
Prophylactic use	4.57	0.290
Constant	1.19	0.537

$R^2 = 0.884$; P = 0.491

5.4 DISCUSSION

This study presents for the first time data from an ecologic study that correlates national antimicrobial consumption from food animals with prevalence of antibiotic-resistant *E.coli* isolates from food animals, and NTS human isolates from Kenyan hospitals. Firstly, this study shows negative correlations between tetracycline and beta-lactams usage as well as tetracycline and aminoglycoside usage. Secondly, the study revealed a strong inverse association between fluorquinolone and sulfonamide usage indicating increasing levels of fluroquinolone consumption with decreasing levels of sulfonamide usage in food animals. This pattern of drug use reflects a shift towards the use of broad-spectrum antimicrobial agents in food animals in Kenya. The ecologic study design chosen allows the evaluation of the effect of antibiotic consumption on resistance rates on a national level. A strong and significant association was documented between total antimicrobial consumption in food animals and prevalence of resistance among *E. coli* isolates from animals. However, a weak relationship was documented between total antimicrobial consumption in food animals and prevalence of resistance among NTS isolates from humans, except for tetracycline. An almost linear association existed between tetracycline use and proportion of tetracycline resistant *E. coli* from food animals. A weaker correlation was found between beta-lactam usage and the proportion of ampicilin-resistant *E. coli* isolates; and between tetracycline usage and the prevalence of multi-drug resistant *E. coli*. However, drug-use factors e.g routes of administration, therapeutic or prophylactic use were not significantly ($P > 0.05$) associated with development of multi-drug resistance in food animals in the study. Perhaps other factors associated

with drug use e.g farm-level (farmer experience, education level etc) and animal-level factors may be significantly associated with antimicrobial resistance. In Europe prevalences of resistance to macrolides and ß-lactams in *Streptococcus pneumoniae*, macrolide resistance in *Streptococcus pyogenes* and resistance to quinolones and co-trimoxazole in *E. coli* were obtained from a number of national and international surveillance studies, and compared with antimicrobial consumption in the participating European countries (Goossens *et al.*, 2005). In these studies, significant correlations between levels of resistance and antibiotic consumption were seen, particularly for *S. pneumoniae*, i.e higher levels of antibiotic prescribing were associated with higher levels of antibiotic resistance. Similar observations were made in this study between antimicrobial consumption and resistance in food animals. However, the present study did not find a direct correlation between AMR among NTS isolates and antimicrobial usage in food animals as reported in the USA and Europe. This is in agreement with the findings of Kariuki *et al.* (2002) that food animals may not be the major vehicles of NTS infection in humans in Kenya. Generally, the findings of the present study are in agreement with those of Austin *et al.* (1999) who modeled the relationship between antimicrobial use and endemic resistance based on epidemiologic observations and the European Antimicrobial Resistance Surveillance Study (EARSS, 2000). However, these ecologic studies have some limitations. First, antibiotic sales data cannot be used synonymously with antibiotic exposure. For example, the data on usage volumes allow comparisons but do not measure individual exposure to antibiotics. In other words, animals receiving antibiotics may not be the same ones from whom antibiotic-resistant bacteria were

isolated. Therefore, antimicrobial consumption data might not account for these differences. Second, differences in antimicrobial consumption rates among various food animal species could not be accounted for. Finally, data are lacking regarding the maximum time lag between antimicrobial consumption and possible changes in resistance patterns on a national level. Antimicrobial use exerts selective pressure on resistance in bacterial pathogens in several ways (Albrich *et al.*, 2004). Any type of recent antimicrobial treatment can select for resistance by eradicating antibiotic-susceptible bacteria, while indirectly promoting the transmission of AMR pathogens in a community or country. However, whether decreasing antibiotic use in the community will have a sustained impact on resistance rates is unclear. Antibiotic resistance may take longer to return to previous levels than the time it took for antibiotic resistance to increase after excessive antibiotic use. In Finland for instance, in the 1990s antibiotic resistance decreased slowly with substantially reduced antibiotic consumption after an initial rapid increase; such situations may lead to different correlations between use and resistance, depending on whether resistance is increasing or decreasing (Houvinen, 2002). The present study observed persistent levels of sulfonamide resistance in Kenya despite decreasing sulfonamide consumption levels in food animals in the last five years. This finding is in agreement with the results of a recent study in the United Kingdom which reported persistence of sulfonamide resistance in *E. coli* despite cessation of sulfonamide usage (Enne *et al.*, 2001). Moreover, There is evidence from the USA (Langlois *et al.*, 1986) and from Norway (Kruse and Simonsen, 2000) that some resistance may persist long after the use of an antibiotic has been discontinued. The persistence of virginiamycin

resistance after its ban has been attributed to the use of penicillins selecting for associated resistance to virginiamycin (Bager *et al.*, 2002), such associated resistance as a result of one antibacterial agent selecting for resistance to another that is unrelated because the two resistance determinants are genetically linked on the same plasmid or transposon (co-resistance). This study found an association between sulfonamide resistance and beta-lactam consumption in food animals. Significant associations between AMR rates for different antibiotics during the study period were observed. A variety of factors could be responsible for this, but perhaps the selective pressure exerted by inappropriately used antibiotics is likely the most important (Albrich *et al.*, 2004). Indeed, amounts of antibiotic use in Kenya varied widely during this period (Mitema *et al.*, 2001). It is possible that socioeconomic, cultural, demographic, attitudinal and behavioral determinants have a major impact on antibiotic consumption practices in food animals in Kenya. Further studies of factors that influence prescription patterns may provide useful information for assessing public health policy strategies aimed at reducing antibiotic use and ultimately levels of antimicrobial resistance. The data presented in this analysis suggest an important association between antibiotic consumption in food animals and resistance among *E. coli* isolates from food animals and slightly weaker association for NTS infections in humans. The relationship between antimicrobial use and AMR are complex; interventions aimed at promoting more rational prescribing patterns should be supported by adequate experimental and epidemiological evidence. Therefore, this study supports the adoption of the precautionary principle in the containment of AMR. Moreover, this study recommends review of relevant legislation, planning and

implementation of a national action plan for the appropriate use of antimicrobial agents in Kenya, whose effectiveness should be monitored and evaluated through prospective and continuous surveillance of antimicrobial resistance and antimicrobial sales data. In conclusion, the findings in this study provide important information on the associations between AMR and antimicrobial usage in food animals. The study has shown that tetracycline usage in food animals leads to increased tetracycline resistance in food animals and also co-selects for sulfonamide resistance in food animals, more so following oral use. The study has identified tetracycline use in food animals as a possible risk factor for tetracycline resistance in humans. In addition, usage of β-lactam antibiotics contributes to ampicillin resistance in food animals, while overall antimicrobial usage is associated with prevalence of tetracycline resistance and MDR in food animals.

CHAPTER SIX

6.0 GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

The use of antibiotics to treat disease in food animals started in the mid-1940s. The introduction of antibiotics in commercial feed for cattle, pigs, and chickens began in the early 1950s. The scientific debate over the potential public health risks from animal use of antibiotics began more than 30 years ago when researchers first reported that the addition of streptomycin to chicken feed increased the growth rate of chickens (Jukes *et al.*, 1956).

The increase in resistance of bacteria to drugs used to treat human infections has raised questions about the role that antimicrobial drug use in food-producing animals plays in the emergence of antimicrobial drug-resistant bacteria. In recent years, concerns about the use of antimicrobial products in food animals have focused on human food safety because foods of animal origin are vehicles of food-borne disease in humans. The selection of drug-resistant bacterial populations is a consequence of exposure to antimicrobial drugs and can occur from human, animal, and agricultural uses. The administration of these compounds to food animals is necessary to maintain their health. However, food animals can become reservoirs of bacteria capable of being transferred to humans through food. Food-carrying resistant bacterial pathogens can cause human illness and subsequent therapeutic failure.

The present study on antimicrobial consumption patterns in food animals has shown that many of the antimicrobials used to treat animals are either identical to or related to drugs used in human medicine, including penicillins, tetracyclines, cephalosporins,

aminoglycosides, macrolides and fluoroquinolones. Most of these drugs are also used to treat food-borne disease in humans. Tetracyclines constituted 70 % of total antimicrobial consumption, fluoroquinolones 22 %, penicillins 4 %, aminoglycosides 2 % and sulfonamides 1 %. However, There was hardly any use of extended spectrum β-lactam (ESBLs) antimicrobial agents in food animals.

Moreover, the study indicated increasing trends in oxytetracycline and fluoroquinolone usage and declining trends in sulfonamides, penicillins aminoglycosides and macrolides consumption in food animals. Interestingly, in Kenya animal consumption of fluoroquinolone appears to be on the increase despite a reduction in levels of consumption in Europe and USA due to legal restrictions.

AMR phenotypes of *E. coli* (as indicator bacteria) in healthy food animals at slaughter were characterized in this study. These results demonstrated that the proportion of MDR in healthy food animals was > 20 % and MDR (to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines) was found in some food animal isolates. With such excessive use of antibiotics it is not surprising that there is evidence for a high prevalence of resistance in commensal bacteria. Multi-drug resistant strains of *E. coli* are increasingly prevalent worldwide (Rodriguez *et al.*, 2001). In some cases this has been linked to antimicrobial use in food animals either as growth promoters or for therapy (van den Bogaard *et al.*, 2001). In an earlier study in Kenya (Kariuki *et al.*, 1999) demonstrated high levels of transferable resistance in commensal *E. coli* from children with diarrhoea although there was no genotypic evidence that these *E. coli* were related with those found in domestic poultry.

Thus, the commensal flora of the intestines can act as reservoirs of antibiotic resistance genes. These genes can then be transferred to pathogenic bacteria in the intestines. More recent studies have confirmed that using antimicrobial drugs in animals increases the risk of selecting for resistant food-borne pathogens, and that these pathogens can then be transferred to humans through direct contact with either contaminated food or animals (van den Bogaard *et al.*, 2001; White *et al.*, 2001). Much of the discussion about the adverse effects on human health associated with the veterinary use of antimicrobial agents has revolved around the clinical significance and sources of antimicrobial-resistant nontyphoidal *Salmonella* infections in humans (Institute of Medicine, 1989; U.S. Congress, Office of Technology and Assessment, 1995). This issue gained prominence in the light of the recent discussions concerning the public health implications of veterinary use of fluoroquinolones (Anonymous, 1994; Beam, 1994), a class of antimicrobials essential for the treatment of several life-threatening infections in humans (Wilcox and Spencer, 1992; Conte, 1995). Because of these public health concerns, the Food and Drug Administration prohibited the use of fluoroquinolones in food animals in the United States in 1997.

There is epidemiological and clinical evidence that the use of antimicrobial agents, at subtherapuetic or therapeutic concentrations, results in antimicrobial resistance. However, in order for an antimicrobial-resistant pathogen to have a public health consequence, there must be both usage of the antimicrobial agent and dissemination of the resistant pathogen.

The role of dissemination has been documented by the consequences of using fluoroquinolones for the treatment of infections in *Salmonella* in humans and food animals in the United Kingdom, where a fluoroquinolone (enrofloxacin) has been widely used in food animals following its approval for veterinary use in 1993. Following the approval decreased susceptibility to fluoroquinolones (MIC >0.25) rapidly emerged among human *Salmonella* isolates, particularly among isolates of multiply-resistant S. Typhimurium DT104 which were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) isolated from humans (Threfall *et al.*, 1997). A parallel rapid emergence of decreased susceptibility to fluoroquinolones, as indicated by antimicrobial resistance to nalidixic acid, has been observed among animal *S.* Typhimurium DT104 R-type ACSSuT isolates in the United Kingdom. In Kenya although increased prevalence of MDR NTS have been reported, fluoroquinolone resistance has not been reported, although decreased susceptibility to nalidixic acid and ciprofloxacin has been observed over the last 10 years (Kariuki *et al.*, 2005).

Most human *Salmonella* infections in developed countries are acquired from ingestion of contaminated foods of animal origin. However, in a previous study in Kenya Kariuki *et al.* (2002), were unable to show any significant association between NTS isolates from humans and those from animals living in close contact. There is supporting evidence in the United States and Europe that human usage of fluoroquinolones has little impact on resistance among *Salmonella* isolates; and since most persons infected with antimicrobial-resistant *Salmonella* do not have a history of recent international travel (Riley *et al.*, 1984; McDonald *et al.*, 1987; Lee *et al.*, 1994)

the only likely cause for the emergence and increasing prevalence of antimicrobial-resistant *Salmonella* in the United States is the use of antimicrobial agents in animals, predominately food animals. Four lines of evidence support the conclusion that most AMR among *Salmonella* isolates in humans results from the use of antimicrobial agents in food animals.

Firstly, several outbreak investigations of antimicrobial-resistant *Salmonella* infections in humans have combined epidemiological studies and laboratory subtyping techniques to trace back antimicrobial-resistant *Salmonella* through the food distribution system to farms, and antimicrobial use on the farms was found to be associated with the antimicrobial resistance (Holmberg *et al.*, 1984; Lyons *et al.*, 1985; Tacket *et al.*, 1985; Spika *et al.*, 1987).

Secondly, emergence of *Salmonella* Typhimurium DT104 R-type ACSSuT with decreased susceptibility to fluoroquinolones in the United Kingdom. The continued emergence of *S.* Typhimurium DT104 with decreased susceptibility to fluoroquinolones in humans in the United Kingdom provides increasingly strong evidence that antimicrobial-resistance among *Salmonella* isolates in humans results from the use of antimicrobial agents in food animals. Decreased susceptibility to fluoroquinolones among human *Salmonella* isolates was rare in the United Kingdom prior to 1993, despite the widespread use of ciprofloxacin in humans since 1987. However, following the 1993 approval and widespread use of enrofloxacin in veterinary medicine, human *Salmonella* isolates (and animal isolates) with decreased

susceptibility to ciprofloxacin rapidly emerged beginning in 1994 (Threfall *et al.*, 1997).

Thirdly, comparisons between resistant phenotypes among isolates of human and animal sources. Arguably, if veterinary use of antimicrobial agents is responsible for the development of antimicrobial resistant *Salmonella* in animals which may be transmitted to humans, then the patterns of antimicrobial resistance observed among *Salmonella* isolates collected from healthy animals and humans should be similar. Several studies from developed countries have shown that NTS from farm animals are multiply resistant to commonly available drugs including both quinolones and extended spectrum β-lactams (Fey *et al.*, 2000; Kruchaga *et al.*, 1998; Wiuff *et al.*, 2000). However, in Kenya, antimicrobial susceptibility tests showed that NTS from humans were multiply resistant to antimicrobial agents commonly available in Kenya whereas NTS from animals were fully susceptible to all antibiotics tested (Kariuki *et al.*, 2002).

Fourthly, the comparison of antimicrobial usage patterns in humans and animals with AMR patterns among humans and animals. Although limited data is available on antimicrobial agent usage in food animals in many countries, where available these data suggests that the patterns of antimicrobial usage in food animals are similar to the spectrum of antimicrobial resistance observed among *Salmonella* isolates from food animals and humans. In the present study, a weaker correlation was found between the prevalence of MDR among NTS from humans and fluoroquinolone consumption in food animals in Kenya. However, there was a stronger correlation

between fluoroquinolone consumption in food animals and NTS susceptibility to fluoroquinolones (MIC >0.25).

With the recognition that foods of animal origin are the source of most human *Salmonella* infections (and most antimicrobial-resistant *Salmonella* infections), and that most antimicrobial resistance among *Salmonella* isolates are caused by the use of antimicrobial agents in food animals, it is possible to evaluate the potential human health consequences of unrestricted veterinary use of fluoroquinolones using the human health risks model developed by the Institute of Medicine in 1988 of the United States (Institute of Medicine, 1989). It is estimated that each year in the United States, most of the approximately 2,400 persons with life-threatening invasive *Salmonella* infections are treated with fluoroquinolones, of whom approximately 500 die. Fluoroquinolones may therefore be life-saving for approximately 1,900 persons each year in the United States.

In Kenya, NTS account for a steadily increasing proportion of human salmonellosis, and often present as bacteremia without diarrhoeal disease (Kariuki *et al.*, 2000; Arthur *et al.*, 2001). MDR salmonella were first documented in Nairobi in 1994 with increasing prevalence over time (Kariuki *et al.*, 1996). For instance, between 1997 and 2003 the prevalence of NTS isolates resistant to ampicillin, co-trimoxazole, streptomycin, chloramphenicol and tetracycline increased from 31 % to 42 %. Although still sensitive to fluoroquinolones, these isolates have 10-fold higher MIC's of nalidixic acid and ciprofloxacin than non-MDR salmonella isolates.

Evidence presented in this study demonstrates that emergent fluoroquinolone resistance among *Salmonella* isolates in Kenya could result from direct fluoroquinolone use in food animals. However, the clinical significance of fluoroquinolone-resistant NTS currently is not precisely known because other antimicrobial treatment options are limited. Treatment failures and serious outcomes, including deaths, would be expected. For instance, using the reported incidence of approximately 1000 cases of salmonellosis per year (Ombui, 1998) if 10 % of *Salmonella* isolates in Kenya were to be fluoroquinolone-resistant as a result of usage in food animals, and 16 % of persons with invasive fluoroquinolone-resistant infections were to die, fluoroquinolone usage in food animals under such a scenario, would result in 16 deaths each year. This study, therefore emphasize the need of prudent use of antimicrobial agents in food animals.

In June 1998, the WHO addressed the use of quinolones in food-producing animals and noted with concern that the use of antimicrobial agents will cause resistance to develop and that there is a potential human health hazard from resistant *Salmonella*, *Escherichia coli*, and *Campylobacter* organisms transferred to humans through the food supply (WHO, 1998). However, the experts also agreed that antimicrobial drugs, including quinolones in certain instances, are needed to treat sick animals and urged more research on the possible human health effects from the use of these drugs in animals (WHO, 1998).

The emergence and increasing prevalence of MDR *Salmonella* complicates the treatment of *Salmonella* infections in humans and animals. In developing countries,

including Kenya increasing rates of resistance to *Salmonella* Typhimurium DT104 R-type ACSSuT which become resistant to commonly available antibiotics have made treatment of clinical salmonellosis a clinical dilemma (Gupta *et al.*, 2002). For example chloramphenicol is the drug of choice in Malawian children since ciprofloxacin and ceftriaxone are less available and much more expensive (Graham *et al.*, 2000). The increasing prevalence of antimicrobial resistance among *Salmonella* isolates, and the consequences, demonstrates the urgent need to develop strategies to reduce antimicrobial agent usage in food animals. Since antimicrobial agent usage can be reduced through the implementation of non-antimicrobial means of controlling infectious diseases, such as improved hygiene and sanitation, such efforts, which will minimize development of antimicrobial resistance and dissemination of antimicrobial-resistant pathogens, should be emphasized (Helmuth and Protz, 1997). Efforts should also be taken to ensure that antimicrobial agents are used prudently in food animals. Prudent usage of antimicrobial agents maximizes the therapeutic effect of the antimicrobial agent and minimizes the development of antimicrobial resistance.

Since subtherapeutic uses of antimicrobial agents do not exert a therapeutic effect, such uses are non-prudent and should be replaced by non-antimicrobial methods of growth promotion. Because of the particular contribution of subtherapeutic tetracycline in the development and dissemination of antimicrobial resistant *Salmonella*, the subtherapuetic use of tetracycline should be restricted in line with WHO recommendations (World Health Organization, Division of Emerging and Other Communicable Diseases Surveillance and Control, 1997). Because fluoroquinolones are a vital class of antimicrobial agents for the treatment of

potentially life-threatening *Salmonella* infections in humans, and widespread usage of fluoroquinolones in food animals will lead to rapid emergence and dissemination of resistance to humans with adverse health consequences, the use of fluoroquinolones in food animals should be restricted until policy guidelines for the prudent use of antimicrobial agents in food animals have been implemented.

Unfortunately in Kenya, there is neither a national veterinary formulary nor an official policy on antimicrobial drug use in food animals. The current general liberalization of antimicrobial agents market in Kenya is the main cause of uncontrolled and ready availability of antimicrobial agents to farmers over-the-counter. Under such circumstances unsupervised sale and misuse of antimicrobial agents occur, thereby increasing the risk of AMR developing. There is an urgent need to review the relevant national legislation in order to address sale and distribution of antimicrobial agents from a Kenyan perspective. Among the legislations that need to be reviewed are the Pharmacy and Poisons Act (Cap 244), Animal Diseases Act (Cap364), the Veterinary Surgeons Act (Cap 366) and the Kenya Industrial Property Act (2002).

Unlike toxicological safety of drug residues in food, no predictive models currently exist to precisely estimate the rate and extent of AMR that may emerge from the use of antimicrobial drugs in food animals. Despite the current lack of such models, certain information can be generated to support a pre-approval and post-approval AMR safety assessment (FDA, 1999). Possible strategies include (a) categorization of antimicrobial drugs based on the importance of the drug for human medicine; (b)

revision of the pre-approval safety assessment for antimicrobial resistance for new animal drug applications to include an evaluation of all uses for microbial safety; (c) post-approval monitoring for the development of antimicrobial drug resistance; (d) collection of food animal antimicrobial drug use data; and (e) the establishment of regulatory thresholds.

The antimicrobial resistance risk assessment should characterize the proposed drug product as to the potential for resistance to emerge in animals, the potential that such resistance would be transmitted from animals to humans, and the potential consequence of that resistance to human health. These factors taken together will be used to characterize the overall risk that the proposed drug use in animals would cause antimicrobial resistance to emerge and to impact human health.

In addition to the risk assessment, applications for antimicrobial drugs for food-producing animals may need to undergo a risk management assessment. The recognition that the use of antimicrobial drugs exerts microbial selection pressure focuses attention on the need for appropriate or prudent use of antimicrobial drugs. A principle consistent with the judicious use concept is the idea that certain antimicrobial agents, or certain antimicrobial use patterns, are more likely than others to exert selection pressures favorable to the emergence of AMR. This risk-based approach should be used by registration and regulatory authorities to approve only limited use conditions for particular antimicrobial agents and to impose certain use restrictions which may include limitations regarding product marketing status, extra-label use provisions, or dosage and administration instructions.

Finally, to facilitate the development of a monitoring system on antimicrobial use in Kenya, the Pharmacy and Poisons Board of the Ministry of Health needs to enhance its regulatory mandate on antimicrobial distribution and determine the points in the distribution system where meaningful and useful data can be collected in a continuous and logistically feasible manner. The Board needs to design and implement a national surveillance program of antimicrobial use in food animals that provides valid data in a timely and methodological fashion. The national surveillance program should support risk analysis related to human health and policy development related to antimicrobial use in Kenya. Surveillance should include indicator (*E. coli* and *Enterococcus faecium*) and pathogenic bacteria (*Salmonella spp, Campylobacter spp* and *Streptococcus spp*) isolated from animals, foods, animal feeds and imported animal products. The bacteria (indicator, zoonotic and pathogenic) chosen for active surveillance and the laboratory methods used within the surveillance program should be comparable to those of DANMAP, EARSS and NARMS, so that Kenya can participate in a global system of surveillance of antimicrobial resistance in bacteria of food-animal origin.

6.2 CONCLUSIONS

In summary this study has led to the following conclusions:

1. Tetracycline still remains the most prescribed antimicrobial agent in in food animals in Kenya.
2. Total antimicrobial usage in food animals was significantly associated with tetracycline resistance in food animals and strongly correlated with tetracycline resistance in humans.
3. Fluoroquinolone was the second most prescribed antimicrobial agent and the most prescribed oral antimicrobial agent in food animal practice in Kenya during the study period (2001-2002).
4. *E coli* isolates from healthy cattle, poultry and swine showed moderate to high levels of resistance to commonly prescribed antimicrobials notably ampicillin, sulfamethoxazole, co-trimoxazole and tetracycline. Multi-drug resistance was much more common among isolates from poultry and swine than from cattle.
5. Genotypic studies showed that most ampicillin-resistant *E. coli* isolates from food animals expressed the TEM- β-lactamase genes.
6. Tetracycline resistance was encoded by either *Tet(A)* or *Tet(B)* genes both of which accounted for about fifty five percent of resistance. Of the two genotypes *Tet(A)* was the most predominant gene.

7. Sulfonamide resistance was mediated by *SulI* and /or *SulII* genes in over ninety five percent of resistant bacteria. However, *SulII* gene was the most predominant gene.
8. The demonstration of conjugational transfer of MDR genes and the presence of mobile class1integrons led to the conclusion that food animals may pose a risk to human health in Kenya.
9. The observation from linear regression analysis model predicts an annual increase of 2.3 % in prevalence of tetracycline resistance per additional 1000 kg of tetracycline usage in food animals.

6.3 RECOMMENDATIONS

1. Because fluoroquinolones are a vital class of antimicrobial agents for the treatment of potentially life-threatening *Salmonella* and *Camplobacter* infections in humans and widespread usage of fluoroquinolones in food animals may lead to rapid emergence and dissemination of resistance to humans with adverse health consequences, The study recommends: (a) for closer surveillance of fluoroquinolone consumption and its effect on emergence of fluoroquinolone resistance in both food animals and humans (b) that the use of fluoroquinolones in food animals (poultry) should be restricted in line with " the precautionary principle" until policy guidelines for the prudent use of antimicrobial agents in food animals have been implemented.

2. Since tetracycline usage in food animals was strongly correlated with tetracycline resistance in both animals and humans; and tetracycline resistance was significantly associated with chloramphenicol resistance in food animals, it is recommended that there should be regulation of tetracycline usage in food animals (poultry and swine) in Kenya in line with WHO recommendations.
3. Noting that β-lactam usage in food animals was significantly associated with the prevalence of ampicillin resistance in food animals and the resistant mechanism is due to β-lactamase production, it is recommended that certain extended spectrum β-lactams (ESBL's) be introduced for food animal use in Kenya in line with WHO guidelines.

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8.0 APPENDICES

Appendix 1: Composition and preparation of culture media, reagents and components of kits

1.1 Culture media

1.1.1 Blood Agar

Composition (g/litre)

'Lab lemco' powder.....10 g

Peptone.....10 g

Sodium chloride.....5 g

Agar.....15 g

PH at 37°C.....7.3 ± 0.1.

This agar was prepared by suspending 40 grammes of the blood agar base (Oxoid) in one litre of distilled water and boiled to dissolve completely. Sterilization was done at 121°C for 15 minutes. The sterile base was cooled to 50°C after which 7 % of sterile defrinated blood was added.

1.1.2 Citrate medium

Composition

Trisodium citrate. $2H_2O$.....1.0 g

Magnesium sulphate. $7H_2O$.....1.2 g

Diammonium hydrogen phosphate.....0.5 g

Potassium chloride.....1.0 g

Agar.....15 g

Distilled water.....980 ml

0.04 % w/v solution of phenol.....20 ml

The above reagents were mixed and dissolved by boiling. The pH was then adjusted to 6.8 and 3 ml of the media dispensed into crew capped bijou bottles. They were then sterilized at 121°C for 15 minutes.

1.1.3 Cooked meat medium

The medium was prepared by adding 15 grammes of cooked meat pellets to 20 ml nutrient broth (Oxoid) in universal bottles and sterilized by autoclaving at 121°C for 15 minutes.

1.1.4 Eosin Methyl Blue Agar (Oxoid)

Composition (g/l)

Peptic digest of animal tissue.....10 g

Dipotassium phosphate.....2 g

Lactose.....10 g

Eosin Y.....0.4 g

Methylene blue.....0.065 g

Agar.....15 g

PH at 37°C.....7.1 $\pm$ 0.1

This agar was prepared by suspending 37.5 grammes of Eosin Methyl blue agar base (Oxoid) in one litre of distilled water and boiled to dissolve completely. Sterilization was done at 121°C for 15 minutes. The sterile base was cooled to 50°C and shaken to oxidize methylene blue.

1.1.5 MacConkey Agar (Oxoid)

Composition (g/l)

Peptone from Casein.....17 g

Peptone from meat .....3 g

Lactose.....10 g

Neutral Red.....0.03 g

Crystal Violet.....0.001g

Agar.....13.5 g

pH .....7.1 $\pm$ 0.1

This was prepared by dissolving 50 grammes of macConkey powder (Oxoid) in one litre of freshly distilled water and boiled to dissolve completely. Sterilization was then done at 121°C for 15 minutes.

1.1.6 Muellar- Hinton Agar (Oxoid)

Composition (g/l)

Beef dehydrated infusion.....300 g

Casein hydrolysate.....17.5 g

Starch.....1.5g

Agar.....17.0 g

pH .....7.4 $\pm$ 0.2

This was prepared by dissolving 35 grammes of muellar- Hinton powder (Oxoid) in one litre of distilled water and boiled to dissolve completely. Sterilization was then done at 121°C for 15 minutes.

1.1.7 Muellar- Hinton broth (Oxoid)

Composition g/l

Beef dehydrated infusion.....300 g

Casein hydrolysate.....17.5 g

Starch.....1.5 g

This was prepared by dissolving 21 grammes of dry medium in one litre of distilled water and sterilized by autoclaving at 121°C for 15 minutes.

1.1.8 Nutrient broth (Oxoid)

Composition (g/l)

Peptone.....10 g

Sodium chloride .....5.0 g

pH.....7.2 ± 0.2

The broth was prepared by adding 15 grammes of dehydrated medium to one litre of distilled water, boiled to dissolve and distributed into universal bottles. They were then sterilized by autoclaving at 121° C for 15 minutes.

1.1.9 Stuart's transport medium

Composition (g/l)

Sodium glycerophoshate.....10 g

Sodium thioglycollate .....0.5 g

Cysteine-Hcl.....0.5 g

Calcum chloride.....0.1 g

Methylene blue.....0.001 g

Agar.....5 g

The broth was prepared by adding 15 grammes of dehydrated medium to one litre of distilled water, boiled to dissolve and distributed into universal bottles. They were then sterilized by autoclaving at 121° C for 15 minutes.

1.1.10 Tryptone soya broth (Oxoid)

Composition (g/l)

Tryptone.....15.0 g

Soya peptone.....5.0 g

NaCl.....5.0 g

Tryptic soy broth was rehydrated by dissolving 30 grammes of the powder in one litre of distilled water, and then five (5) ml amounts dispensed into screw capped tubes and then sterilized by autoclaving at 121°C for 15 minutes.

1.2. Preparation of solutions

1.2.1 10 X TBE Buffer (Tris Borate EDTA buffer)

108 g tris base, 55 g boric acid and 40 ml 0.05 M EDTA (pH 8.0) were dissolved in one litre of distilled water.

1.2.2 1 % Agarose gel in TBE

1.25 grammes of agarose electrophoresis grade was added to 1.25 ml distilled water and 12.5 ml of 10 X TBE was then added to make a final volume of 15 ml. The mixture was heated to dissolve, then cooled to 60°C and 7.5µl ethidium bromide (10 mg/ml) was added and thoroughly shaken to mix.

1.2.3 Ethidium bromide stock solution (10 mg/ml)

One gramme of ethidium bromide was added to 100 ml distilled water, and then stirred with a magnetic stirrer until it dissolved. The solution was then transferred to a bottle container and stored at 4°C.

1.2.3 Loading dye (Stop mix)

2.5 grammes of sodium dodecyl sulphate (SDS), 25 ml glycerol and 0.0125 g bromothymol blue were mixed in 50 ml distilled water and warmed to 37 °C to dissolve the SDS.

1.3 KIT COMPONENTS

1.3.1 PCR core Kit components

a). Taq DNA polymerase

One vial contains 250 units, 5 units per ul in storage buffer: 20 mM Tris/ Hcl, 100 mM dithiothreitol, 0.1 mM EDTA, nonidet P40, 0.55 (v/v), Tween 20 (0.5 % v/v), glycerol 50 % (v/v), pH 8.0 (4 °C).

b). dNTP- Stock solution

One vial 200 ul, containing each 10 mM dATP, dCTP, dGTP and dTTP in sterile distilled water, pH 7.0.

c). PCR reaction buffer

One vial with 1.0 ml 100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3.

Appendix 2: Results of disc diffusion susceptibility test for *E. coli* isolates from food animals

ISOLATE	TET	COT	CHL	AMP	SULF	STR	KANA	GENTA	SPECIE	SOURCE
10C	S	R	S	S	R	S	S	S	PORCINE	CARCASS
10F	S	S	S	S	S	S	S	S	PORCINE	FECAL
11C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
12C	R	R	S	R	R	IR	S	S	PORCINE	CARCASS
12F	S	S	S	S	S	S	S	S	PORCINE	FECAL
13C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
15C	S	S	S	IR		S	S	S	PORCINE	CARCASS
16C	R	R	S	IR	R	S	S	S	PORCINE	CARCASS
17C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
18C	IR	R	S	R	R	R	S	S	PORCINE	CARCASS
1F	IR	R	S	S	R	S	S	S	PORCINE	FECAL
1OOC	S	S	S	S	S	S	S	S	PORCINE	CARCASS
20C	R	R	S	R	R	R	S	S	PORCINE	CARCASS
21C	R	R	S	S	R	S	S	S	PORCINE	CARCASS
22C	R	S	S	S	R	R	S	S	PORCINE	CARCASS
23C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
23F	S	S	S	S	S	S	S	S	PORCINE	FECAL
24C	IR	S	S	IR	S	S	S	S	PORCINE	CARCASS
24F	S	S	S						PORCINE	FECAL
25C	R	R	S	S	R	S	S	S	PORCINE	CARCASS
25F	S	R	S	S	R	S	S	S	PORCINE	FECAL
26C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
26F	S	S	S						PORCINE	FECAL
27C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
27F	R	R	S		S	S	S	S	S PORCINE	FECAL
28C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
29C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
29F	R	R	S	R	R	R	S	S	PORCINE	FECAL
2C	S	S	S						PORCINE	CARCASS
2F	S	S	S	S	S	S	S	S	PORCINE	FECAL
30C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
31C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
31F	IR	S	S	R	S	S	IR	S	PORCINE	FECAL
32C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
32F	S	S	S	S	S	S	S	S	PORCINE	FECAL
33C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
33F	R	R	S	R	R	R	S	S	PORCINE	FECAL
34C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
34F	S	S	S	S	S	S	S	S	PORCINE	FECAL
35C	S	S	S	R	S	S	S	S	PORCINE	CARCASS
35F	R	R	R	R	R	IR	S	S	PORCINE	FECAL
36F	S	S	S	S	S	S	S	S	PORCINE	FECAL

9C 3	S	S	S	S	S	S	S	S	PORCINE	CARCASS
39F	S	S	S	S	S	S	S	S	PORCINE	FECAL
3C	R	R	S	R	R	R	S	S	PORCINE	CARCASS
3F	IR	R	R	R	R	IR	R	IR	PORCINE	FECAL
40C	R	S	S	R	S	S	R	S	PORCINE	CARCASS
40F	S	S	S	S	S	S	S	S	PORCINE	FECAL
42F	S	S	S	S	S	S	S	S	PORCINE	FECAL
42F	S	S	S	S	S	S	S	S	PORCINE	FECAL
43C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
44C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
45F	R	S	S	S	S	S	S	S	PORCINE	FECAL
46C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
46F	S	S	S	S	S	S	S	S	PORCINE	FECAL
48C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
49F	S	S	S	S	S	S	S	S	PORCINE	FECAL
4C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
4F	R	R	R	R	R	R	S	S	PORCINE	FECAL
51F	S	S	S	S	S	S	S	S	PORCINE	FECAL
52C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
52F	IR	R	S	R	IR	R	S	S	PORCINE	FECAL
52F	S	S	S	S	S	S	S	S	PORCINE	FECAL
54F	S	S	S	S	S	S	S	S	PORCINE	FECAL
55C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
5C	IR	R	S	IR	R	S	S	S	PORCINE	CARCASS
5F	R	R	S	IR	R	S	S	S	PORCINE	FECAL
60C	R	R	R	R	R	R	S	S	PORCINE	CARCASS
61C	S	S	S	IR	S	S	S	S	PORCINE	CARCASS
62C	R	S	S	S	S	S	S	S	PORCINE	CARCASS
63C	S	S	S	IR	S	S	S	S	PORCINE	CARCASS
63F	S	S	S	S	IR	IR	S	S	PORCINE	FECAL
64C	S	S	S						PORCINE	CARCASS
64F	S	S	S	S	S	S	S	S	PORCINE	FECAL
65C	S	S	S	S	R	S	S	S	PORCINE	CARCASS
65F	R	R	R	R	R	IR	R	S	PORCINE	FECAL
66C	S	S	S	S	S	R	S	S	PORCINE	CARCASS
66C	S	S	S	S	IR	R	S	S	PORCINE	CARCASS
67F	S	S	S	IR	S	S	S	S	PORCINE	FECAL
68F	S	S	S	S	S	S	S	S	PORCINE	FECAL
67F										
69C	S	S	S	S	S	S	S	S	PORCINE	CARCASS

69F	S	S	S	R	S	S	S	S	PORCINE	FECAL
6C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
6F	R	S	S	S	S	S	S	S	PORCINE	FECAL
70C	S	S	S	R	S	S	S	S	PORCINE	CARCASS
72C	R	R	R	IR	R	R	S	S	PORCINE	CARCASS
73F	S	S	S	S	S	S	S	S	PORCINE	FECAL
74C	IR	S	S	R	S	R	S	S	PORCINE	CARCASS
74F	S	S	S	R	S	S	S	S	PORCINE	FECAL
75C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
75F	IR	S	IR	R	S	S	R	S	PORCINE	FECAL
76C	R	R	S	IR	R	S	S	S	PORCINE	CARCASS
76F	S	S	S	S	S	S	S	S	PORCINE	FECAL
77C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
77F	IR	S	S	S	S	S	S	S	PORCINE	FECAL
78C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
78F	S	S	S	S	S	S	S	S	PORCINE	FECAL
79C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
79F	S	S	S	S	S	S	S	S	PORCINE	FECAL
7C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
7F	IR	R	S	R	R	IR	S	S	PORCINE	FECAL
80C	IR	S	S	IR	S	S	S	S	PORCINE	CARCASS
80F	R	R	R	R	R	R	R	S	PORCINE	FECAL
81C	S	S	S	S	S	S	S	S	PORCINE	CARCASS
81F	S	S	S	S	S	S	S	S	PORCINE	FECAL
82F	S	S	S	S	S	S	S	S	PORCINE	FECAL
84F	S	S	S	S	S	S	S	S	PORCINE	FECAL
8C	R	R	S	IR	R	S	S	S	PORCINE	CARCASS
8F	S	S	S	S	S	S	S	S	PORCINE	FECAL
9C	R	R	R	R	R	S	IR	S	PORCINE	CARCASS
B10Ci	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B10Cii	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B10Fii	S	S	S	S	S	S	S	S	BOVINE	FECAL
B11Ci	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B11Cii	S	S	S	R	S	IR	S	S	BOVINE	CARCASS
B12Ci	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B12Cii	S	S	S	IR	S	IR	S	S	BOVINE	CARCASS
B12Ciii	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B12Ciii	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B12Civ	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B14Fi	S	S	S	S	S	S	S	S	BOVINE	FECAL
B14Fiii	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B15Ciii	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B16Ci	S	S	S	R	S	IR	S	S	BOVINE	CARCASS
B16Cii	S	S	S	IR	S	S	S	S	BOVINE	CARCASS

B16Fi	S	S	S	S	S	IR	S	S	BOVINE	FECAL
B17Fii	S	S	S	S	S	S	S	S	BOVINE	FECAL
B18Ci	S	S	S	IR	S	IR	S	S	BOVINE	CARCASS
B18Fi	S	S	S	S	S	IR	S	S	BOVINE	FECAL
B19Ci	S	S	S	IR	S	IR	S	S	BOVINE	CARCASS
B19Fi	S	S	S	R	S	S	S	S	BOVINE	FECAL
B1Ciii	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B21Ciii	S	S	S	IR	S	S	S	S	BOVINE	CARCASS
B23Cii	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
B23F	S	S	S	S	S	S	S	S	BOVINE	FECAL
B2Fi	S	S	S	S	S	IR	S	S	BOVINE	FECAL
B2Fii	S	S	S	IR	S	IR	S	S	BOVINE	FECAL
B2Fii	S	S	S	S	S	S	S	S	BOVINE	FECAL
B2OC	S	S	S	R	S	S	S	S	BOVINE	CARCASS
B3C	S	S	S	R	S	S	S	S	BOVINE	CARCASS
B3Cii	S	S	S	R	S	S	S	S	BOVINE	CARCASS
B5Ci	S	S	S	S	S	S	S	S	BOVINE	CARCASS
B6Fi	S	S	S	IR	S	IR	S	S	BOVINE	FECAL
B6Fii	S	S	S	S	S	S	S	S	BOVINE	FECAL
B7Fi	S	S	S	S	S	S	S	S	BOVINE	FECAL
B7Fii	S	S	S	S	S	S	S	S	BOVINE	FECAL
B8Fci	IR	R	S	S	R	S	S	S	BOVINE	CARCASS
B8FiiC	S	S	S	R	S	IR	S	S	BOVINE	CARCASS
B9Fi	S	S	S	S	S	IR	S	S	BOVINE	FECAL
BJOPC	S	S	S	S	S	IR	S	S	BOVINE	CARCASS
|C\\P	S	S	S	S	S	S	S	S	POULTRY	PHARYNGX
C02	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C14	S	S	S	R	S	S	S	S	POULTRY	CLOACA
C15	S	S	S	S	S	IR	S	S	POULTRY	CLOACA
C16B	S	S	S	S	S	IR	S	S	POULTRY	CLOACA

C19	R	R	S	S	R	S	S	S	POULTRY	CLOACA
C1K	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C20	IR	R	S	S	R	S	S	S	POULTRY	CLOACA
C21	S	R	S	S	R	S	S	S	POULTRY	CLOACA
C22	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C23	IR	R	S	S	R	S	S	S	POULTRY	CLOACA
C40D	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C42	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C45D	R	R	R	R	R	R	S	S	POULTRY	CLOACA
C49	S	R	S	S	R	S	S	S	POULTRY	CLOACA
C50	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C51a	S	S	S	S	S	IR	S	S	POULTRY	CLOACA
C57	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C57b	S	S	S	S	S	IR	S	S	POULTRY	CLOACAL
C58	S	S	S	S	S				POULTRY	CLOACAL
C60	S	S	S	S	S	IR	S	S	POULTRY	CLOACA
C61a	S	S	S	S	S	IR	S	S	POULTRY	CLOACA
C61b	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C61C	R	R	IR	IR	R	S	S	S	POULTRY	CLOACA
C63	S	S	S	IR	S	S	S	S	POULTRY	CLOACA
C64C	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C64P	S	S	S	S	S	IR	S	S	POULTRY	PHARYNGX
C68C	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C70C	S	R	S	R	R	R	S	S	POULTRY	CLOACA
C70P	S	R	S	R	R	R	S	S	POULTRY	PHARYNGX
C71	S	S	S	R	S	S	S	S	POULTRY	CLOACA
C72D	R	R	S	S	R	IR	S	S	POULTRY	CLOACA
C73D	S		S						POULTRY	CLOACA
C74D	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C77K	R	S	S	S	S	S	S	S	POULTRY	CLOACA
C82K	S	R	S	R	R	S	S	S	POULTRY	CLOACA

C82P	S	R	S	S	R	S	S	S	POULTRY	PHARYNGX
C83K	IR	R	S	R	R	R	S	S	POULTRY	CLOACA
C83P	S	S	S	S	S	S	S	S	POULTRY	PHARYNGX
C84P	S	S	S	S	S	S	S	S	POULTRY	PHARYNGX
C85P	S	R	S	S	R	S	S	S	POULTRY	PHARYNGX
C86K	S	R	S	S	R	S	S	S	POULTRY	CLOACA
C87K	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C87P	S	S	S	S	S	S	S	S	POULTRY	PHARYNGX
C89K	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C89P	IR	S	S	R	S	S	S	S	POULTRY	PHARYNGX
C90K	S	S	S	S	S	S	S	S	POULTRY	CLOACA
C90P	S	S	S	S	S	S	S	S	POULTRY	PHARYNGX
C91K	IR	S	S	S	S	S	S	S	POULTRY	CLOACA
Cc16	S	S	S	S	S	S	S	S	POULTRY	CLOACA
B10Fi	S	S	S	S	S	S	S	S	BOVINE	FECAL
B15C	S	S	S	S	S	S	S	S	BOVINE	CARCASS

Key: TET = TETRACYCLINE, COT = COTRIMOXAZOLE, CHL = CHLORAMPENICAL, AMP = AMPICILLIN, SULF = SULFAMETHOXAZOLE, ST = STREPTOMYCIN, KANA = KANAMYCIN, GENTA = GENTAMYCIN.

S = Sensitive, IR = Intermediate resistance, R = Resistance.

Appendix 3: Genotypic characterization of ampicillin resistant *E. coli* isolates from food animals using PCR

Isolate	Source	Phenotype	*Bla*$_{TEM}$ gene
3C	Porcine	Resistant	+
65C	Porcine	Resistant	+
80F	Porcine	Resistant	-
74F	Porcine	Resistant	+
40C	Porcine	Resistant	+
33F	Porcine	Resistant	+
29F	Porcine	Resistant	+
18C	Porcine	Resistant	+
69F	Porcine	Resistant	-
82K	Poultry	Resistant	+
45D	Poultry	Resistant	+
70C	Poultry	Resistant	+
70P	Poultry	Resistant	+
89P	Poultry	Resistant	+
19Ci	Bovine	Intermediate	+
3Cii	Bovine	Resistant	+
2Fii	Bovine	Intermediate	+
3C	Bovine	Intermediate	+
8F2C	Bovine	Intermediate	+

Isolate	Source	Phenotype	*Bla*$_{TEM}$ gene
11Cii	Bovine	Resistant	+
21Ciii	Bovine	Intermediate	+
12Cii	Bovine	Intermediate	+
16Ci	Bovine	Resistant	+
18Ci	Bovine	Intermediate	+
C16ii	Bovine	Intermediate	+
63	Poultry	Intermediate	+
61C	Poultry	Intermediate	+
14	Poultry	Resistant	+
71	Poultry	Resistant	+

Appendix 4: Genotypic characterization of tetracycline resistant genes among *E. coli* isolates from food animals using PCR

Isolate	Source	Phenotype	*Tet(A)*	*Tet(B)*
31F	Porcine	Intermediate	-	-
7F	Porcine	Intermediate	-	-
5F	Porcine	Resistant	+	-
9C	Porcine	Sensitive	-	-
75F	Porcine	Intermediate	+	-
33F	Porcine	Intermediate	-	-
65F	Porcine	Resistant	+	-
72C	Porcine	Resistant	-	-
18C	Porcine	Intermediate	+	-
20C	Porcine	Intermediate	-	-
29F	Porcine	Resistant	+	-
60C	Porcine	Resistant	-	+
4F	Porcine	Resistant	-	-
40C	Porcine	Resistant	-	-
5C	Porcine	Intermediate	-	+
74C	Porcine	Intermediate	+	-
35F	Porcine	Sensitive	-	-

Appendix 4 continued

Isolate	Source	Phenotype	*Tet(A)*	*Tet(B)*
3C	Porcine	Resistant	+	-
62C	Porcine	Resistant	-	-
24C	Porcine	Sensitive	-	-
16C	Porcine	Intermediate	-	-
12C	Porcine	Sensitive	-	-
91K	Poultry	Intermediate	-	-
20	Poultry	Intermediate	-	-
89P	Poultry	Intermediate	+	-
23	Poultry	Intermediate	+	-
19	Poultry	Resistant	+	-
72D	Poultry	Resistant	-	-
45D	Poultry	Resistant	+	-
61C	Poultry	Resistant	-	-

Appendix 5: Genotypic characterization of sulfonamide resistance genes and class 1 integrons among *E. coli* isolates from food animals using PCR.

Isolate	Source	Phenotype	*SulI*	*SulII*	Class1 integron
20	Poultry	Resistant	+	-	-
23	Poultry	Resistant	-	+	-
19	Poultry	Resistant	-	+	-
72D	Poultry	Resistant	-	-	-
45D	Poultry	Resistant	+	+	-
61C	Poultry	Resistant	-	ND	-
29F	Porcine	Resistant	+	+	-
4F	Porcine	Resistant	-	+	-
9C	Porcine	Resistant	-	+	-
33F	Porcine	Resistant	-	-	-
65F	Porcine	Resistant	-	+	+
85P	Poultry	Resistant	+	-	+
5F	Porcine	Resistant	-	+	-
16C	Porcine	Resistant	+	+	-
12C	Porcine	Resistant	-	-	-
35F	Porcine	Resistant	-	-	-
3C	Porcine	Resistant	-	+	-
72C	Porcine	Resistant	-	+	+
60C	Porcine	Resistant	+	-	-

Key: + = positive; - = negative, ND = not done.