CHARACTERIZATION OF AMINOGLYCOSIDE AND CHLORAMPHENICOL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA ENTERICA* SEROTYPES FROM SOME FOOD ANIMALS IN KENYA

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

To my wife Catherine Wachuka and daughter Rebecca Mwende who shared with me the day to day troubles and lifted my spirits when my energy ran low and were patient to miss my attention during the tedious task of writing this thesis. I thank my wife for her love, energetic support and understanding, which helped me to pursue my academic dreams and aspirations.

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ABBREVIATIONS

3'CS	3'conserved segment
5'CS	5'conserved segment
59-be	59 base element
AAC	Aminoglycoside acetyltransferase
AFLP	Amplified Fragment Length Polymorphism
ANT	Aminoglycoside adenyltransferase
APH	Aminoglycoside phosphorylases
API	Analytical Profile Index
ATCC	American Type Culture Collection
bp	Base pair(s)
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAT	Chloramphenicol acetyltransferase
CCC	Covalently closed circular
CDC	Centers for Disease Control and Prevention
CFU/ml	Colony forming units per millilitre
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLDT	Cytolethal distending
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetre
CNF	Cytotoxic necrotising factor
DAEC	Diffusely adherent *Escherichia coli*
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme

dATP	Deoxyadenosine triphophate
DCA	Deoxycholate citrate agar
dCTP	Deoxycytidine triphosphate
ddNTP	Dideoxynucleotide
dfr	Dihydrofolate reductase gene
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DT	Definitive Type
eae	*Escherichia coli* attaching effacing
EAggEc	Enteroaggregative *Escherichia coli*
EDP	Energy dependent phase
EDTA	Ethylene diamine tetraacetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EMB	Eosin Methylene Blue
EMBL	European Molecular Biology Laboratory
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
E-test	Epsilon test
EU	European Union
F	Fimbrial
FAO	Food and Agriculture Organisation
g	Gram
H antigen	Flagellar antigen
h	Hour
H_2S	Hydrogen Sulphide

HC	Hemorrhagic colitis
HCL	Hydrochloric acid
Hly	Hemolysin
HUS	Hemolytic uremic synderome
IMViC	Indole, Methyl red, Voges-Proskauer and Citrate
Int	Integrase
IS	Insertion sequence
K antigen	Capsular antigen
kb	Kilo base pairs
KDa	Kilodalton
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide
LT enterotoxin	Heat labile enterotoxin
M	Molar
MDR	Multidrug resistant
MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
mob	Mobilisation gene(s)
MOPS	Morpholinopropasulfonic
MUCAP	Methylumbelliferyl caprylate

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nanogram
nm	Nanometre
NORM/NORM-VET	Norwegian monitoring program for Resistance in Microbes/Norwegian monitoring program for Resistance in Microbes in veterinary and food production sectors
NTEC	Necrotizing *Escherichia coli*
NTS	Non-typhoid *Salmonella* species
O antigen	Somatic antigen
OC	Open circle
OD	Optical density
OIE	Office International des Epizootics (World Organisation for Animal Health)
ORF	Open reading frame
*ori*T	Origin of transfer
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Random Amplification of Polymorphism DNA
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium deodecyl sulphate
ser.	Serotype
SPI	*Salmonella* pathogenicity island
spp.	Species
SRF	Substracted Restriction Fingerprinting
ST	Heat stable
STEC	Shiga toxin *Escherichia coli*
Stx	Shiga toxin
subsp.	Subspecies
TAE	Tris- Acetate-EDTA
TES	Tris-EDTA-Sodium chloride
Tn	Transposon
tra	Transposition gene(s)
tRNA	Transfer RNA
Tsh	Temperature sensitive hemagglutinin
TSI	Triple Sugar Iron
TTSS	Type three secrection system
U	unit
USA	United States of America
v/v	Volume per volume
Vi antigen	Capsular antigen
WHO	World Health Organisation
ZI	Zone of inhibition
α	Alpha

β	Beta
ε	Epsilon
μ	Microns
μg	Microgram
μg/ml	Microgram per millilitre
μl	Microlitre

ABSTRACT

The emergence of antimicrobial resistance among pathogenic and commensal bacteria has become a serious problem worldwide and an understanding of the molecular mechanisms by which it emerges and spreads is crucial in designing intervention strategies to reduce its progression. In this study, the prevalence and antimicrobial susceptibility of *Escherichia coli* and *Salmonella* isolates from food animals were determined. Thirty *E. coli* and three *Salmonella enterica* isolates were characterized for the molecular basis of aminoglycoside and chloramphenicol resistance. A total of 235 (82.5%) and 16 (5.6%) of 285 samples were positive for *E. coli* and *Salmonella* isolates, respectively. The isolation rate for *E. coli* was 72.7%, 84.7% and 90.5% in samples from cattle, chickens and pigs, respectively and ranged from 0.0% in cattle and chickens to 13.8% in pigs for *Salmonella*. Three *Salmonella* serotypes were identified, with *Salmonella enterica* subsp. *enterica* serotype Saintpaul (*S.* Saintpaul) being the predominant serotype (64.3%) followed by *S.* Heidelberg and *S.* Braenderup.

Among the *E. coli* isolates, resistance was found in 65.5% and multidrug-resistance (resistance to ≥ 2 antimicrobials) in 37.9% of the isolates. Resistance was more frequently observed in chicken isolates. The most common resistances were to ampicillin, streptomycin, tetracycline, sulphamethoxazole/trimethoprim or kanamycin (42.5-11.9%). Resistances to kanamycin, sulphamethoxazole/trimethoprim, and tetracycline were significantly lower in isolates from cattle (2.5-7.5%) than from the other species (12.0-40.0%) ($p < 0.01$). Resistance to streptomycin was significantly higher in cattle and chicken than in pigs while resistance to ampicillin was higher in pigs ($p = 0.001$). Chloramphenicol resistance varied from 2.0% in chickens to 9.5% in pigs. Similar antimicrobial resistance rates were observed among the faecal (29.9%) and carcass swab (33.1%) *E. coli* isolates from both cattle and pigs. Forty resistance patterns were recorded. For *Salmonella*, resistance to at least ampicillin, chloramphenicol, streptomycin or tetracycline was observed in 37.5% and multidrug resistance in 12.5% of the *Salmonella* isolates belonging to serotypes Saintpaul and

Braenderup. Twenty three (76.7%) of 30 *E. coli* isolates characterized for the molecular basis of aminoglycoside and chloramphenicol resistance harboured plasmids ranging in size from 2 to 106 kb while all three *Salmonella* isolates were plasmid-free. Twenty eight (96.6%) of the 29 *E. coli* and two of the *Salmonella* isolates resistant to streptomycin contained at least one of the two streptomycin resistance genes tested. All *Salmonella* isolates were positive for *strA* gene only. Among the *E. coli* isolates, 21 (72.4%) were positive for *strA* and *aadA1* while the remaining seven isolates (24.1%) were positive for *strA* gene only. The *catA1* gene was detected in all the chloramphenicol-resistant *E. coli* and *Salmonella* isolates. All the kanamycin- and/or gentamicin-resistant *E. coli* isolates were negative for the two genes analysed.

Sequence analysis of an unusual large *strA* amplicon of ~1.2 kb from *E. coli* isolates revealed disruption of the *strA* gene by the insertion of a functionally active trimethoprim resistance gene cassette *dfrA14*. Such amplicons were observed in 10 isolates, all of which harboured also the *aadA1* gene. Two small plasmids of 6 and 8 kb mediating resistance to streptomycin and sulphonamides, and to streptomycin, sulphonamides and tetracycline, respectively, were identified. The *strA* gene was physically linked to the sulphonamide resistance gene *sul2* in both plasmids. The *strA* and *catA1* genes were conjugally co-transferred with resistances to ampicillin, tetracycline and sulphamethoxazole/trimethoprim. The *aadA1* gene was found as cassette-borne gene within class 1 integrons and was linked to trimethoprim resistance gene cassette *dfrA1*.

This study shows that multidrug resistant (MDR) *E. coli* isolates are prevalent in cattle, pigs and chickens and on fresh cattle and pig carcasses in Kenya. Secondly, *Salmonella* is present in pigs at slaughter and on pork carcasses, and pigs are a potential source of single and multiple antimicrobial-resistant non-typhoidal *Salmonella enterica* subsp. *enterica* serotypes that could pose a public health hazard in human infections. To my knowledge this is the first

report of *S.* Heidelberg among food animals in Kenya. The most prevalent resistance patterns observed in this study were towards antimicrobial agents commonly used in food animals.

The data suggest that selection pressure imposed by the use of tetracycline derivatives, aminoglycosides, sulphonamides and penicillins in food animals is a key driving force in the selection of antimicrobial resistance in *E. coli* and non-typhoidal *Salmonella enterica* subsp. *enterica* serotypes. This study further revealed that *E. coli* and *Salmonella* serotypes Braenderup and Saintpaul from food animals in Kenya may represent a reservoir of streptomycin and chloramphenicol resistance genes which may be transferable to other bacteria. The physical linkage of streptomycin resistance gene *strA* to sulphonamide resistance gene *sul2* offers the possibility of co-selection of either of these genes when there is selective pressure imposed by the use any one of the antimicrobials. The location of *strA* and *catA1* genes on conjugative plasmids and the *aadA1* gene within class 1 integrons constitute an effective way to spread streptomycin and chloramphenicol resistances among bacteria from different ecosystems.

It is recommended that prudent use of antimicrobials in food animals should be encouraged, slaughter hygiene improved and adequate heat processing of foods of animal origin in order to minimize the risk of transfer of antimicrobial resistant bacteria to humans. A national antimicrobial resistance surveillance program in food animals and detailed epidemiological and molecular studies on acquisition of resistance genes and distribution of antimicrobial-resistant *E. coli* and *Salmonella* isolates among food animals, food products and humans in Kenya are necessary.

CHAPTER ONE

1.0: GENERAL INTRODUCTION

1.1. Introduction

The emergence of antimicrobial resistance among pathogenic and commensal bacteria has become a serious problem worldwide that affects treatment of infectious diseases both in humans and in animals resulting in decreased productivity, increased morbidity and mortality, and increased costs (Bischoff *et al.*, 2002). The major influences on the amplification and spread of antimicrobial-resistant bacteria are the use of antimicrobial agents in human medicine and their use in livestock for therapy, metaphylaxis, prophylaxis and growth promotion (Hart *et al.*, 2004). The World Health Organization (WHO) has recommended that, unless a risk-based evaluation demonstrates their safety, the use of antimicrobial agents in food animals for growth promotion that belong to classes of antimicrobials used in humans should be terminated (WHO, 2000). Similar recommendations to discontinue the use of human antimicrobial agents as growth promoters in food animals have been made in the United States of America (USA) and the European Union (EU) (Anderson *et al.*, 2003). In 1998, the EU withdrew approval of four growth promoters (tylosin, spiramycin, bacitracin, and virginiamycin) because of their structural relatedness to antimicrobial agents used in human medicine (Anderson *et al.*, 2003).

Resistant bacteria and resistance genes from domestic animals can be transmitted to man indirectly via the food chain or directly from the animals (Helmuth and Hensel, 2004) and potentially result in food-borne illness in humans that is less responsive to treatment with conventional antimicrobial drugs. Commensal bacteria such as *Enteroccus* spp. and *E. coli* can develop resistance and thus be a source of resistance genes that can be further spread to pathogenic bacteria (NORM/NORM-VET, 2003). *E. coli* is the most common enterobacterium found in different animal species (Kijima-Tanaka *et al.*, 2003) and can serve

as an indicator bacterium that easily acquires antimicrobial resistances. The prevalence of resistance in commensal *E. coli* is a good indicator of the selective pressure of antibiotic use and resistance problems to be expected in pathogenic bacteria. In food-producing animals, a low prevalence and degree of antibiotic resistance in the intestinal flora should be considered a distinguishing quality and safety mark (van den Bogaard *et al.*, 2000). The intestinal flora in food animals in Kenya could be exposed to a great selective pressure since more than 95% of the antimicrobials used in these animals are orally administered (Mitema *et al.*, 2001).

In regard to zoonotic bacteria, such as *Salmonella* spp. and *Campylobacter* spp., it has been documented that resistant isolates can be transferred from animals to humans through food and in this way contribute to the resistance problem in human medicine (NORM/NORM-VET, 2003). Salmonellosis is considered as one of the most widespread foodborne zoonoses in industrialized as well as developing countries, even though the incidence seems to vary between countries (Molla *et al.*, 2003a). Farm animals often carry *Salmonella*, affecting meat, dairy products and eggs (Cabrera *et al.*, 2004) and so act as sources of contamination, which is of paramount epidemiological importance in non-typhoid human salmonellosis (Acha and Szyfres, 2001). In the USA and Europe, farm animals are the major reservoir and foods of animal origin are major vehicles of non-typhoidal *Salmonella* spp. (NTS) infection in humans (Threlfall, 2000; Ahmed *et al.*, 2000). In contrast, Kariuki *et al.* (2002) found in Kenya that the majority of NTS studied, including *S.* Typhimurium and *S.* Enteritidis from animals, were different genotypes from NTS strains isolated from humans.

Given the association of certain *Salmonella* serotypes with food poisoning and the likehood that some strains may be multiply resistant to various antimicrobials, a complete understanding of the risk posed by these pathogens during processing of foods of animal origin requires that the serotype and antimicrobial resistance profile of the isolates be determined in addition to their prevalence (McEvoy *et al.*, 2003). *Salmonella* isolates display high natural susceptibility levels to the most commonly used antimicrobial agents (Stock and

Wiedemann, 2000). However, the occurrences of *Salmonella* strains showing resistance to one or more antibacterial agents have steadily increased, probably due to continuous antibiotic pressure in human and veterinary medicine (Orman *et al.*, 2002; Molla *et al.*, 2003b). Of particular concern is the isolation of ceftriaxone- and ciprofloxacin-resistant *Salmonella*, because of the importance of these two agents in treating *Salmonella* infections in children and adults (Chiu *et al.*, 2002; Fey *et al.*, 2000), respectively. This problem is especially relevant in developing countries like Kenya, where lack of economic resources does not allow a wide antibacterial armentarium (Cabrera *et al.*, 2004).

While the antimicrobial resistance of commensal *E. coli* isolates of avian origin in Kenya has been reported (Bebora *et al.*, 1994, Kariuki *et al.*, 1999), data on the prevalence and patterns of resistance of *E. coli* from other food-producing animals are unavailable. Moreover, to date, only a single study (Kariuki *et al.*, 2002) has extensively analysed the levels of resistance to antimicrobial agents in *Salmonella* isolated from food-producing animals in Kenya. The WHO, the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) have emphasized the importance of monitoring antimicrobial resistance in veterinary medicine and have published several reports and recommendations in this regard (NORM/NORM-VET, 2003). Consequently, several European countries (Wray and Gnanou, 2000) as well as Canada (CIPARS, 2002) and the USA (CDC, 2001) have established national surveillance programmes to assess bacterial susceptibility to antimicrobials among enteric bacteria from healthy animals. However, no such monitoring program exists for the antimicrobial resistance in food animals in Kenya.

Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the anti-infective armamentarium (Kotra *et al.*, 2000) to treat a broad range of life-threating infections in humans and animals (Gonzalez-Zorn *et al.*, 2005). On the other hand chloramphenicol is a broad-spectrum antibiotic that has been used extensively to treat bacterial infections in many countries for many years and in less developed settings, it retains

a major role in the treatment of bacterial meningitis in humans (Shultz *et al.*, 2003). In Kenya, as in the EU or the USA, chloramphenicol is not approved for use in food animals.

Resistance against aminoglycosides and chloramphenicol in *E. coli* and *Salmonella* serotypes of food animal origin has been reported in various parts of the world (Bywater *et al.*, 2004; Bischoff *et al.*, 2005). One of the most common resistance mechanisms against aminoglycosides is the production of aminoglycoside acetyltransferases (AACs), aminoglycoside phosphorylases (APHs), and aminoglycoside adenyltransferases (ANTs) (Shaw *et al.*, 1993), which are mainly mediated by transferable large plasmids (Yamane *et al.*, 2005). The phosphotransferase *aph(3'')-Ib* and *aph(6)-Id* genes (also known as *strA* and *strB*, respectively) and the adenyltransferase gene *ant(3'')-Ia* (also designated *aadA1*) are the most frequently encountered streptomycin resistance genes in *E. coli* (Reyes *et al.*, 2003), *Salmonella spp.* and other Gram-negative bacteria (Gebreyes and Altier, 2002). The genes *aph(3')-Ia* and *ant(2'')-Ia* are among the commonly reported genes mediating resistances to kanamycin and neomycin, and to kanamycin, tobramycin and gentamicin in *E. coli* (Sandvang and Aarestrup, 2000; Guerra *et al.*, 2003; Saenz *et al.*, 2004) and diverse *Salmonella* serotypes (Frech *et al.*, 2003).

Resistance to chloramphenicol is known to be mediated enzymatically by the plasmid-located chloramphenicol acetyltransferases (CATs) (Cannon *et al.*, 1990) or by the nonenzymatic chloramphenicol resistance genes *cmlA* (Dorman and Foster, 1982), or *floR* that encode efflux pumps (Cloeckaert *et al.*, 2001). The *floR* gene is similar in primary structure to *cmlA* and confers resistance to both chloramphenicol and florfenicol (Bischoff *et al.*, 2005). The emergence of *Salmonella* spp. and *E. coli* isolates with multiple-antibiotic-resistance phenotypes, involving co-resistance to four or more unrelated families of antibiotics, has been previously reported and is considered a serious health concern (Gebreyes and Altier, 2002; Maynard *et al.*, 2003; Saenz *et al.*, 2004). Resistance phenotypes may arise from many different genetic determinants and each determinant may present specific epidemiological

features (Lanz *et al.*, 2003). Therefore, the assessment of the resistance situation at the genetic level is an important aspect in the understanding and control of antimicrobial resistance (Lanz *et al.*, 2003).

Transfer of resistance genes by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons (Schwarz and Chaslus-Dancla, 2001; Carattoli, 2001) is an important factor that can contribute to the increase in MDR bacteria (Saenz *et al.*, 2004). Integron-borne gene cassettes conferring resistance to aminoglycosides and chloramphenicol are much diffused in MDR *E. coli* (Sandvang and Aarestrup, 2000; Bischoff *et al.*, 2005) and *Salmonella* (Gebreyes and Altier, 2002; Nogrady *et al.*, 2005) isolates.

Integrons are genetic units containing elements for site-specific recombination, capture and mobilization of gene cassettes (Hall and Stokes, 1993). The class 1 integrons are the best characterized (Reyes *et al.*, 2003) and the most frequently detected integrons among *Enterobacteriaceae* (Guerra *et al.*, 2003), and are important in the proliferation of bacterial multidrug resistance in these species (Chen *et al.*, 2004). Integrons of this class comprise two conserved segments of variable length, within which are found antibiotic resistance gene cassettes (Recchia and Hall, 1995). The 5'conserved end (5'CS) encodes a DNA integrase (IntI1) that mobilizes and inserts gene cassettes through a site-specific recombinational mechanism at a specific site (*attI*) adjacent to the *IntI* gene (Hall and Stokes, 1993). The 5'CS also contains a promoter sequence, P_{ant}, needed for the expression of most of the genes carried on cassettes (Collis and Hall, 1995). The 3'conserved end (3'CS) of class 1 integrons includes a truncated antiseptic resistance gene (*qacEΔ1*), a sulphonamide resistance gene (*sul1*) and an open reading frame (*orf5*) of unknown function (Recchia and Hall, 1995)

Gene cassettes consist of a gene coding region (or opening reading frame) and a recombination site known as a 59-base element which is located downstream of the gene in the linear integrated form (Hall and Stokes, 1993). The 59-base elements vary in sequence and length, but are all imperfect inverted repeats and are related to a consensus sequence at

their outer ends (Hall *et al.*, 1991). The 59-base element plays an essential role in the process of gene acquisition, because it is recognized by the integron-encoded DNA integrase (Int) (Hall *et al.*, 1991) and the recombination crossover occurs between the G and the first T of the GTT triplet of the seven-base core site found in each 59-base element at the end distal to the 3` end of the gene (Hall *et al.*, 1991).

Previous studies in Kenya have reported aminoglycoside and/or chloramphenicol resistance in bacteria of animal origin (Gakuya *et al.*, 2001; Kariuki *et al.*, 2002), but none of them has analysed the genetic background underlying these resistance phenotypes and the mechanisms responsible for their wide diffusion in *E. coli* and *Salmonella* strains. The determination of the genetic location and the potential linkage of the genes responsible for aminoglycoside or chloramphenicol resistance with other antimicrobial resistance genes among MDR *E. coli* and *Salmonella* isolates are important in predicting the risk of further spread or persistence of the resistance.

1.2: OBJECTIVES

1.2.1. General objective

The overall objective of this study was to determine the phenotypic and genetic characteristics, gene location and transferability of aminoglycoside and chloramphenicol resistance among *E. coli* and *Salmonella* isolates from cattle, pigs and chickens.

1.2.2. Specific objectives

1. To isolate and determine the prevalence of *E. coli* and *Salmonella* from cattle, pigs and chickens
2. To determine the antimicrobial susceptibility of the isolates
3. To investigate the presence and distribution of streptomycin resistance genes *strA* and *aadA1*, chloramphenicol resistance genes *catA1*, *catA3* and *cmlA*, kanamycin resistance gene *aph(3)-1a* and gentamicin resistance gene *ant(2)-1a* among the isolates
4. To determine the location of these genes in genome of bacterial isolates and whether they are transferable by conjugation and/or transformation

CHAPTER TWO

2.0: LITERATURE REVIEW

2.1. *ESCHERICHIA COLI*

Escherichia coli (*E. coli*) is a member of the family *Enterobactericeae* and is commonly found in the intestinal tract of humans and warm-blooded animals and, as a result of faecal contamination during slaughter, is often found in soil, water, and foods (Schroeder *et al.*, 2002). The bacterial species *E. coli* includes a variety of different types that range from avirulent, commensal strains that are present in the normal intestinal flora to highly virulent strains that cause a variety of severe infections in both humans and animals (Bean *et al.*, 2004).

2.1.1. Identification of *E. coli*

2.1.1.1. Physical and biochemical characteristics

E. coli grows readily in media commonly used in microbiology laboratories (Michael *et al.*, 1985). On eosin methylene blue (EMB) agar they have a characteristic appearance of a greenish metallic sheen, which is a helpful property for identification (Michael *et al.*, 1985). On MacConkey agar, colonies are reddish and a red zone is produced in the media around the colonies. Morphologically, *E. coli* is a short Gram-negative, non-spore forming and usually peritrichuous and fibriate bacillus (Holts *et al.*, 1994), 0.5 μm by 10 μm by 30 μm varying from coccoid bipolar shaped to long filamentous forms (Michael *et al.*, 1985). *E. coli* forms acid and gas from a wide variety of fermentable carbohydrates (Sooka *et al.*, 2004). Although lactose is often fermented, some strains utilise it slowly.

The majority of the *E. coli* strains decarboxylate lysine and ornithine, form acid from Sodium mucate and frequently utilise Sodium acetate as a sole source of carbon (Sooka *et al.*, 2004). Ninety nine percent of *E. coli* isolates are indole-positive, making this an ideal test for

differentiation from other enteric bacteria (Sooka *et al.*, 2004). *E. coli* is also identified using the Analytical Profile Index (API) 20E. The API 20E system uses 20 miniature reaction compartments (cupules) and is a standardized, miniaturized version of conventional procedures for rapid identification of *Enterobacteriaceae* and other Gram-negative bacteria. The major advantage of the API 20E system is that it is a standardized system that is more convenient and easier to use in identification of Gram-negative bacteria than the conventional tests (Juang and Morgan, 2001).

2.1.1.2. Serotyping

Kauffmann *et al.* (1946) proposed an antigenic scheme for *E. coli* based on three types of surface antigen [(somatic (O), capsular (K) and flagella (H] produced by this organism. The somatic or O antigens are thermostable surface antigens found in all smooth Enterobacteriaceae (Sooka *et al.*, 2004). The O antigen is the O-specific polysaccharide of the cell wall lipopolysaccharide (LPS) (Michael *et al.*, 1985). The O antigen is demonstrated by agglutination with type specific sera (Sooka *et al.*, 2004). K or capsular antigens are composed of polysaccharide except for K88 and K99, which are proteins (Sooka *et al.*, 2004). The K antigens are detected by immunoelectrophoresis (Ewing *et al.*, 1986). The H or flagella antigens are heat-labile and proteinaceous in nature (Ewing *et al.*, 1986). Detection of H antigen is achieved by agglutination with specific sera (Sooka *et al.*, 2004). A specific combination of O and H antigens defines the serotype of an isolate (Sooka *et al.*, 2004) and over 700 serotypes have been recognized (Kaper *et al.*, 2004).

2.1.1.3. Molecular detection

Multiplex PCR assays have been successfully developed and can be used to detect virulent *E. coli* in clinical settings and in food and environmental samples (Sooka *et al.*, 2004).

Its specificity and sensitivity has been demonstrated in the presumptive identification of *E. coli* O157:H7 using PCR that targerts the *eaeA* gene (Oberst *et al.*, 1998) and is increasingly being used for rapid and quantitative detection of virulent *E. coli* in food samples (Sooka *et al.*, 2004).

2.1.2. Virulence factors

Although normally commensal in nature, certain strains of *E. coli* are associated with a variety of infections in humans and animals (Yang *et al.*, 2004). The pathogenicity of *E. coli* strains is considered to be mainly determined by specific virulence factors often organized in large blocks, called pathogenic islands, which are either located on the chromosome or large plasmids or are transmitted by bacteriophages (Bean *et al.*, 2004). The virulence factors for *E. coli* strains include adhesins and several exotoxins (Frydendahl, 2002). Fimbrial types F4 (K88), F5 (K99), F6 (987P), and F107 and intimin, an outer membrane protein encoded by the *E. coli* attaching effacing (*eae*) gene, play a role in adhesion to mucosal surfaces (Imberechts *et al.*, 1992). The exotoxins produced by *E. coli* include heat-stable (STa and STb) or heat-labile (LT) enterotoxins, Shiga toxins (Stx1 and Stx2), cytotoxic necrotizing factors (CNF1 and CNF2) (Sarrazin *et al.*, 2000) and hemolysins (α-Hly and ε-Hly) (Salvadori *et al.*, 2003). Other virulence factors include the lipopolysaccharide, temperature-sensitive hemagglutinin (Tsh), and increased serum survival factor (ISS) (La Ragione and Woodward, 2002; Mellata *et al.*, 2003).

2.1.3. *E. coli* infections

In chickens, *E. coli* may cause infections of the respiratory tract and soft tissues, resulting in collibacilosis, air sacculitis, and cellulitis (Gross, 1991). Avian pathogenic *E. coli* most commonly belong to O1, O2, or O78 (Mellata *et al.*, 2003). In swine, pathogenic *E. coli* causes neonatal and postweaning diarrhoea and oedema (Bertschinger, 1999). The *E. coli* serogroups

associated with neonatal and postweaning diarrhoea and oedema belong to a limited number of serogroups including, O8, O138, O139, O141, O147, and O157 (Nagy and Fekete, 1999). *E. coli* is an important pathogen in bovine neonates and causes intestinal and extraintestinal infections (Salvadori *et al.*, 2003). In dairy cows, *E. coli* is regarded as opportunistic and environmental pathogen that can cause infection and inflammation of the mammary gland (Bean *et al.*, 2004). In humans, *E. coli* causes such infections as gastroenteritis, urinary tract infections, neonatal meningitis, septicaemia and haemorrhagic colitis (Sussman, 1997). The strains that cause diarrhoea, acute gastroenteritis or colitis in humans are referred to as enterovirulent *E. coli* (Sooka *et al.*, 2004).

2.1.3.1. Classification of enterovirulent strains of *E. coli*

2.1.3.1.1. Enteropathogenic *E. coli* (EPEC)

The principal feature of infections caused by enteropathogenic *E. coli* (EPEC) is the attaching-and-effacing (A/E) histopathology observed in intestinal biopsies from patients or infected animals (Nataro and Kaper, 1998). The intimate adherence of enteropathogenic *E. coli* to epithelial cells is mediated by a 94-97 KDa outer membrane protein known as intimin first reported by Jerse *et al.* (1990). The fimbrial adhesin F5 (K99) plays a role in the colonization of epithelial cells in the small intestine of calves (Acres, 1985) and occasionally piglets (Moon *et al.*, 1977). EPEC illness is characterised by profuse watery diarrhoea, fever, malaise and vomiting (Sooka *et al.*, 2004).

2.1.3.1.2. Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) or Shiga toxin (Stx)-producing strains are characterised by their ability to produce Shiga-like toxins and to induce attaching and effacing lesions (Gyles, 1994). The Shiga toxin produced by *E. coli* strains (STEC) is similar to Shiga-

toxin produced by *Shigella dysenteriae* type 1 (Salvadori *et al.*, 2003). *E. coli* producing Stx-1 and/or Stx-2 is a cause of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in humans (Sooka *et al.*, 2004). Most cases of HC and HUS are caused by ingestion of contaminated foods and drinks. Less frequent modes of transmission of the infection are cattle-to-person or person-to-person direct contact (Mainil, 1999). Cattle frequently excrete strains of STEC in their faeces and this may represent a source of infection (Blanco *et al.*, 1996) however, asymptomatic STEC infection in household contacts represents a potential source of infection via person-to-person transmission (Ludwig *et al.*, 2002).

2.1.3.1.3. Enterotoxigenic *E. coli* (ETEC)

Enterotoxin-producing *E. coli* (ETEC) have been identified as the causative agent of several important diarrheal diseases in animals and humans. These bacteria may produce thermolabile (LT-I and LT-II) and thermostable (STa and STb) enterotoxins (Elwell, 1980) that result in a secretory diarrhoea (travellers' diarrhoea) similar to that of *Vibrio cholerae* (Sooka *et al.*, 2004). Cytotoxic necrotising factor (CNF)-producing *E. coli*, known as necrotizing *E. coli* (NTEC), have been isolated from animals with enteritis (De Rycke *et al.*, 1987) and from humans with extraintestinal infections (Caprioli *et al.*, 1987). NTEC can produce two types of CNF (CNF1 or CNF2) that can be distinguished by the morphological alterations induced in HeLa cells, by cross-neutralization assays, by the specific necrotizing activity of CNF2 in mouse footpads, and by the presence of other virulence factors associated with NTEC (De Rycke *et al.*, 1987). CNF1 is produced by α-hemolysin-positive *E. coli* strains that cause extraintestinal infections in humans (Blanco *et al.*, 1992).

2.1.3.1.4. Enteroinvasive *E. coli* (EIEC)

EIEC strains penetrate the intestinal epithelial cells to produce inflammatory or dysenteric diarrhoea and have been implicated in foood-borne outbreaks as a cause of diarrhoeal disease (Sooka *et al.*, 2004).

2.1.3.1.5. Other enterovirulent *E. coli*

Other enterovirulent *E. coli* strains include the enteroaggregative *E. coli* (EAggEC) strains associated with acute or persistent diarrhoea (Vila *et al.*, 2000), diffusely adherent *E. coli* (DAEC) strains that exhibit a diffuse pattern of adherence to Hep-2 cells but their pathogenic ability is not well understood and cytolethal distending (CLDT)-producing *E. coli* (Sooka *et al.*, 2004). CLDT *E. coli* isolates are not associated with diarrhoea, however the toxin causes elogation of cells at 24 h, followed by cellular distension and cytotoxicity (Sooka *et al.*, 2004).

2.1.4. Antimicrobial resistance in *E. coli*

In animals, antimicrobial resistance in normal gut flora has been observed in cattle, chickens and pigs in Japan (Kijima-Tanaka *et al.*, 2003), Europe (Bywater *et al.*, 2004), and in turkeys in The Netherlands (van den Bogaard *et al.*, 2001). In Kenya, resistance has been reported in *E. coli* isolates from poultry (Bebora *et al.*, 1994; Kariuki *et al.*, 1997), rats (Gakuya *et al.*, 2001) and animal products (Ombui *et al.*, 1995). Antimicrobial-resistant *E. coli* has also been isolated from healthy humans (Okeke *et al.*, 2000). Varying frequencies of resistance to aminoglycoside and chloramphenicol in *E. coli* strains have been reported (Guerra *et al.*, 2003; Bywater *et al.*, 2004). *E. coli* isolates from clinical specimens are mostly resistant to multiple antimicrobials (Yu *et al.*, 2004) and strains with decreased susceptibility to fluoroquinolones have been observed (Kijima-Tanaka *et al.*, 2003).

2.2: *SALMONELLA*

2.2.1. Classification and sources of *Salmonella*

The genus *Salmonella* is a typical member of the family *Enterobactericeae* and consists of only two species, *S. bongori* and S. *enterica*, with S. *enterica*, being divided into six subspecies: *enterica*, *salamae*, *arizonae*, *houtenae, diarizonae* and *indica* (Velge *et al.*, 2005). *Salmonella* nomenclature is complex and scientists use different systems to refer to this genus. For example, *Salmonella enterica* subsp. *enterica* serotype/serovar Enteritidis is often abbreviated as *Salmonella* serotype (ser.) Enteritidis or *Salmonella* Enteritidis. Serovars of *S. enterica* subsp. *enterica* are given names (usually geographical names) whereas serovars of other subspecies are designated by their O:H formula (Grimont *et al.*, 2000). Most types of *Salmonella* live in the intestinal tracts of farm animals and as a result of contamination, are also found in soil, water, and foods (van Duijkeren *et al.*, 2002). These serotypes are referred to as nontyphoid *Salmonella* species (NTS) and are capable of producing disease in both animals and in humans. The typhoidal *Salmonella* species are adapted to humans and they are the cause of typhoid fever.

2.2.2. Identification of *Salmonella*

2.2.2.1. Phenotypic methods

2.2.2.1.1. Physical and biochemical characteristics

The genus *Salmonella* consists of Gram-negative, nonspore-forming bacilli (Velge *et al.*, 2005), which are generally motile with peritrichous flagella, grow on nutrient agar, facultative anaerobes, ferment glucose, often with production of gas. They reduce nitrates to nitrites and the oxidase test is negative (Grimont *et al.*, 2000). The following biochemical characteristics are used for *Salmonella* identification: urea not hydrolysed, tryptophan and phenylalanine not deaminated, acetoin is produced, lactose, adonitol, sucrose, salicin and 2-ketogluconate not

fermented, hydrogen sulphide (H_2S) produced from thiosulphate, lysine and ornithine decarboxylated, growth on Simmons citrate agar, 4-methylumbelliferyl caprylate (MUCAP) hydrolysed. Noteworthy, *S.* Typhi never decarboxylates ornithine and fails to grow in Simmons citrate agar. *S.* Paratyphi fails to produce H_2S, decarboxylates lysine and fails to grow in Simmons citrate agar (Grimont *et al.*, 2000). Both manual and automated API 20E systems are also widely used in identification of *Salmonella.*

2.2.2.1.2. Serotyping

Identification of various serovars of *Salmonella* is based on the presence of lipopolysaccharide (somatic or O antigen), flagella (H antigen, phase I and II) and capsular (Vi) antigen on the bactertial cell surface as determined by serum agglutination (Popoff *et al.*, 2003). Each *Salmonella* serogroup has a group-specific O-antigen and within each O-group, different serovars are distinguished by a combination of O and H antigens that are present. Within the *S. enterica* subsp. *entericae*, the most common O-antigen serogroups are A, B, C1, C2, D and E. Strains within these serogroups cause approximately 99% of the *Salmonella* infections in humans and warm-blooded animals (Uzzau *et al.*, 2000). Serotypes in other subspecies are usually isolated from cold-blooded animals and the environment but rarely from humans (Uzzau *et al.*, 2000).

2.2.2.1.3. Biotyping

A biotype is the biochemical variation between two microbes of the same serotype (Ekperigin and Nagaraja, 1998). Biotypes have been described in serovar Typhimurium (Descamp *et al.*, 1982). However, the choice of tests for biotyping has often been empirical (Grimont *et al.*, 2000). Utilization of *d*-tartrate is used to separate two biotypes in serovar Paratyphi B. Biotype Paratyphi B cannot utilize d-tartrate whereas biotype Java can. Biotype

Java commonly associated with diarrhoea and isolated from stools, whereas biotype Paratyphi B is often asociated with paratyphoid and isolated from blood or stools (Grimont *et al.*, 2000).

2.2.2.1.4. Phage typing

Phage typing evaluates the susceptibility or resistance of isolates to a set of selected bacteriophages (Grimont *et al.*, 2000). Thus, the phage type reflects differences between two organisms within the same serotype with different susceptibilities to a lytic bacteriophage (Varnam and Evan, 1993). Phage typing has played a central role in epidemiological studies in *S.* Typhimurium and in understanding the evolution of the *S.* Enteritidis pandemic (Ekperigin and Nagaraja, 1998). Although *Salmonella* phage-typing is cheap and requires no expensive equipment, its requirement for well-trained personel has limited it to reference laboratories (Grimont *et al.*, 2000).

2.2.2.2. Genotypic methods

These are molecular typing techniques that are useful in defining clonal relationship between strains. They have been used for epidemiological investigation of salmonellae (Ebner and Mathew, 2001) and in assessing the distribution of *Salmonella* strains within food-processing environments (Giovannacci *et al.*, 2001).

2.2.2.2.1. Plasmid profiling and plasmid restriction profiling

This method compares the plasmid content of bacterial isolates. Plasmid DNA is extracted and plasmids of different sizes separated by agarose gel electrophoresis and viewed after ethidium bromide staining under UV illumination. Isolates derived from the same epidemic strain will have plasmids with identical sizes (Grimont *et al.*, 2000). This test requires

the presence of at least one plasmid type. More precise results are obtained when plasmid DNA is extracted, digested by restriction endonuclease and the fragments separated by agarose gel electrophoresis. Identical plasmids should have the same restriction pattern (Tacket, 1989). However, due to the mobility of plasmids, plasmid profiles are not considered to be stable strain characteristics (Terletski *et al.*, 2004). This method has been applied to *Salmonella* isolates in various studies and proved to be particularly suitable for studies of the transfer of antimicrobial resistance or virulence genes. Determination of antimicrobial resistance patterns have also been widely used as additional tools in epidemiological studies (Terletski *et al.*, 2004).

2.2.2.2.2. Ribotyping

Ribotyping is performed by hybridisation of bacterial DNA with molecular probes specific for rDNA (Stull *et al.*, 1988). The bacterial DNA is extracted, purified, digested by a restriction endonuclease and the fragments separated by gel electrophoresis. The fragments in the gel are transferred to a nylon membrane, to maintain their relative positions and hybridized with a labelled mixture of 16S + 23S rRNA. Depending on the label, autoradiography or immunoenzymatic reaction yields a fragment pattern, often referred to as ribotypes (Grimont and Grimont, 1986). For ribotyping of *Salmonella* isolates, an automated RiboPrinter™ has been used successfully (Terletski *et al.*, 2004).

2.2.2.2.3: IS*200* typing

IS*200* typing is based on hybridisation of restriction endonuclease digested whole cell DNA with a gene probe specific for IS*200*, an insertion element that preferentially occurs in *Salmonella* isolates (Stanley and Saunders, 1996). The *Salmonella* isolates can be differentiated by comparing the restriction patterns of bacterial DNA after hybridisation with the IS*200*

probe. Strains differ by the number of visualized fragments (IS*200* number of copies) and the size of fragments (Grimont *et al.*, 2000). This method has proved to be a valuable additional tool for the analysis of isolates of various *Salmonella enterica* subsp. *enterica* serovars usually involved in food-borne outbreaks, such as Typhimurium (Stanley *et al.*, 1993).

2.2.2.2.4. Random Amplification of Polymorphic DNA (RAPD)

RAPD also known as AP-PCR (Arbitrarily Primed PCR) is a rapid genomic typing method of broad application (Lin *et al.*, 1996). This technique is based on the amplification of anonymous sequences with short random primers (Terletski *et al.*, 2004). The resulting PCR products are visualised by agarose gel electrophoresis and the profiles of amplified products are characteristic of the template DNA. This method has been applied to differentiate *S.* Enteritidis isolates (Lin *et al.*, 1996). The major disadvantage of the method is the poor agreement between laboratories and reproducibility of the RAPD patterns (Meunier and Grimont, 1993).

2.2.2.2.5. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE uses restriction endonucleases, which have infrequently occurring restriction sites in a given bacterial DNA (Grimont *et al.*, 2000). The advantage of this method is that it samples the entire genome avoiding the problem of differential mutation rates between various classes of genomic sequences (Terletski *et al.*, 2004). Although PFGE is highly discriminative for *Salmonella* with *Xba*I, *Bln*I or *Spe*I (Murase *et al.*, 1995), studies show that different restriction endonucleases exhibit variable discriminative power for the different *Salmonella* serovars (Terletski *et al.*, 2004). Disadvantages of this method include limitation in the resolution of the fragment patterns and the relatively high costs for equipment and consumables required (Terletski *et al.*, 2004).

2.2.2.2.6. Amplified Fragment Length Polymorphism (AFLP)

AFLP combines digestion with two suitable restriction endonucleases, ligation of suitable adapters and subsequent PCR amplification. The resulting amplicons may be labelled by fluorescent tags and analysed using an automated sequencer (Terletski *et al.*, 2004). Costs of equipments and consumables are also a major disadvantage in this method (Terletski *et al.*, 2004).

2.2.2.2.7. Subtracted Restriction Fingerprinting (SRF)

The SRF technique is based on digestion of genomic DNA with two restriction enzymes, fragment end biotin labelling followed by selective capture and removal of biotin-tagged DNA fragments by streptavidin-coated magnetic particles (Terletski *et al.*, 2004). SRF has been applied for typing of *Salmonella enterica* subsp. *enterica* serovars Typhimurium (Frech *et al.*, 2003). Some advantages of this method are; high reproducibility, does not require extensive time and labour, comparatively easy interpretation of results and does not require sophisticated equipments (Terletski *et al.*, 2004).

2.2.3. Virulence factors

Differences in virulence among *Salmonella* serovars and in the course of *Salmonella* infections in various host species have been attributed to the variable acquisition and evolvement of virulence factors (Falkow, 1996). A large part of these determinants are clustered in *Salmonella* pathogenicity islands (SPIs) (Marcus *et al.*, 2000). The virulence factors include surface appendages such fimbriae (Edwards *et al.*, 2000) and flagellae (Chilcott and Hughes, 2000) and the type-III-secretion system (TTSS), which facilitate initial adherence and invasion, respectively (Kubori *et al.*, 1998). *Salmonella* strains exhibit starvation-stress response mechanisms that enable them to survive environmental challenges such as nutrient

starvation, oxidative stress or digestive enzymes (Spector, 1998) and produce toxins (Singh *et al.*, 2004).

2.2.4. *Salmonella* spp., host range and infections

The degree of host adaptation varies between *Salmonella* serotypes and affects the pathogenicity for man and animals. Serotypes adapted to man, such as *S.* Typhi and *S.* Paratyphi, usually cause severe diseases in humans such as septicaemic typhoidic syndrome (enteric fever). These serotypes are not usually pathogenic to animals (Velge *et al.*, 2005). Serotypes that are highly adapted to animal hosts, such as *S.* Gallinarum (poultry) or *S.* Abortus-ovis (sheep), usually produce very mild symptoms in man. However, *S.* Choleraesuis which has the pig as the primary host causes severe systemic illness in humans. *S.* Typhi and other human-adapted salmonellae are rarely – if at all – transmitted by food/animals compared to ubiquitous/nontyphoid serotypes (Velge *et al.*, 2005).

Non-typhoidal serotypes, such as *S.* Enteritidis or *S.* Typhimurium, which affect both man and animals, generally cause gastrointestinal infections usually less severe than enteric fever. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonisation in chickens (Cowden *et al.*, 1989). Large outbreaks of human infection have been associated with food-borne transmission including that from contaminated poultry and poultry products, meat and milk and other dairy products (Threlfall, 2000; Ahmed *et al.*, 2000). In Kenya, NTS frequently cause bacteraemic infections among immunocompromised individuals and the very young (Kariuki *et al.*, 2002).

2.2.5. Antimicrobial resistance in *Salmonella*

Salmonella isolates display high natural susceptibility to the most commonly used antimicrobial agents (Stock and Wiedemann, 2000). However, the occurrences of *Salmonella*

strains showing resistance to one or more antibacterial agents have steadily increased, probably due to continuous antibiotic pressure in human and veterinary medicine (Orman *et al.*, 2002; Molla *et al.*, 2003b). Of particular concern is the isolation of ceftriaxone- and ciprofloxacin-resistant *Salmonella*, because of the importance of these two agents in treating *Salmonella* infections in children and adults (Chiu *et al.*, 2002; Fey *et al.*, 2000), respectively. MDR *Salmonella* isolates have been often associated with specific phage types, for example, *Salmonella* Typhimurium DT 104 which is commonly pentaresistant, exhibiting resistance to ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, and tetracycline (Gebreyes and Altier, 2002). Multidrug resistant NTS have become increasingly important bacterial pathogens in developing countries causing bacteraemia and other invasive disease (Graham *et al.*, 2000; Kariuki *et al.*, 2002).

2.2.6. *Salmonella enterica* subsp. *enterica* serotype Heidelberg (*S.* Heidelberg)

S. Heidelberg is group B *Salmonella* which apparently accounts for a small proportion of cases of diseases in humans (Mammina *et al.*, 2003). However, a high prevalence of *S.* Heidelberg in both human and non-human sources, mainly food and livestock, has been reported in the USA and Canada (Demczuk *et al.*, 2003). Isolates of *S.* Heidelberg alternate with those of *S.* Enteritidis to be the second or third most prevalent *Salmonella* serotype found in human infections in Canada and the USA (Demczuk *et al.*, 2003). In Canada, serovar Heidelberg is the most common *Salmonella* serovar obtained from non-human sources and is most often found in poultry, eggs (Chambers *et al.*, 1998) and ground beef (Sorensen *et al.*, 2002).

Similarly, serovar Heidelberg has been found most often in Danish turkeys, though it was not often the cause of human infections (Pedersen *et al.*, 2002). In Italy, a nationwide clonal spread of this serotype has been associated with poultry (Mammina *et al.*, 2003) whereas in

Ethiopia *S.* Heidelberg has been reported in camels (Molla *et al.*, 2003a). So far, its sources and distribution in Kenya have not been documented. Serovar Heidelberg infections have been associated with severe disease symptoms, including extraintestinal infections (Wilmshurst and Sutcliffe, 1995), septicemia, and myocarditis (Burt *et al.*, 1990). *S.* Heidelberg has been reported to show increasing resistance to antimicrobial agents and mimics the multidrug resistance observed in *S.* Typhimurium strain DT 104 (Demczuk *et al.*, 2003). Isolates characterized by resistance to ampicillin, streptomycin, tetracycline, and nalidix acid, with additional resistance to kanamycin in two cases, have been reported (Mammina *et al.*, 2003).

2.2.7. *Salmonella enterica* subsp. *enterica* serotype Saintpaul (*S.* Saintpaul)

S. Saintpaul is amongst the top 10 serovars in Australia and the predominant serotypes isolated from food animals, slaughterhouse personnel and retail meat products in Ethiopia (Molla *et al.*, 2003a). *S.* Saintpaul (antigenic formula, 4: eh: 1, 2) has become the most frequent serotype in Japan (Hata *et al.*, 2003). In Australia, *S.* Saintpaul isolates from non-human sources show a wide range of animal sources, including reptilian, bovinek ovine, porcine, equine, canine, avian and marsupial species (Taylor *et al.*, 2000). In Kenya, *S.* Saintpaul has been isolated from humans in small number (Kariuki *et al.*, 2002). This serotype has been associated with foodborne outbreaks due to contaminated paprika in Germany (Lehmacher *et al.*, 1995), water supply in Australia (Taylor *et al.*, 2000) and mangoes in the USA (Beatty *et al.*, 2004).

2.2.8. *Salmonella enterica* subsp. *enterica* serotype Braenderup (*S.* Braenderup)

S. Braenderup has been recognized since 1978 as a cause of human salmonellosis and has also been isolated in India, in faeces of rapaces and cockroaches (Peng, 1992). To date, a number of outbreaks of gastroenteritis due to this serovar have been described, including

association with a contaminated meal served in an airliner in Finland (Hatakka and Asplund, 1993), contamination of meat pies and terrines (Urfer *et al.*, 2000) and recently with eating Roma tomatoes in the United States and Canada (CDC, 2005). *S.* Braenderup has been reported in chickens in Kenya (Kariuki *et al.*, 2002) and as a dominant serotype from mutton and pork in Ethiopia (Ejeta *et al.*, 2004). Molla *et al.* (2003b) reported multiple-drug resistance to up to eight different antimicrobials in *S.* Braenderup isolates from chicken carcass and giblets in Ethiopia. A study undertaken in Canada also indicated that *S.* Braenderup strains were multiple resistant to gentamicin, sulfisoxazole and tetracycline (Poppe *et al.*, 1995).

2.3: AMINOGLYCOSIDE ANTIBIOTICS

2.3.1. Sources of aminoglycosides

Aminoglycoside antibiotics are produced by different species of *Streptomyces* (streptomycin, neomycin, paromomycin, tobramycin, apramycin and kanamycin) or by the genus *Micromonospora* (gentamicin and sisomicin). It is thought that these compounds may have originated as intermediates in certain biosynthetic pathways in the fungi (Smith and Baker, 2002). Streptomycin was the first aminoglycoside to be isolated in 1943 (Smith and Baker, 2002) and was thereafter followed by the successive introduction of a series of milestone compounds including kanamycin, gentamicin, and tobramycin (Mingeot-Leclercq *et al.*, 1999). In the 1970s, the semisynthetic aminoglycosides dibekacin, amikacin, and netilmicin were obtained by chemical modification of kanamycin B, kanamycin A and sisomicin antibiotics, respectively (Mingeot-Leclercq *et al.*, 1999). The aminoglycosides are named after the production organisms, where the '–mycin' suffix is used to refer to the drugs isolated from genus *Streptomyces* while the '–micin' suffix refers to the antibiotics isolated from *Micromonospora* spp. (Mingeot-Leclercq *et al.*, 1999).

2.3.2. Chemical structures and properties of aminoglycosides

The aminoglycosides are a family of molecules containing a molecular nucleus, an aminocyclitol ring that can be streptidine or 2-deoxystrepamine and two or more aminosugars linked by glycosidic bonds to the nucleus (Smith and Baker, 2002). There are two distinct sub-families recognized based upon the structure of the aminocyclitol ring, the streptomycin group which contains a streptidine derivative and the larger kanamycin/neomycin group which contains a less-substituted deoxystreptamine derivative (Smith and Baker, 2002). The kanamycin/neomycin group consists of a central aminocyclitol ring (typically known as the B ring) with two or three substituted aminoglycan rings (A, C and D) linked either at 4 and 5

hydroxyls of the B ring (neomycin, butirosin, lividomycin and paromomycin) or at the 4 and 6 hydroxyls (kanamycin, amikacin, tobramycin and gentamicin) (Smith and Baker, 2002). The structures of several aminoglycoside drugs are shown in Fig. 2.1.

Aminoglycosides are multifunctional hydrophilic sugars that posses several amino and hydroxyl functionalities (Kotra *et al.*, 2000). The amine moieties are mostly protonated in biological media; hence these antibiotics are polycationic and show binding affinity for nucleic acids (Kotra *et al.*, 2000). The aminoglycosides are commonly used as injectable or topical preparations because they are poorly absorbed from the gastrointestinal tract (Hu *et al.*, 2001). When used parenterally, adequate drug concentrations are typically found in bone, synovial fluid and peritoneal fluid (Gonzalez and Spencer, 1998). Penetration of biologic membranes is poor and intracellular concentrations are usually low, with the exception of the proximal renal tubule (Gonzalez and Spencer, 1998).

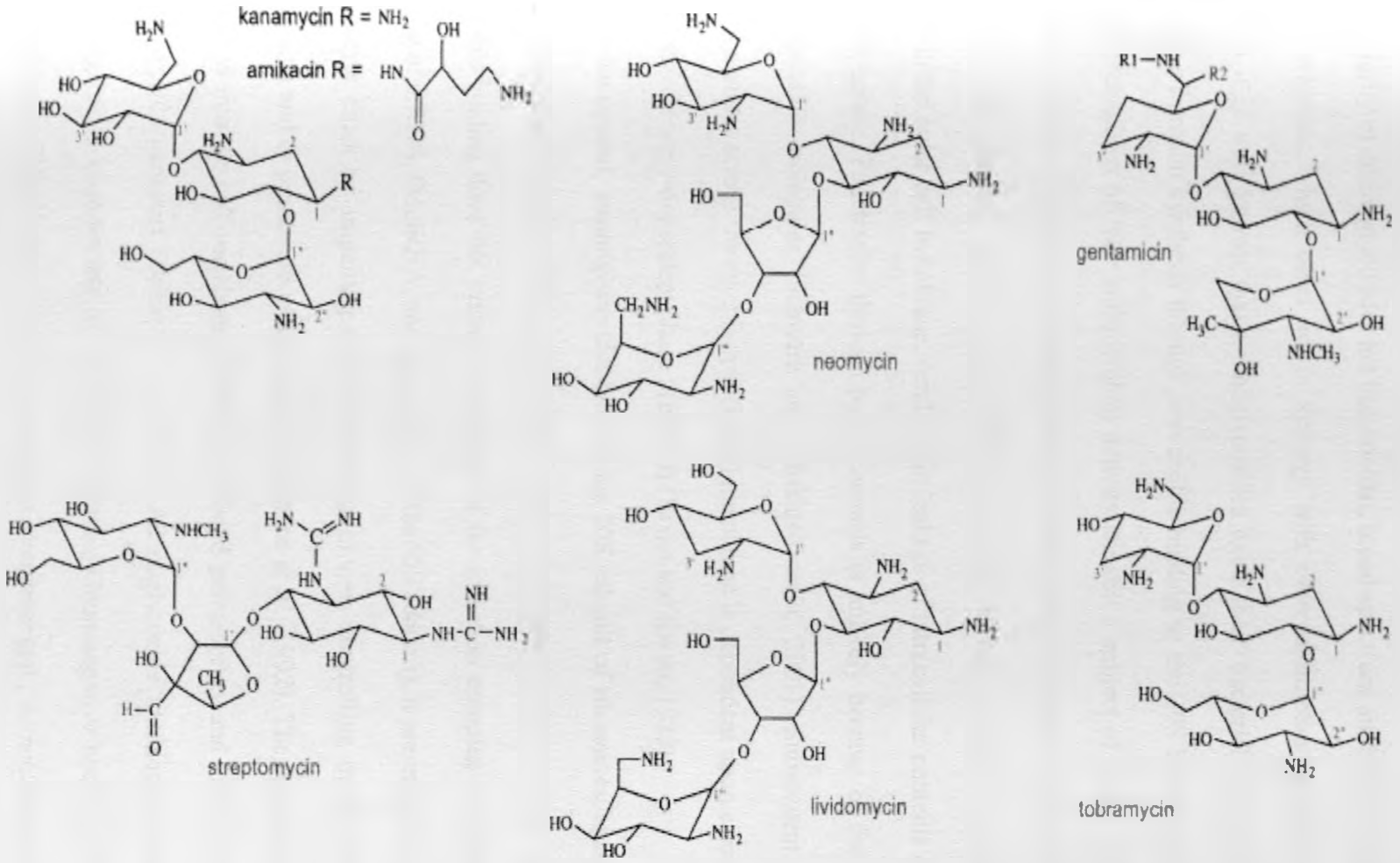


Fig. 2.1: The structures of some of the common aminoglycoside antibiotics. The two variable groups on gentamicin, designated R1 and R2, specify three different forms of gentamicin, C1 (R1 = R2 = CH3), C1A (R1 = R2 = H) and C2 (R1 = H, R2 = CH3).

2.3.3. Mechanisms of action and spectrum of activity

Majority of aminoglycosides are bactericidal, broad-spectrum antibiotics with predictable pharmacokinetics, which often act in synergy with other antibiotics (Kotra *et al.*, 2000). Although it is well known that aminoglycosides exert their bacterial activity primarily by inhibition of protein synthesis through irreversible binding to the 30S bacterial ribosome, the precise mechanisms of their antimicrobial activity are still a subject of study (Vakulenko and Mobashery, 2003). Studies have shown that the initial site of action is the outer bacterial membrane (Vakulenko and Mobashery, 2003) where the cationic antibiotic molecules create fissures in the outer cell membrane, resulting in leakage of intracellular contents and enhanced antibiotic uptake. Penetration through porin channels is unlikely because of the large size of aminoglycoside molecules (Schwarz and Chaslus-Dancla, 2001). Subsequent transport of aminoglycosides across the cytoplasmic (inner) membrane is dependent upon electron transport and is termed energy-dependent phase I (EDP-I) (Bryan and Kwan, 1983).

In the cytosol, aminoglycosides bind to the 30S subunit of ribosomes, again through an energy-dependent process (energy-dependent phase II [EDP-II]) (Bryan and Kwan, 1983). While this binding does not prevent formation of the initiation complex of peptide synthesis (binding of mRNA, fMetRNA, and association of the 50S subunit), it prevents the elongation of the nascent chain by impairing the proofreading process controlling translational accuracy (misreading and/or premature termination) (Melancon *et al.*, 1992). The aberrant proteins may be inserted into the cell membrane, leading to altered permeability and further stimulation of aminoglycoside transport (Busse *et al.*, 1992). Aminoglycoside antibiotics exhibit in vitro activity against a wide variety of clinically important Gram-negative bacilli such as *E. coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp. as well as *Staphylococcus aureus* and some streptococci (Vakulenko and Mobashery, 2003).

Their activity against enterococci is adequate only when they are used synergistically with a cell wall-active antibiotic, such as β-lactams and vancomycin (Gilbert, 2000). Aminoglycosides are also active against *Yersinia pestis* (Frean *et al.*, 1996), *Francisella tularensis* (Baker *et al.*, 1985) and *Brucella* spp. (Akova *et al.*, 1999), the etiological agents of plague, tularemia, and brucellosis, respectively. Arbekacin demonstrates the widest spectrum of antibacterial activity and has remarkable activity against methicillin-resistant *Staphylococcus aureus* (Aoki, 1994), including isolates that exhibit resistance to other aminoglycosides (Cordeiro *et al.*, 2001).

2.3.4. Therapeutic uses

Despite their potential nephrotoxicity and ototoxicity and problems associated with aminoglycoside-resistant organisms, aminoglycosides remain valuable and sometimes indispensable for the treatment of various infections and prophylaxis in specific situations (Kotra *et al.*, 2000; Vakulenko and Mobashery, 2003). Aminoglycosides exhibit several characteristics that make them useful as antimicrobial agents. Among them are concentration-dependent bactericidal activity, postantibiotic effect, relatively predictable pharmacokinetics, and synergism with other antibiotics (Vakulenko and Mobashery, 2003). Aminoglycoside antibiotics are useful for empirical treatment of febrile neutropenic patients and patients with serious infections caused by aerobic Gram-negative microorganisms, including *Enterobacteriaceae* and *P. aeruginosa* (Gilbert, 2000).

Combination with antipseudomonal penicillins or cephalosporins is recommended for treatment of systemic pseudomonal infections. For urinary tract infections, monotherapy with an aminoglycoside is possible because intravenously administered antibiotic is excreted exclusively by the kidneys (Vakulenko and Mobashery, 2003). Aminoglycosides are often used in combination with β-lactams and glycopeptides for the treatment of patients with bacterial endocarditis caused by enterococci and less often for streptococcal endocarditis (Graham and

Gould, 2002). Aminoglycoside-β-lactam combinations are also widely used in mastitis therapy (Mitema *et al.*, 2001). Streptomycin shows excellent activity against *Mycobacterium tuberculosis* and remains a first-line drug in combination chemotherapy for drug-resistant tuberculosis (Gillespie, 2002).

2.3.5. Mechanisms of resistance

Acquired resistance to the aminoglycosides can occur via three different mechanisms: (i) modification of the ribosomal target (ii) decreased antibiotic uptake and accumulation, or (iii) enzymatic modification of the drug leading to inactivation (Vakulenko and Mobashery, 2003).

2.3.5.1. Modification of the ribosomal target

Modifications of the target that produces aminoglycoside resistance include mutational changes in the ribosomal proteins or 16S rRNA and enzymatic methylation of the rRNA (Vakulenko and Mobashery, 2003). Point mutations within the 30S subunit confer resistance to aminoglycosides by altering the binding affinity of the tRNAs (Smith and Baker, 2002). For example, some streptomycin-resistant *M. tuberculosis* strains have been detected with a point mutation in the *rpsl* gene (Davies and Wright, 1997). Streptomycin resistance due to mutational changes in 16S rRNA or ribosomal proteins has occasionally been demonstrated for other microorganisms (Prammananan *et al.*, 1998). A series of special methylases, namely RmtA, RmtB and ArmA that protect microbial 16S rRNA, the main target of aminoglysides, have been identified (Gonzael-Zorn *et al.*, 2005).

2.3.5.2. Decrease in drug uptake and accumulation

The bacterial uptake of aminoglycoside antibiotics requires respiration, which generates an electrical potential across the cytoplasmic membrane (Vakulenko and Mobashery, 2003). A

low level of transmembrane potential or even its absence is responsible for the intrinsic resistance of anaerobic bacteria (Vakulenko and Mobashery, 2003) and decreased susceptibility of facultative anaerobes such as enterococci to aminoglycosides (Moellering, 1991). Active efflux has been evidenced for neomycin, kanamycin, and hygromycin A in *E. coli* (Edgar and Bibi, 1997) protein Mdfa, a member of the family of multidrug resistance proteins. Multidrug active efflux systems have been identified as mechanisms of natural resistance to aminoglycoside antibiotics in Gram-negative bacteria such as *P. aeruginosa* (Westbrock-Wadman *et al.*, 1999) and *E. coli* (Rosenberg *et al.*, 2000).

2.3.5.3. Aminoglycoside-modifying enzymes

Three families of enzymes that perform cofactor-dependent aminoglycoside modification in the bacterial cytoplasm have been recognized; these are aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs) (Vakulenko and Mobashery, 2003). Each of the three families of enzymes is further divided into classes, designated by the site of modification, which is indicated in parentheses. They are further subdivided into enzyme types (designated by Roman numerals) that specify unique resistance phenotypes (Vakulenko and Mobashery, 2003). Individual enzymes of the same class and type that produce the same phenotype but are encoded by different genes are designated by a lowercase letter (Shaw *et al.*, 1993). For example, the AAC(6′)-I enzymes AAC(6′)-Ia, AAC(6′)-Ib, AAC(6′)-Ic, etc., are aminoglycoside acetyltransferases that modify the antibiotic at position 6′ and produce the same phenotype (inactivation of tobramycin, amikacin, netilmicin, kanamycin, and dibekacin), but are encoded by different genes (Vakulenko and Mobashery, 2003).

2.3.5.3.1. Aminoglycoside phosphotransferases

Aminoglycoside phosphotransferases are able to phosphorylate specific hydroxyl groups in all classes of aminoglycoside antibiotics. Seven classes of enzymes, APH(3′), APH(2"), APH(3"), APH(4), APH(7"), APH(6), and APH(9) have been identified in clinical isolates and aminoglycoside-producing organisms (Shaw *et al.*, 1993). Seven different types of APH(3′), APH(3′)-I to APH(3′)-VII, have been identified among Gram-negative and Gram-positive bacteria and also aminoglycoside-producing microorganisms (Shaw *et al.*, 1993). APH(3′)-I produces resistance to kanamycin, neomycin, lividomycin, paromomycin, and ribostamycin (Vakulenko and Mobashery, 2003). The gene for the first APH(3′)-I enzyme (*aph(3′)-Ia*) has been identified on plasmids and transposons in many Gram-negative bacteria (Vakulenko and Mobashery, 2003) and Gram-positive opportunistic human pathogen *Corynebacterium striatum* (Tauch *et al.*, 2000).

The gene for APH(3′)-IIb has been identified in the chromosome of *P. aeruginosa* (Hachler *et al.*, 1996). APH(3′)-III produces resistance to kanamycin, neomycin, lividomycin, paromomycin, butirosin, and ribostamycin (Vakulenko and Mobashery, 2003). Often, *aph(3′)-IIIa* in Gram-positive bacteria is found in combination with genes for other aminoglycoside-modifying enzymes (Udo and Dashti, 2000) or is genetically linked with resistance genes for other classes of antibiotics (Werner *et al.*, 2001). The genes for APH(3′)-IV and -V were detected only in antibiotic-producing microorganisms (Vakulenko and Mobashery, 2003). APH(3′)-VI and APH(3′)-VII produce resistance to amikacin, isepamicin, kanamycin, neomycin, paromomycin, butirosin, and ribostamycin and kanamycin and neomycin, respectively (Shaw *et al.*, 1993). The *aph(2")-Ia* gene, which is found downstream from *aac(6′)-Ie*, encodes the C-terminal part of the bifunctional enzyme AAC(6′)-Ie-APH(2")-Ia which encodes for resistance to gentamicin, tobramycin, and kanamycin (Vakulenko and Mobashery, 2003).

APH(3") and APH(6) modify the 3"- and 6-hydroxyl groups of streptomycin, respectively. The genes *aph(3")-Ib-aph(6)-Id* encoding these two different phosphotransferases (referred to in the literature as *strA* and *strB*) are linked and have been reported in both pathogenic and environmental microorganisms usually associated with the Tn*5393* or Tn*5393*-like transposons that reside on large conjugative plasmids, although the small nonconjugative plasmid RSF1010 that carries this tandem set of genes has also been detected in plant pathogens (Sundin, 2000) and in a fish pathogen (L'Abee-Lund and Sorum. 2000). Table 2.1 summarizes the substrate profiles of the aminoglycoside phosphotransferases.

Table 2.1: Substrate profiles of aminoglycoside phosphotransferases

Phosphotransferase	Substrate(s)
APH (3′)	
I	Kanamycin, neomycin, lividomycin, paromomycin, ribostamycin
II	Kanamycin, neomycin, butirosin, paromomycin, ribostamycin
III	Kanamycin, neomycin, lividomycin, paromomycin, ribostamycin, butirosin, amikacin, isepamicin
IV	Kanamycin, neomycin, butirosin, paromomycin, ribostamycin
V	Neomycin, paromomycin, ribostamycin
VI	Kanamycin, neomycin, paromomycin, ribostamycin, butirosin amikacin, isepamicin
VII	Kanamycin, neomycin
APH(2″)	
Ia (bifunctional enzyme)	Kanamycin, gentamicin, tobramycin, sisomicin, dibekacin
Ib, Id	Kanamycin, gentamicin, tobramycin, netilmicin, dibekacin
Ic	Kanamycin, gentamicin, tobramycin
APH(3″)-Ia, -Ib	Streptomycin
APH(7″)-Ia	Hygromycin
APH(4)-Ia, -Ib	Hygromycin
APH(6)-Ia, -Ib, -Ic, -Id	Streptomycin
APH(9)-Ia, -Ib	Spectinomycin

Adapted from Vakulenko and Mobashery (2003).

2.3.5.3.2. Aminoglycoside Acetyltransferases

Aminoglycoside acetyltransferases comprise four classes of enzymes: AAC(1), AAC(3), AAC(2′), and AAC(6′). Aminoglycoside 6′-acetyltransferases are broad-spectrum enzymes capable of modifying most of the clinically important aminoglycosides (Vakulenko and Mobashery, 2003). Genes for at least 24 AAC(6′)-I enzymes have been identified in both Gram-negative and Gram-positive microorganisms (Vakulenko and Mobashery, 2003). The only two known AAC(6′)-II enzymes confer resistance to gentamicin, tobramycin, netilmicin, and sisomicin, but not to amikacin. Of all the known AAC(6′)s, AAC(6′)-Ib is the most prevalent among various Gram-negative microorganisms (Shaw *et al.*, 1993). The genes for several AAC(6′)-I enzymes have been identified in the chromosomes of Gram-negative bacteria (Vakulenko and Mobashery, 2003). AAC(3)s are widely distributed among different bacterial genera, including aminoglycoside producers (Vakulenko and Mobashery, 2003).

The gene for AAC(3)-Ia was detected on conjugative plasmids and transposons and within gene cassettes in integrons from enterobacteria and *P. aeruginosa* (Poirel *et al.*, 2001). The *aac(3)-Ib* gene has been found fused to another aminoglycoside resistance gene, *aac(6′)-Ib*, located in an integron from *P. aeruginosa* (Dubois *et al.*, 2002). Three *aac(3)-II* genes encoding AAC(3)-IIa, -IIb, and -IIc have been identified in various clinical isolates of Gram-negative bacteria (Vakulenko and Mobashery, 2003). AAC(3)-IV that acetylates gentamicin, tobramycin, netilmicin, sisomicin, apramycin, and dibekacin have been observed, in clinical isolates of *E. coli*. The genes for AAC(3)-VII, -VIII, -IX, and -X have been discovered in aminoglycoside-producing actinomycetes (Vakulenko and Mobashery, 2003). AAC(1) confers resistance to apramycin, paromomycin, lividomycin, and ribostamycin and has been identified in animal isolates of *E. coli* (Lovering *et al.*, 1987). The genes for AAC(2′)-Ib, -Ic, -Id, and -Ie have been detected in mycobacterial species (Ainsa *et al.*, 1997). Table 2.2 summarizes the substrate profiles of the aminoglycoside acetyltransferases.

Table 2.2: Substrate profiles of aminoglycoside acetyltransferases

Acetyltransferase	Substrate(s)
AAC(6′)	
I (at least 24 different enzymes)	Tobramycin, amikacin, netilmicin, dibekacin, sisomicin, kanamycin, isepamicin
II	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, kanamycin
AAC(3)	
Ia, Ib	Gentamicin, sisomicin, fortimicin
IIa, IIb, Iic	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin
IIIa, IIIb, IIIc	Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin, neomycin, paromomycin, lividomycin
IV	Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, apramycin
VII	Gentamicin
AAC(1)	Paromomycin, lividomycin, ribostamycin, apramycin
AAC(2′)-Ia	Tobramycin, gentamicin, netilmicin, dibekacin, neomycin

Adapted from Vakulenko and Mobashery (2003).

2.3.5.3.3. Aminoglycoside Nucleotidyltransferases

Aminoglycoside nucleotidyltransferases (ANTs) comprise five classes, ANT(2"), ANT(3"), ANT(4'), ANT(6), and ANT(9). They utilize ATP as the second substrate and modify aminoglycoside antibiotics by transferring AMP to their hydroxyl group at positions 2", 3", 4', 6, and 9, respectively (Vakulenko and Mobashery, 2003). ANT(2")-Ia produces resistance to gentamicin, tobramycin, sisomicin, dibekacin, and kanamycin (Cameron *et al.*, 1986). The *ant(2")-Ia* gene was found within various genetic backgrounds, including small nonconjugative plasmids, conjugative plasmids (Carattoli *et al.*, 2002), and various transposons and integrons (Centron and Roy, 2002). The gene *ant(3")-I* for ANT(3")-I confers resistance to streptomycin by modifying its 3"-hydroxyl group and to spectinomycin by modifying it at position 9 (Hollingshead and Vapnek, 1985) and are widely distributed among Gram-negative microorganisms (Vakulenko and Mobashery, 2003). The *ant(3")-Ia* gene has been detected within transposons and plasmids among various bacterial species (Vakulenko and Mobashery, 2003). Class 1 integrons harboring *ant(3")-I* genes have been identified frequently among various clinical isolates of *Enterobacteriaceae* (White *et al.*, 2001). The *aadA1* has recently been found in a class 2 integron (Saenz *et al.*, 2004).

The *ant(4')-Ia* genes (often referred to in the literature as *aadD*) encodes resistance to amikacin, tobramycin, dibekacin, isepamicin, and kanamycin (Vakulenko and Mobashery, 2003) and have been detected on the large conjugative plasmid from *S. aureus* (Berg *et al.*, 1998). The gene for ANT(4')-IIa adenyltransferase was originally cloned from *P. aeruginosa* (Jacoby *et al.*, 1990). ANT(6)-I produces resistance to streptomycin, and the *ant(6)-Ia* gene has been found in *Enterococcus faecalis* and *Enterococcus faecium* isolates (Kobayashi *et al.*, 2001). Table 2.3 summarizes the substrate profiles of the aminoglycoside nucleotidyltransferases.

Table 2.3: Substrate profiles of aminoglycoside nucleotidyltransferases

Nucleotidyltransferase	Substrate(s)
ANT(2″)-I	Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin
ANT(3′)-I	Streptomycin, spectinomycin
ANT(4′)-Ia	Tobramycin, amikacin, dibekacin, kanamycin, isepamicin
ANT(4′)-Iia	Tobramycin, amikacin, kanamycin, isepamicin
ANT(6′)-I	Streptomycin
ANT(9)-I	Spectinomycin

Adapted from Vakulenko and Mobashery (2003).

2.4: CHLORAMPHENICOL

2.4.1. Chemical structure and properties of chloramphenicol

Chloramphenicol originally referred to as chloromycetin, was first isolated from a fungus *Streptomyces venezuelae* in 1947 (Ehrlich *et al.*, 1947) and shown to be a broad spectrum antibiotic with a novel structure (Fig. 2.2), remarkable both for p-nitrophenyl group (at C-1) and an N-dichloroacetly substituent (at C-2) attached to a 1,3-propanediol with two chiral centers (C-1 and C-2) (Shaw, 1983). It was the first naturally occurring substance to contain a nitro group ($-NO_2$), which was considered to be responsible for the dose-unrelated aplastic anemia. The relative simplicity of chloramphenicol made it the first naturally occurring substance to be marketed as the product of chemical synthesis and has been produced exclusively this way since 1950 (Schwarz *et al.*, 2004). Chloramphenicol is a highly stable antibiotic which can be stored for prolonged times at room temperature. It is amphiphilic and unionized at physiological pH (Shaw, 1983). Chloramphenicol can pass biological membranes to reach intracellular bacteria and is able to readily traverse the blood-brain barrier (Simon and Stille, 2000).

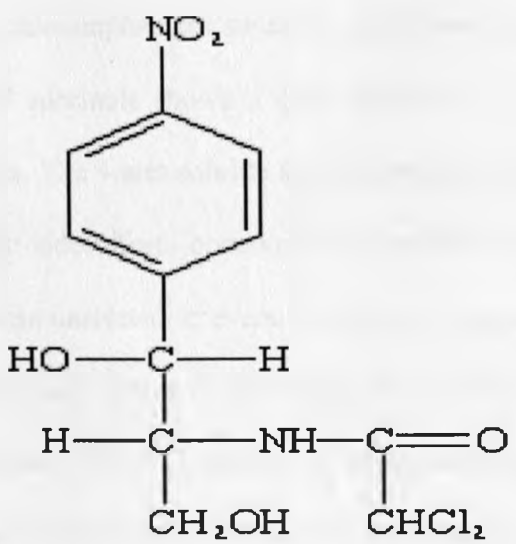


Fig. 2.2. Chemical structure of chloramphenicol

2.4.2. Mode of action and spectrum of activity

In prokaryotes, chloramphenicol is a highly specific and potent inhibitor of protein biosynthesis (Schwarz *et al.*, 2004). Chloramphenicol inhibits bacterial protein biosynthesis by prevention of peptide chain elongation. Its bacteriostatic activity is based on a reversible binding to the peptidyltransferase centre at the 50S ribosomal subunit of 70S ribosomes (Schlünzen *et al.*, 2001). The substrate spectrum of chloramphenicol includes Gram-positive and Gram-negative, aerobic and anaerobic bacteria, but also chlamydiae, mycoplasmas, and rickettsiae (Shaw, 1983). Intrinsic resistance to chloramphenicol has not been observed although members of different bacterial species and genera may differ in their basic levels of susceptibility to chloramphenicol (Schwarz *et al.*, 2004).

2.4.3. Therapeutic use in human and veterinary medicine

Chloramphenicol and some derivatives, such as thiamphenicol and azidamfenicol, have been used over the years in human medicine (Schwarz *et al.*, 2004) and were considered as the drugs of choice to treat salmonellosis in human and veterinary medicine over a long period of time (Nogrady *et al.*, 2005). Certain esters of chloramphenicol, such as chloramphenicol palmitate or chloramphenicol succinate, have been produced for therapeutic applications. Chloramphenicol succinate shows a good solubility in water and therefore is used for parenteral applications. The water soluble azidamphenicol is only used in eye drops (Simon and Stille, 2000). The side-effects observed in connection with the application of chloramphenicol include a dose-unrelated irreversible aplastic anaemia (Simon and Stille, 2000), a dose-related reversible bone-marrow suppression, or the Gray syndrome in neonates and infants (Yao and Moellering, 1999). Occasionally, hypersensitivity to chloramphenicol ranging from skin rashes to anaphylaxis has been observed, too (Yao and Moellering, 1999).

Based on these adverse effects and on the availability of less toxic antimicrobial agents with a similar spectrum of activity, the use of chloramphenicol in humans is nowadays limited to the therapy of a small number of life threatening infections (Shaw, 1983). Since chloramphenicol readily crosses the blood–brain barrier, it remains an alternative therapeutic agent for the treatment of meningitis caused by susceptible strains of *Haemophilus influenzae*, *Neisseria meningitidis* or *Streptococcus pneumoniae* when no other antimicrobial agents can be used, e.g. in penicillin-allergic patients (Mascaretti, 2003). The use of chloramphenicol in veterinary medicine in EU is currently limited to pets and non-food-producing animals. It was banned in 1994 from use in any food-producing animals in the EU. The main reason for this ban was protection of the consumer from potential adverse effects arising from chloramphenicol residues in carcasses of food animals (Schwarz *et al.*, 2004).

2.4.4. Bacterial resistance to chloramphenicol

The frequently encountered mechanism of bacterial resistance to chloramphenicol is enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases (CATs) (Murray and Shaw, 1997). However, there are also reports on other mechanisms of chloramphenicol resistance, such as the non-enzymatic chloramphenicol resistance genes *cmlA* (Dorman and Foster, 1982), or *floR* that encode efflux pumps (Cloeckaert *et al.*, 2001). The *floR* gene confers resistance to both chloramphenicol and florfenicol and is structurally similar to *cmlA* (Bischoff *et al.*, 2005). Inactivation by phosphotransferases, mutations of the target site and permeability barriers have also been reported (Murray and Shaw, 1997).

2.4.4.1. Chloramphenicol acetyltransferases (CATs)

The CAT enzymes encoded by the *cat* family of genes are widespread in Gram-negative bacteria (Nogrady *et al.*, 2005). The *cat* genes are sub-categorised into *catA* and *catB* groups, which are not significantly related to each other (Schwarz *et al.*, 2004). The type A CATs have been detected in a wide variety of bacteria (Schwarz *et al.*, 2004). There are at least 16 distinct groups, A-1 – A-16, of the *catA* genes. The prototype *cat* gene A-1, *catA1*, which is a part of transposon Tn*9* (Frech *et al.*, 2003) has been detected on variety of resistance plasmids of Gram-negative bacteria (Luck *et al.*, 2001). Some groups of *cat* genes including, members of group A-3 and A-7 – A-9 have been found on plasmids or conjugative transposons. The *cat* genes located on plasmids pC221, pC223 or pC194 are inducibly expressed via translational attenuation, with chloramphenicol itself acting as an inducer (Schwarz *et al.*, 2004). Inducible *cat* genes mediate high level resistance to chloramphenicol (Schwarz *et al.*, 2004).

There are at least five different groups of type B *cat* genes (Schwarz *et al.*, 2004) and these genes are often associated with either multiresistance transposons or plasmid-borne multiresistance integrons and have been detected in a variety of enterobacterial species (Pai *et al.*, 2003). Translational attenuation has been proposed as the regulatory mechanism for the chloramphenicol *catB1* gene (Rodgers *et al.*, 2002) which has been reported to confer only low level chloramphenicol resistance (Murray and Shaw, 1997).

2.4.4.2. Chloramphenicol exporters

The export of chloramphenicol from bacterial cells can be mediated by either specific transporters and/or multidrug transporters (Schwarz *et al.*, 2004). Specific transporters have a limited substrate spectrum whereas that multidrug transporters often includes a wide range of unrelated substances. At least eight different groups of specific exporters are currently known (Schwarz *et al.*, 2004). These include the non-enzymatic chloramphenicol resistance due to the

cmlA or *floR* gene genes (Bischoff *et al.*, 2005). The genes referred to as *pp-flo*, *cmlA*-like, *floSt* or *floR*, mediate combined resistance to chloramphenicol and florfenicol (Schwarz *et al.*, 2004). A novel type of exporter gene, designated *fexA* mediating resistance to chloramphenicol and florfenicol has recently been identified on the 34- kb plasmid pSCFS2 from *Staphylococcus lentus* (Kehrenberg and Schwarz, 2004).

A number of multidrug transporter systems have been identified whose substrate spectrum includes chloramphenicol but the levels of chloramphenicol resistance mediated by these systems are generally lower than those mediated by specific exporters (Schwarz *et al.*, 2004). The AcrAB-TolC multidrug efflux system is able to export chloramphenicol at low levels (Schwarz *et al.*, 2004). Overproduction of this system, due to mutation at regulator loci, however, leads to clinical levels of resistance to chloramphenicol and other antimicrobials by active efflux (Lee *et al.*, 2000). The MdfA multidrug transporter which also exports chloramphenicol has been identified in *E. coli* (Edgar and Bibi, 1997).

2.4.4.3. Other resistance mechanisms

Other chloramphenicol resistance mechanisms such as non-enzymatic chloramphenicol resistance based on permeability barriers have been described in various bacteria (Schwarz *et al.*, 2004). The *mar* locus which is present in bacteria of many enterobacterial genera has also been reported to contribute to chloramphenicol resistance (Schwarz *et al.*, 2004). Mutations in the major ribosomal protein gene cluster of *E. coli* and *Baccilus subtilis* (Anderson *et al.*, 1984) as well as in the 23S rRNA gene of *E. coli* (Ettayebi *et al.*, 1985) are known to confer resistance to chloramphenicol. Recently, an rRNA methylase has been described which mediates combined resistance to chloramphenicol, florfenicol and clindamycin by preventing ribosomal binding of these drugs (Kehrenberg *et al.*, 2005). The corresponding gene, *cfr*, has been detected on plasmid pSCFS1 from *Staphylococcus sciuri* (Schwarz *et al.*, 2004).

2.5: METHODS USED FOR THE CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE

2.5.1. Conventional methods for antimicrobial susceptibility testing

2.5.1.1. Disc diffusion susceptibility test

This method measures the ability of drugs to inhibit the growth of microorganisms. A standardized inoculum of the organism is swabbed onto the surface of a Mueller-Hinton (MH) agar plate and the filter paper discs impregnated with specific antimicrobial agents are placed on the agar (Washington, 1985). After overnight incubation at 35 ^{o}C, the diameter of the zone of inhibition (ZI) of bacterial growth around each disc is measured. The size of the ZI is inversely proportional to the minimum inhibitory concentration (MIC) of the organism. This method is an indirect measure of the susceptibility based on MIC zone size correlation. The sizes of the growth inhibition determine the level of resistance. These zonal sizes vary with the molecular characteristics of different drugs. Tablets may also be used in place of discs. Each tablet is made aseptically and contains a standard concentration of the desired drug (Boucassals, 1980). The method is suitable for most rapidly growing pathogens.

2.5.1.2. MIC dilution test

The MIC of a particular drug to a particular organism can be quantitatively measured in-vitro through the agar or broth agar micro/ or macro-dilution test. Agar or broth MIC tests are often considered to be the standard reference method for the evaluation of antibiotic resistance according to Waterworth, (1980). Generally dilution tests are considered satisfactory if the MICs vary no more that ±1 dilution step around the mean on repeated tests. For most purposes a concentration of 128 μg/ml is a satisfactory upper limit for routine testing with any antimicrobial agent. The lowest concentration varies according to the antimicrobial agent. The

range of concentrations should include the end point for appropriate standard strains such as *E. coli* ATCC 25922 to permit adequate control (Washington, 1985).

2.5.1.2.1. Broth dilution MIC determination method

In the broth dilution MIC method, various concentrations of an antimicrobial drug are inoculated with a standard suspension of test bacteria. Following an overnight incubation at 37 °C, the MIC is determined by observing the lowest concentration of the drug that will inhibit visible growth of the test bacteria (Andrew, 2001). For full range MIC testing, 5-8 concentrations representing a therapeutically achievable range for an antimicrobial agent are usually tested. The method can be done using the test tube dilutions (macrodilution) or the plastic microdilution tray (microdilution) (Andrew, 2001).

2.5.1.2.2. Agar dilution MIC determination method

For the agar dilution method different concentrations of antimicrobial agents are incorporated onto MH agar plates (Andrew, 2001). These are inculated with an inoculum of test organisms adjusted to match 0.5 McFarland standards. Inoculation is done either by a calibrated loop or an inoculum-replicating device (replicator). Plates are incubated at 35 °C overnight and read by observing the lowest drug concentration that inhibits visible bacterial growth. This concentration is reported as the MIC. The agar dilution method has advantages of being able to test a number of strains simultaneously, detect microbial heterogeneity or contamination, and has a slightly better reproducibility than broth dilution method (Ericsson and Sherris, 1971).

2.5.1.3. Epsilon test (E-test)

The test provides E-test (Bolmstrom *et al.*, 1988) plastic strips for direct quantification of antimicrobial susceptibility of microorganisms. E-test combines the ease of disc diffusion and accuracy of the MIC broth dilution techniques. It utilizes a rectangular plastic strips that contain predefined, continuous exponential gradient of antibiotic concentrations that correspond to MIC dilutions. This plastic test strips have a reading and interpretive scale corresponding to the 2-fold MIC dilutions indicated on the surface of an inoculated MH plate. The drug on the strip is immediately released and diffused in the agar. After 24 hours of incubation, an elliptical zone of inhibition of bacterial growth is seen around the test strip. The zone edge intersects the plastic strip at a specific level corresponding to the inhibitory concentration of the drug that inhibits the microorganism.

2.5.1.4. Chromogenic disc method

Isolated bacteria can also be screened for antimicrobic-modifying enzymes by commercially available chromogenic disc method (Cockerill III, 1999). This method has been used in virtually all known β-lactamase producing bacteria. The method relies on the visualization of a colored product that results from the hydrolysis of the substrate β-lactam molecule nitrocefin, contained in the paper disc (Cockerill III, 1999). The nitrocefin substrate is also available as a solution which is added to the bacteria lysate containing the enzyme.

2.5.2. Molecular techniques used for the characterization of antimicrobial resistance

2.5.2.1. Plasmid profiling

Plasmids can be isolated from an overnight bacterial broth by a phenol-chloroform extraction method (Kado and Liu, 1980). The principal involves the disruption of the bacterial cell wall by treatment with lysozyme, lysis of the internal cell membranes with detergent and denaturation of the chromosomal DNA by alkaline pH. Plasmid DNA is recovered by ethanol precipitation in the cold. It is electrophoresed through vertical or horizontal agarose slab gel that separates it on the basis of molecular mass during migration towards the anode. Most of the plasmid DNA exists in the covalently closed circular (CCC) form (Hardy, 1981). When one of the two polynucleotide strands of CCC plasmids is broken, an open circle (OC) is formed. Application of shearing forces during plasmid DNA isolation results in the formation of OC forms. Excessive shearing forces results in L forms. Large plasmids are difficult to keep in CCC form during isolation and purification.

Since plasmids often carry antibiotic resistance determinants, plasmid fingerprinting can be used for epidemiological purposes (Aarts *et al.*, 2001). The relatedness of isolated bacterial strains is determined from the number and size of plasmids and their restriction profiles (Aarts *et al.*, 2001). For example *Aeromonas salmonicida* was characterized by restriction enzyme analysis of the high-molecular-weight R-plasmid mediating resistance to oxytetracycline resistance (Adams *et al.*, 1998). Llanes *et al.* (1999) used plasmid profiling in combination with hybridisation analysis and pulsed field gel electrophoresis to characterize the spread of amoxycillin resistance among *Salmonella* spp. The weakness of the analysis of bacterial strains by plasmid profiling is inherent in the fact that plasmids are mobile extrachromosomal elements that can be lost or acquired easily, thus epidemiologically related strains can exhibit different plasmid profiles (Aarts *et al.*, 2001).

2.5.2.2. Restriction endonuclease DNA cleavage pattern analysis

This involves use of enzymes that recognize unique DNA sequences (target) to cleave the double stranded DNA at specific sites within the targets. These restriction enzymes (endonucleases) recognize specific palindromic, base sequences of four or more bases within the DNA molecule and cleave the DNA either within or close to these restriction sequences at defined positions (Platt *et al.*, 1986). The resulting fragments are then resolved electrophoretically in an agarose gel depending on their relative mobility, with smaller fragments moving faster compared to larger ones (Platt *et al.*, 1986). More bands are usually visible after restriction digestion. By analyzing the gel patterns it is possible to identify related genomes (Roberts, 1978). Both chromosomal and plasmid DNA can be digested by restriction endonucleases. Identical chromosomes and plasmids could have identical targets and would be cleaved into identical DNA fragments or if they are different they yield different fragments (Roberts, 1977).

2.5.2.3. Polymerase Chain Reaction (PCR)

PCR is based on repeated cycles in which the amount of a specific target DNA fragment is exponentially increased (Aarts *et al.*, 2001) and involves cycles of heating the sample for denaturing, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase (Fluit *et al.*, 2001). In theory, each round of amplification gives a doubling of the number of DNA target molecules, but the process is seldom 100% efficient because of the presence of inhibitors, and in later rounds of amplification DNA polymerase may become limited (Fluit *et al.*, 2001). Standard PCRs are useful for identifying genes which encode antimicrobial resistance (Cockerill III, 1999). These assays are highly specific, especially if there are no other nucleic sequences harbored by the organism which share significant homology with the target genetic material and large quantities of target nucleic acid are

amplified (Cockerill III, 1999). The latter condition exists when organisms are first propagated by culture and then isolated colonies are used as template for the PCR. Amplifying such large quantities of target nucleic acid reduces the sensitivity requirements for such assays, making it less likely that contamination with extraneous nucleic acid will be a significant problem.

Therefore, provided that negative controls are used, specialized contained specimen processing areas and/or amplicon "sterilization" may be unnecessary (Cockerill III, 1999). Amplicons can also be sterilized, that is, chemically or enzymatically modified, such that they cannot serve as a template for subsequent PCR assays which use the same oligonucleotide primers (Cockerill III, 1999). The PCR amplicons can be confirmed as the desired target nucleic acid (ie., part or all of a resistance-associated genetic material) by electrophoretic mobility determinations, probe hybridisation assays (Southern blotting of electrophoretic gels, slot, dot blot, enzyme linked immunosorbent assay or liqiud hybridisation formats), restriction fragment length polymorphism (RFLP) analysis, or DNA sequencing (Cockerill III, 1999). Several PCR protocols have been adapted to improve gene identification, sensitivity and/or specificity ("nested" PCR assay) and to detect long DNA fragments that may contain several antibiotic resistance genes or to simultaneous detect several antibiotic genes at different chromosomal loci (multiplex PCR) (Aarts *et al.*, 2001). PCR followed by restriction analysis has been used for the detection of mutations associated with resistance to antibiotics (Heisig, 1996) based on the fact that mutations leading to resistance can modify or create recognition sites for endonucleases (Suzuki *et al.*, 1988).

2.5.2.3.1. Cloning of PCR products

The cloning of PCR products provides one with a stable form of the amplified segment with restriction enzyme sites or other sequences useful for subsequent studies of the inserts (Lohff and Cease, 1992). The effeciency of cloning PCR-amplified fragments into plasmid vectors depends on the fragment size, insert toxicity and the complexity of the insert. The larger the DNA fragment, the lower the cloning efficieny. Optimization of the molar concentration ratios of the vector to insert is critical to ensure efficient cloning. A 1:10 dilution of the PCR product in commonly used. The use of fresh PCR products is recommended due to the potential presence of exonucleases that degrade the nucleotide.

The techniques used in cloning PCR products include, TA cloning, blunt-end cloning and directional cloning. TA cloning is used to clone PCR products generated by polymerases like *Taq* polymerase which have terminal transferase activity that preferentially adds adenine to the 3′ ends of the PCR products into a vector containing complementary 3′ thymidine overhangs (TA cloning) (Borokov and Rivkin, 1997). Blunt-end cloning is used to clone PCR products produced by proofreading polymerases such as *Pwo* or *Pfu* (Lohff and Cease, 1992) or polishing *Taq* polymerase products. Proofreading polymerases possess 3′→5′ exonuclease activity that removes the 3′-A over-hangs to generate blunt-end PCR products. Polishing protocols use enzymes to remove the single nucleotide extension from PCR products generated with *Taq* polymerase (Costa and Weiner, 1992). Directional cloning involves the introduction of additional restriction sites at the 5′ end of the primers. The amplified DNA fragment is digested with the appropriate restriction enzyme and ligated into the multiple cloning sites of a linearized vector (Scharf *et al.*, 1986)

2.5.2.4. DNA Hybridisation

Hybridisation is based on the fact that in nucleic acids a cytosine forms base pairs with a guanine and an adenine forms base pairs with either a thymidine (in DNA) or a uracil (in

RNA). In hybridisation, the DNA in a sample is rendered single-stranded and allowed to combine with a single-stranded probe (Fluit *et al.*, 2001). Early hybridisations were performed with target DNA immobilized on a nitrocellulose membrane, but nowadays a variety of different solid supports, including magnetic beads, are used. Other variations include the binding of a capture probe to a solid support. After binding of the target, the probe can hybridize. Probes can be labeled with a variety of reporters, including radioactive isotopes, antigenic substrates, enzymes or chemiluminescent compounds (Fluit *et al.*, 2001). Hybridisations are required to confirm the lack of the targeted gene in the case of negative PCR results due to primer mismachting (Aarts *et al.*, 2001). Moreover, they have successfully been used for the analysis of MDR *E. coli* strains (Aarts *et al.*, 2001).

2.5.2.5. DNA sequencing

Sequencing of DNA can be accomplished by either chemical or enzymatic means. The original (chemical) technique for sequencing, the Maxam and Gilbert sequencing method, relies on the nucleotide-specific chemical cleavage of DNA (Maxam and Gilbert, 1977) and is not routinely used any more (Graham and Hill, 2001). The enzymatic technique, Sanger sequencing, involves the use of dideoxynucleotides (2', 3'-dideoxy) that terminate DNA synthesis and is, therefore, also called dideoxy chain termination sequencing. It utilizes dideoxynucleotides (ddNTPs) to terminate chain elongation during the in vitro synthesis of DNA from a cloned template. Synthesis is initiated using a specific oligonucleotide primer. During the synthesis reaction a radioactive nucleotide (usually dATP) is incorporated into the elongating strands.

Nowadays, labelling of the sequence reaction is done non-radioactive but rather with fluorescent coloring (Graham and Hill, 2001). Four separate reactions are carried out simultaneously, each of which contains all 4 dNTPs and a single ddNTP. The higher the concentration of ddNTP the more frequently chain elongation will terminate. Therefore, one

can regulate the extent of sequence information obtainable by varying the dNTP/ddNTP ratio. Following the extension reactions the products are resolved by electrophoresis in a denaturing (urea) polyacrylamide gel. The results are obtained when the gel is dried and exposed to x-ray film (autoradiography). The size of each fragment is determined by its terminal dideoxynucleotide, so the DNA sequence corresponds to the order of fragments read from the gel. Large-scale DNA sequencing is frequently performed using automated systems, which use fluorescence-labeled primers in dideoxynucleotide sequencing reactions (Graham and Hill, 2001). As the newly synthesized DNA strands are electrophoresed through a gel, they pass through a laser beam that excites the fluorescent label.

The resulting emitted light is then detected by a photomultiplier, and a computer collects and analyzes the data. Automated DNA sequencing remains the "gold standard" for identifying the products of amplification reactions. DNA sequencing of PCR products has become a much cheaper and faster method by virtue of automation (Cockerill III, 1999). Instruments are now available for semi-automated running and analyzing of sequence gels. Therefore, any resistance gene or resistance mutation can be determined relatively easily and economically by direct DNA sequencing (Cockerill III, 1999).

2.5.2.6. Bioinformatics and resources for nucleic acids analysis

To date a large number of antibiotic resistance genes have been discovered and described. For the development of tests for the detection and characterization of antibiotic resistance genes, Internet services are available to retrieve the necessary genetic information and to explore the already sequenced bacterial genomes (Aarts *et al.*, 2001). These services include but are not limited to, database similarity searches such as Blast, sequence retrieval (e.g. Entrez), alignment interfaces and comparative genomics (Aarts *et al.*, 2001). Websites such as www.genome.ad.jp/kegg/kegg.html, www.tigr.org, www.embl-heidelberg.de/services and

http://gonow.to/genomes;www.ncbi.nlm.gov provide sequence and service information (Aarts *et al.*, 2001).

2.6: TRANSFER OF ANTIMICROBIAL RESISTANCE GENES

2.6.1. Elements involved in horizontal transfer of resistance genes

The rapid spread of antimicrobial resistance genes between bacteria of the same and of different species and genera is mainly the result of horizontal transfer events of mobile genetic elements carrying one or more resistance genes (Schwarz and Chaslus-Dancla, 2001). Among them, plasmids, transposons and integrons/gene cassettes play a major role (Schwarz and Chaslus-Dancla, 2001).

2.6.1.1. Plasmids

Plasmids are extrachromosomal DNA elements which have been detected in virtually all bacterial genera of medical or veterinary importance, but also in bacteria which constitute the physiological flora of the skin and the various mucosal surfaces in humans and animals (Schwarz and Chaslus-Dancla, 2001). Plasmids are capable of autonomous replication and vary in size from < 2 kb to > 100 kb. Plasmids belonging to different incompatibility groups can stably coexist in the same bacterial cell (Schwarz and Chaslus-Dancla, 2001). Plasmid-borne properties are not essential for the survival of the bacteria under physiological conditions, but may be of benefit for the bacterium under specific conditions (Schwarz and Chaslus-Dancla, 2001).

These accessory properties include resistance to antimicrobial agents, disinfectants, heavy metal cations, anions, nucleic acid binding substances or bacteriocins. In addition to resistance properties, various other traits are known to be plasmid-borne, such as metabolic properties, virulence properties, and fertility functions (Stanisich, 1988). Plasmids may form

cointegrates with other plasmids, may integrate or be integrated, either in part or in toto, into the chromosomal DNA or can act as vectors for transposons and integrons/gene cassettes (Bennet, 1995). Large plasmids can carry genes (*tra* gene complex) which enable them to move on their own from one host cell to another. Such plasmids are referred to as conjugative plasmids (Schwarz and Chaslus-Dancla, 2001).

2.6.1.2. Transposons

Transposons do not possess replication systems and therefore must integrate for their stable maintenance into replication-proficient vector molecules such as chromosomal DNA or plasmids in the cell. Transposons also vary in size (< 1 kb to 60 kb) and structure. The smallest transposons, also known as insertion sequences, solely carry the gene for a transposase which is responsible for the movement of the element. Larger transposons usually carry one or more additional genes, most of which code for antibiotic resistance properties. Many transposons have little or no target specificity and therefore can insert themselves at various positions in the chromosomal or plasmid DNA. Large conjugative transposons may also harbour *tra* genes (Bager and Helmuth, 2001)

2.6.1.3. Integrons

Integrons are genetic units containing elements for site-specific recombination, capture and mobilization of gene cassettes (Hall and Stokes, 1993) and have primarily been found located within transposons Tn402 and Tn21, which reside on broad host-range plasmids or the IncF plasmids (Villa *et al.*, 2000). Integrons most often represent intact or defective transposons (Schwarz and Chaslus-Dancla, 2001). Based on the sequences of the integrase genes, three classes of integrons, with clinical and epidemiological relevance for antibiotic resistance have been described (Reyes *et al.*, 2003). Class 1, the best characterized integrons

comprise of two conserved segments flanking another, of variable length, within which are found antibiotic resistance gene cassettes (Reyes *et al.*, 2003). The 5' conserved end (5'CS) encodes a DNA integrase (*Int1*) that mobilizes and inserts gene cassettes through site-specific recombinational mechanism at the specific site (*att*I) adjacent to the *IntI* gene (Hall and Stokes, 1993) and also harbours the promoter for the expression of the cassette-borne genes (Schwarz and Chaslus-Dancla, 2001). The 3' conserved end (3' CS) of class 1 integrons includes a truncated antiseptic resistance gene (*qacEΔ1*), a sulphonamide resistance gene (*sul1*) and an open reading frame (*orf5*) of unknown function (Hall and Stokes, 1993). The class 1 integrons are the most frequently detected integrons among *Enterobacteriaceae* (Guerra *et al.*, 2003), and are important in the proliferation of bacterial multidrug resistance in these species (Chen *et al.*, 2004). Class 2 integrons include Tn*7* and its relatives and are associated with resistance to trimethoprim, streptomycin and spectinomycin (Yu *et al.*, 2004).

2.6.1.4. Gene cassettes

Gene cassettes represent small mobile elements of less than 2 kb and, to date, have only been detected in Gram-negative bacteria (Schwarz and Chaslus-Dancla, 2001). They consist of a gene coding region (or opening reading frame) and a recombination site known as a 59-base element (59-be) or *attC* (Carattoli, 2001) which is located 3` to the gene in the linear integrated form (Hall *et al.*, 1991; Hall and Stokes, 1993). Gene cassettes move by site-specific recombination and are usually present at specific sites within an integron (Schwarz and Chaslus-Dancla, 2001). They differ from plasmids by the lack of replication systems, and from transposons by the lack of transposition systems (Schwarz and Chaslus-Dancla, 2001).

2.6.1.4.1. The 59-base element

The 59-be's of the different gene cassettes vary in sequence and length, but are all imperfect inverted repeats and are related to a consensus sequence at their outer ends (Hall *et al.*, 1991). Although several 59-be's conform closely to the 60 bp consensus sequence, many are considerably longer and the longest known is 141 bp (Schwarz and Chaslus-Dancla, 2001). The 59-be's play an essential role in the process of gene acquisition, because they are recognized by the integron-encoded DNA integrase (Int) (Hall *et al.*, 1991) and the recombination crossover occurs between the G and the first T of the GTT triplet of the 7 bp core site with the consensus GTTAGGC or GTTRRRY found in each 59-base element at the end distal to the 3` end of the gene (Stokes *et al.*, 1997). The 59-be's have a twofold axis of symmetry. The most highly conserved feature within the 59-be's are the 7 bp core sites with the consensus GTTAGGC or GTTRRRY and an inverse core site with consensus GCCTAAC or RYYYAAC located at the end proximal to the 3` end of the gene (Stokes *et al.*, 1997).

2.6.2. Resistance transfer mechanisms

Plasmids, transposons and gene cassettes/integrons are spread vertically during the division of the host cell, but can also be transferred horizontally between bacteria of the same or different species and genera via transduction, conjugation/mobilisation or transformation (McManus, 1997; Schwarz and Chaslus-Dancla, 2001).

2.6.2.1. Transduction

Transduction describes a bacteriophage-mediated transfer process. Bacteriophages are also referred to as "bacterial viruses". They infect bacteria by injection of their DNA. In the new host cell, the phage DNA can direct the production of new phage particles which includes expression of phage-borne genes, replication of the phage DNA and packaging of

this DNA into new phage particles which are released from the bacterial cell (lytic cycle). On the other hand, the phage DNA may integrate into the chromosomal DNA of the host cell as a "prophage" and remain there for long periods in an inactive state (lysogenic cycle). External factors such as UV-irradiation can activate the prophage and initiate a lytic cycle (Schwarz and Chaslus-Dancla, 2001).

Chromosomal resistance genes that are located close to the integration site of the prophage may become part of the phage genome when the prophage is not excised precisely from the chromosomal DNA. In this case, the resistance genes spread with the phage particles to new host cells (Schwarz and Chaslus-Dancla, 2001). During phage assembly, resistance plasmids may accidentally be packaged into phage heads instead of phage DNA. The resulting "pseudophages" are able to infect new host cells as the regular phages do. However, since they lack phage DNA, they can only inject the plasmid DNA and thus promote the spread of resistance plasmids to new host cells (Schwarz and Chaslus-Dancla, 2001). The spread of resistance genes via transduction is strongly influenced by the limited amount of DNA that can be packaged into a phage head and the requirement of specific receptors for phage attachment on the surface of the new host cell. For staphylococci, it has been reported that 45 kb is the upper size limit of DNA that can be transduced. While smaller plasmids are transduced as linear concatemers, larger plasmids cannot be packaged into a phage head. Since only host cells that are phylogenetically closely related carry the same receptors for phage attachment, transduction is commonly observed between bacteria of the same species, but rarely seen between bacteria of different species and genera (Schwarz and Chaslus-Dancla, 2001).

2.6.2.2. Conjugation

Conjugation describes the self-transfer of a conjugative plasmid or transposon from a donor cell to a recipient cell (Bennett, 1995). Close contact between donor and recipient is

one of the major requirements for efficient conjugation. The *tra* gene complex whose gene products represent components of the transfer apparatus spans at least 15 kb in Gram-positive bacteria and 30 kb in Gram-negative bacteria and thus cannot be located on small resistance plasmids commonly seen among bacterial pathogens (Schwarz and Chaslus-Dancla, 2001). Small non-conjugative plasmids which coreside in the same host cell may use the transfer apparatus provided by the conjugative element, as long as they have an *ori*T region (origin of transfer) but possibly also possess mobilisation (*mob*) genes. This process is known as mobilisation. Conjugation and mobilisation are believed to be of major importance for the spread of resistance genes between bacteria of different species and genera in bacterial mixed populations as seen on the skin and mucosa of the alimentary, respiratory, and genital tract of humans and animals (Schwarz and Chaslus-Dancla, 2001).

2.6.2.3. Transformation

Transformation describes the transfer of free DNA into competent recipient cells. Transformation is the major way of introducing plasmids into new host bacteria under *in vitro* conditions (McManus, 1997). Under in vivo conditions, transformation is considered to play only a limited role in the transfer of resistance genes (Bennett, 1995). On the one hand, free DNA originating from lysed bacteria is usually rapidly degraded under most environmental conditions. On the other hand, only a few bacteria, such as *S. pneumoniae* or *Bacillus* spp., exhibit a natural ability to take up DNA from their environment (Schwarz and Chaslus-Dancla, 2001).

CHAPTER THREE

3.0: PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF *ESCHERICHIA COLI* AND *SALMONELLA* SEROTYPES FROM CATTLE, PIGS AND CHICKENS

ABSTRACT

The emergence and spread of antimicrobial resistance remains a global health concern. In this study, the prevalence and antimicrobial susceptibility of *E. coli* and *Salmonella* isolates from cattle, pigs and chickens were determined. A total of 235 (82.5%) and 16 (5.6%) of 285 samples were positive for *E. coli* and *Salmonella* isolates, respectively. The isolation rate for *E. coli* was 72.7%, 84.7% and 90.5% in samples from cattle, chickens and pigs, respectively and ranged from 0.0% in cattle and chickens to 13.8% in pigs for *Salmonella*. Salmonellae were isolated from 19.0% of carcass swabs and 8.6% of faecal samples from pigs. More than three quarters (78%) of the pigs, positive for *Salmonella* had culture-positive carcass swab samples and culture negative faeces. Three *Salmonella* serotypes were identified, with *S.* Saintpaul being the predominant serotype (64.3%) followed by *S.* Heidelberg (21.4%) and *S.* Braenderup (14.3%). Among the *E. coli* isolates, resistance was found in 65.5% and multidrug resistance (resistance to ≥ 2 antimicrobials) in 37.9% of the isolates.

Resistance was more frequently observed in chicken isolates. The most common resistances were to ampicillin, streptomycin, tetracycline, sulphamethoxazole/trimethoprim, and kanamycin (42.5-11.9%). Resistances to kanamycin, sulphamethoxazole/trimethoprim, and tetracycline were significantly lower in cattle (2.5-7.5%) than in the other species (12.0-40.0%) ($p < 0.01$). Resistances to streptomycin and ampicillin were significantly higher in cattle and pigs, respectively. Chloramphenicol resistance varied from 2.0% in chickens to 9.5% in pigs. Similar antimicrobial resistance rates were observed among the faecal (29.9%) and carcass swab (33.1%) *E. coli* isolates from both cattle and pigs. Forty resistance patterns

were recorded. For *Salmonella*, resistance to at least ampicillin, chloramphenicol, streptomycin or tetracycline was observed in 37.5% and multidrug resistance in 12.5% of the *Salmonella* isolates. Resistance was found in isolates belonging to serotypes Saintpaul and Braenderup.

This study showed that multidrug resistant *E. coli* isolates are prevalent in cattle, pigs and chickens and on fresh cattle and pig carcasses in Kenya. Secondly, *Salmonella* is present in pigs at slaughter and on pork carcasses and pigs are a potential source of single and multiple antimicrobial-resistant non-typhoidal *Salmonella* serotypes that could pose a public health hazard in human infections. This is the first report of *S.* Heidelberg among food animals in Kenya. The data suggest that selection pressure imposed by the use of tetracycline derivatives, aminoglycosides, sulphonamide drugs and penicillins in food animals is a key driving force in the selection of antimicrobial resistance in *E. coli* and non-typhoidal *Salmonella* serotypes.

It is recommended that the use of antimicrobial agents in food animals should follow prudent use guidelines to minimize the selection and the spread of resistant bacteria and that microbial contamination of carcasses during the slaughter process should be reduced to minimize the risk of transfer of antimicrobial resistant bacteria to humans. The establishment of a national antimicrobial resistance surveillance program in food animals is necessary to identify the emergence of resistant bacteria.

3.1. INTRODUCTION

The use of veterinary antimicrobials has many benefits to the livestock industry ensuring animal health and welfare, but use at subtherapeutic levels exerts great selective pressure on emergence of resistant bacteria (Hart *et al.*, 2004). The emergence of antimicrobial resistance among pathogenic and commensal bacteria has become a serious problem worldwide that affects treatment of infectious diseases both in humans and in animals resulting in decreased productivity, increased morbidity and mortality, and increased costs (Bischoff *et al.*, 2002). The major influences on the amplification and spread of antimicrobial-resistant bacteria are the use of antimicrobials in human medicine and their use in livestock for therapy, metaphylaxis, prophylaxis and growth promotion (Hart *et al.*, 2004).

The WHO has recommended that, unless a risk-based evaluation demonstrates their safety, the use of antimicrobial agents in food animals for growth promotion that belong to classes of antimicrobials used in humans should be terminated (WHO, 2000). Similar recommendations to discontinue the use of human antimicrobial agents as growth promoters in food animals have been made in the USA and the EU (Anderson *et al.*, 2003). In 1998, the EU withdrew approval of four growth promoters (tylosin, spiramycin, bacitracin, and virginiamycin) because of their structural relatedness to antimicrobial agents used in human medicine (Anderson *et al.*, 2003).

The intestinal flora in food-producing animals in Kenya could be exposed to a great selective pressure since more than 95% of the antimicrobials used in these animals are orally administered (Mitema *et al.*, 2001). Resistant bacteria and resistance genes from domestic animals can be transmitted to man indirectly via the food chain or directly from the animals (van den Bogaard *et al.*, 2000; Helmuth and Hensel, 2004) and potentially result in food-borne illness in humans that is less responsive to treatment with conventional antimicrobial drugs. Commensal bacteria such as *Enteroccus* spp. and *E. coli* can develop resistance and

thus be a source of resistance genes that can be further spread to pathogenic bacteria. *E. coli* is the most common enterobacterium found in different animal species (Kijima-Tanaka *et al.*, 2003) and can serve as an indicator bacterium that easily acquires antimicrobial resistances. The prevalence of resistance in commensal *E. coli* is a good indicator for the selective pressure by antibiotic use and resistance problems to be expected in pathogenic bacteria. In food animals, a low prevalence and degree of antibiotic resistance in the intestinal flora should be considered a distinguishing quality and safety mark (van den Bogaard *et al.*, 2000).

Resistant zoonotic bacteria, such as *Salmonella* spp. and *Campylobacter* spp., can be transferred from animals to humans through food and in this way contribute to the resistance problem in human medicine (NORM/NORM-VET, 2003). Salmonellosis is considered as one of the most widespread foodborne zoonoses in industrialized as well as developing countries, even though the incidence seems to vary between countries (Molla *et al.*, 2003a). Farm animals often carry *Salmonella* isolates, affecting meat, dairy products and eggs (Cabrera *et al.*, 2004) and so act as source of contamination, which is of paramount epidemiological importance in non-typhoid human salmonellosis (Acha and Szyfres, 2001). Given the association of certain *Salmonella* serotypes with food poisoning and the likehood that some isolates may be multiply resistant to antimicrobials, a complete understanding of the risk posed by these pathogens during processing of foods of animal origin requires that the serotype and antimicrobial resistance profile of the isolates be determined in addition to their prevalence (McEvoy *et al.*, 2003).

Salmonella isolates display high natural susceptibility to the most commonly used antimicrobial agents (Stock and Wiedemann, 2000). However, the occurrences of *Salmonella* strains showing resistance to one or more antibacterial agents have steadily increased, probably due to continuous antibiotic pressure in human and veterinary medicine (Orman *et al.*, 2002; Molla *et al.*, 2003b). This is an important public health problem that may be related to either therapeutic failure (Zahurul *et al.*, 2003) or the transfer of multidrug -resistant

Salmonella strains and/or the resistance genes from food animals to humans through consumption of contaminated food and food products (Molla *et al.*, 2003b). Of particular concern is the isolation of ceftriaxone- and ciprofloxacin-resistant *Salmonella*, because of the importance of these two agents in treating *Salmonella* infections in children and adults (Chiu *et al.*, 2002; Fey *et al.*, 2000), respectively. This problem is especially relevant in developing countries like Kenya, where lack of economic resources does not allow a wide antibacterial armentarium (Cabrera *et al.*, 2004).

The WHO, the FAO and the OIE have emphasized the importance of monitoring antimicrobial resistance in veterinary medicine and have published several reports and recommendations in this regard (NORM/NORM-VET, 2003). Consequently, several European countries (Wray and Gnanou, 2000) as well as Canada (CIPARS, 2002) and the USA (CDC, 2001) have established national surveillance programmes to assess bacterial susceptibility to antimicrobials among enteric bacteria from healthy animals. However, no monitoring program exists for the antimicrobial resistance in food animals in Kenya.

While the antimicrobial resistance of commensal *E. coli* isolates of avian origin in Kenya has been reported (Bebora *et al.*, 1994, Kariuki *et al.*, 1999), data on the prevalence and patterns of resistance of *E. coli* from other food-producing animals are unavailable. Moreover, to date, only a single study (Kariuki *et al.*, 2002) has extensively analysed the levels of resistance to antimicrobial agents in *Salmonella* serotypes isolated from food-producing animals in Kenya

3.1.1. Objectives

The aims of this study were:

1. To determine and compare the prevalence of *E. coli* and *Salmonella* serotypes in apparently healthy slaughtered cattle, pigs and chickens
2. To determine the susceptibility of *E. coli* and *Salmonella* isolates to various antimicrobial agents.

3.2: MATERIALS AND METHODS

3.2.1. Collection of samples

Fresh faecal and carcass swab samples were collected from individual animals from unrelated herds at the Dagoretti slaughterhouse complex (cattle) and Ndumbuini slaughterhouse (pigs) in Nairobi from June to December 2001. Cattle slaughtered at Dagoretti slaughterhouse complex originate from all parts of the country (Kithuka *et al.*, 2002). Pigs are sent to the abattoir from farms in Kiambu and Nairobi districts which are among the main pig farming districts in Kenya. A single animal was selected at random as being representative of a herd and about 5 g of faeces aseptically removed from the large bowel after evisceration at the slaughtering line. The carcasses were sampled using sterile cotton wool swabs. The samples were immediately placed into Stuart's transport medium (Oxoid, Basingstoke, United Kingdom), maintained on ice while being transported to the laboratory and processed on the same day. In addition, cloacal and pharyngeal swab samples collected from indigenous free range chickens at slaughter at various markets in Nairobi were added to this collection and used for the study.

3.2.2. Isolation and identification of *E. coli*

The samples were inoculated into peptone water (Oxoid) and incubated at 37 °C for 18 h. Subsequently, the cultures were subcultured onto Eosin Methylene Blue (EMB) agar (Oxoid) and incubated overnight at 37 °C. Indole, Methyl red, Voges-Proskauer reaction and Simons citrate (IMViC) tests were performed for the colonies that showed green metallic sheen on EMB agar. Analytical profile index (API) 20E strips (bioMérieux, Marcy-l'Etoile, France) were also used to confirm the identification of the isolates as *E. coli*. One isolate per sample was selected for antimicrobial resistance testing. The *E. coli* isolates selected for resistance testing were restreaked onto blood agar (Oxoid), incubated overnight at 37 °C, and

stored at 4 °C until *in-vitro* susceptibility tests were performed. *E. coli* ATCC 25922 was used as a reference strain for quality control of the antimicrobial susceptibility testing.

3.2.3. Isolation and identification of *Salmonella*

Faecal and swab (carcass, cloacal and pharyngeal) samples were pre-enriched in peptone water. The pre-enriched samples were incubated at 37 °C for 18 h. A 5 ml portion of the pre-enrichment broth was transferred aseptically into enrichment selective tetrathionate broth (Oxoid) and incubated overnight at 37 °C. A loopful of tetrathionate broth culture was streaked onto Desoxycholate citrate agar (DCA, Oxoid) plates, and incubated overnight at 37 °C. The DCA plates were examined for the presence of *Salmonella*-like colonies (colourless with black centres). Single colonies were tested for the appropriate reactions on Triple Sugar Iron agar (TSI, Oxoid) and urea agar (Oxoid) and their identification was confirmed biochemically using API 20E strips (bioMérieux, Marcy-l'Etoile, France).

3.2.4. Serotyping and phage typing of *Salmonella*

Salmonella isolates were sero- and phage-typed at the Robert Koch Institute, National Reference Centre for *Salmonella* and other Enteric Pathogens in Wernigerode, Germany. Serotyping was done based on O- and H- group antigens according to the Kauffmann-White Scheme (Popoff *et al.*, 2003) using slide and microtitre agglutination. The antigenic formulae of *Salmonella* serotypes as listed by LeMinor and Popoff (1997) were used to identify *Salmonella* serotypes. To phage type the isolates; routine test dilutions of each of the typing phages were applied to nutrient agar (Difco, Detroit, USA) plates with a lawn of the respective bacterial strain using a multipoint inoculator. These were incubated overnight at 37°C until the phage lysis could be read. The phage patterns (phage types) and the readings were interpreted according to the Anderson phage-typing scheme (Anderson *et al.*, 1977).

3.2.5. Antimicrobial susceptibility testing

3.2.5.1. Disc diffusion tests

E. coli ATCC 25922 was used as a control for growth of bacteria and potency of the antibiotics. The various bacterial isolates were tested for their susceptibility to commonly used antimicrobial agents on MH agar (MH, Oxoid, Wesel, Germany) by the disc diffusion technique according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004 and NCCLS, 1997) recommendations. The antimicrobial discs used were: ampicillin (10 µg), tetracycline (30 µg), streptomycin (10 µg), kanamycin (30 µg), gentamicin (10 µg), sulphamethoxazole/trimethoprim (23.75/1.25 µg) and chloramphenicol (30 µg) (Himedia Laboratories Ltd, Mumbai, India). The bacterial strains including *E. coli* ATCC 25922 were subcultured onto Luria Bertani (LB) agar (Oxoid) and then incubated at 37°C for 18-24 h. After incubation 4 or 5 colonies were suspended in 0.85% sterile saline to conform to the density of a McFarland standard 0.5.

One hundred microlitres of this bacterial suspension were spread on MH agar plates as evenly as possible using sterile cotton swabs. After 5 to 10 min the antimicrobial discs were placed using a sterile forceps and the plates left on the bench for about 15 min before incubation. After incubation for 18 h at 35 °C, the diameter of the inhibition zone for each antimicrobial disc was measured using a vier calliper or a pair of divider and a ruler to the nearest millimetre. The zone diameters of all the discs except the streptomycin disc were interpreted according to the NCCLS document M31-A2 (NCCLS, 2004), the breakpoints used for streptomycin were those recommended by NCCLS document M2-A6 (NCCLS, 1997). Multidrug resistance was defined as simultaneous resistance to at least two of the antimicrobials tested, with sulphamethoxazole plus trimethoprim considered as one unit since the testing was done in combination. Resistant isolates were stored at -20 °C until further analysis. Aliquots of the same resistant isolates were also lyophilized and stored.

3.2.5.2. Minimum Inhibitory Concentration (MIC) determination

The MICs of ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole/trimethoprim (19:1) and tetracycline among 154 *E. coli* isolates showing resistance on disc diffusion test and 14 *Salmonella* isolates were determined using the standard broth doubling dilution method on MH (Oxoid) medium according to the (NCCLS, 2004) recommendations. Only *Salmonella* isolates were tested against nalidixic acid. The experiments were carried out at the Institute for Animal Breeding, Federal Agricultural Research Centre, Neustadt-Mariensee, Germany. The antimicrobial standards (powders) were obtained from Sigma (Deisenhofen, Germany). Four or five colonies were suspended in 0.85% sterile saline to conform to the density of a McFarland standard 0.5. The suspensions were diluted in cation-supplemented MH broth to yield a final concentration of 10^5 CFU/ml. Serial dilutions of the antimicrobial agents were made in MH broth after which the standardized bacterial inoculums were added.

Antimicrobials were serially diluted from 128 to 2 µg/ml except for sulphamethoxazole/trimethoprim, where 9.5/0.5 – 608/32 µg/ml concentrations were used. After incubation for 18 at 35 °C the tubes were visually examined for turbidity. MIC was recorded as the lowest concentration of the antibiotic in the two-fold dilution series that inhibited visible growth. Reference *E. coli* ATCC 25922, was tested in parallel as a quality control. MICs were interpreted according to breakpoints given by NCCLS (NCCLS, 2003; NCCLS, 2004) except for streptomycin. The breakpoints used for streptomycin were those recommended by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP, 2001). MIC_{50} and MIC_{90} values as well as rates of resistance were calculated and presented.

3.2.6. Statistical analysis

Chi-square test was used to compare the difference between the proportions of the isolates from cattle, pigs and chickens that were resistant to various antibiotics. A value of $p < 0.05$ was considered as significant. The correlation between the standard broth dilution method and disc diffusion method was analysed by means of regression analysis.

3.3: RESULTS

3.3.1. Prevalence of *E. coli*

Two hundred and thirty five (82.5%) *E. coli* were obtained from the 285 samples processed. The prevalence of *E. coli* per animal species was 90.5%, 84.7% and 72.7% in pigs, chickens and cattle, respectively. *E. coli* was present in 38 (69.1%) of 55 and 52 (89.7%) of 58 carcass swabs and in 42 (76.4%) of 55 and 53 (91.4%) of 58 faecal samples from cattle and pigs, respectively (Table 3.1). For chickens, *E. coli* was isolated from 12 (80.0%) and 38 (86.4%) of 15 pharyngeal and 44 cloacal swab samples, respectively.

3.3.2. Prevalence of *Salmonella* serotypes

Out of the total 285 samples examined 16 (5.6%) were *Salmonella* positive. The isolation rate for *Salmonella* per animal species ranged from 0.0% for both cattle and chickens to 13.8% for pigs (Table 3.1). Faeces from cattle, swabs from beef carcasses and chickens cloacae and pharynx did not yield any *Salmonella*. *Salmonella* was isolated from 11 (19.0%) of carcass swab and 5 (8.6%) of faecal samples from pigs. *Salmonella* was isolated from faeces and carcass swab of the same animal only in two cases. For nine pigs, *Salmonella* was found on the carcass but not in the faeces as compared to only one occasion in which *Salmonella* was isolated from faecal, but not from the carcass swab sample taken from the same animal.

Table 3.1: Prevalence of *E. coli* and *Salmonella* isolates per animal species and sample type.

Sample type	Number of samples per animal species positive for *E. coli* and *Salmonella.*					
	Cattle (n = 110)		Pigs (n = 116)		Chickens (n = 59)	
	E. coli	*Salmonella*	*E. coli*	*Salmonella*	*E. coli*	*Salmonella*
Faeces	76.4% (42/55)	0	91.4% (53/58)	8.6% (5/58)	-	-
Carcass swab	69.1% (38/55)	0	89.7% (52/58)	19.0% (11/58)	-	-
Pharyngeal swab	-	-	-	-	80.0% (12/15)	0
Cloacal swab	-	-	-	-	86.4% (38/44)	0
Total	72.7% (80/110)	0	90.5% (105/116)	13.8% (16/116)	84.7% (50/59)	0

3.3.2.1. Distribution of *Salmonella* serotypes

Only 14 of the 16 *Salmonella* strains were able to grow from the lyophilised cultures and these strains were therefore serotyped and phage typed. Three serotypes were identified with *S.* Saintpaul being the predominant serotype (64.3%) followed by *S.* Heidelberg (21.4%) and *S.* Braenderup (14.3%). All the three *S.* Heidelberg isolates were of the same phage type (Table 3.2) while the other serotypes were untypable. The highest proportion (77.8%) of *S.* Saintpaul was identified from carcass swabs as compared to faecal samples (22.2%). Only on one occasion was *S.* Saintpaul identified from faeces and on carcass swab samples obtained from the same animal. Both *S.* Braenderup isolates were from the same animal, of which one was obtained from faeces while the other came from carcass swab sample. All the three *S.* Heidelberg isolates were obtained from carcass swabs. On no occasion was more than one serotype isolated from sample materials from the same animal.

Table 3.2: *Serotype, phage type and antimicrobial resistance profiles of* ***Salmonella*** *isolates from pig faecal and carcass samples.*

Pig No.	Source	Serotype	Antigenic structure			Phage type	Resistance patterns
			O-antigen	H-1	H-2		
1	carcass	*S.* Heidelberg	4, 5, 12	r	1, 2	HB PT 02	-$^{\Phi}$
2	carcass	*S.* Heidelberg	4, 5, 12	r	1, 2	HB PT 02	-
3	carcass	*S.* Heidelberg	4, 5, 12	r	1, 2	HB PT 02	-
4	carcass	*S.* Braenderup	6, 7	e, h	e, n, z15	-$^{\Psi}$	$Amp^{R}Sm^{R}Tet^{R}$
	faeces	*S.* Braenderup	6, 7	e, h	e, n, z15	-	-
5	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	-
6	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	-
7	faeces	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	Amp^{R}
8	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	-
9	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	Tet^{I}
	faeces	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	Cm^{R}
10	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	Sm^{R}
11	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	-
12	carcass	*S.* Saintpaul	4, 5, 12	e, h	1, 2	-	-

Key: Amp, ampicillin; Cm, chloramphenicol; Sm, Streptomycin; Tet, tetracycline; ^{R}resistant, ^{I}intermediately resistant; $^{\Phi}$Susceptible to all antimicrobials in the test panel; $^{\Psi}$Untypable

3.3.3. Antimicrobial susceptibility of bacterial isolates

3.3.3.1. Antimicrobial susceptibility and MIC of *E. coli* isolates

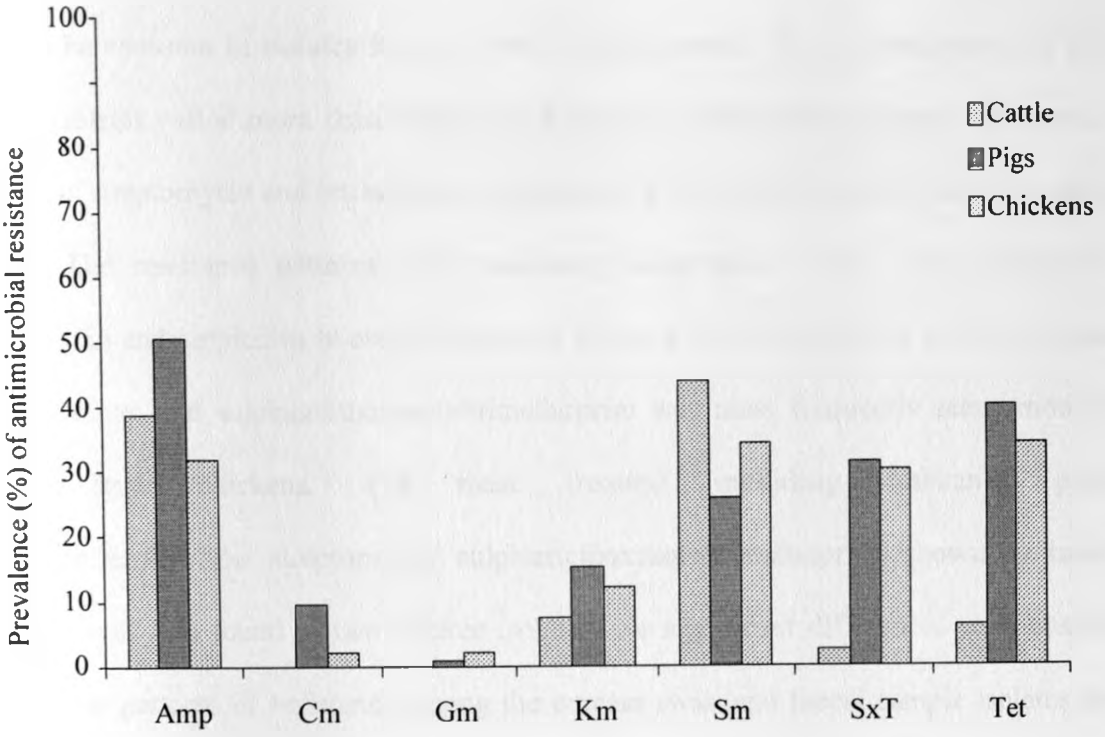
One hundred and fifty four (65.5%) of the *E. coli* isolates (pigs, 28.9%, cattle, 20.9% and chicken, 15.7%) were resistant to at least one of the antimicrobial agents tested by MIC. Overall, 89 (37.9%) of the isolates were multidrug resistant (resistant to ≥2 antibiotics). Resistance was highest in the isolates from chicken (74.0%), followed by pigs (64.8%) and cattle (61.3%). Multidrug resistance was significantly higher ($p < 0.05$) in the isolates from pigs (42.9%) and chickens (40.0%) than in those from cattle (30.0%) (Table 3.3). One isolate from a pig was resistant to all the seven antibiotics tested. The most prevalent resistances among the isolates from the three animal species sampled were to ampicillin (42.5%), streptomycin (33.6%), tetracycline (27.2%), and sulphamethoxazole/trimethoprim (21.3%). For the other antimicrobial agents tested the prevalence was less than 12.0% (Figure 3.1).

Among the isolates obtained from cattle, only resistance to streptomycin or ampicillin reached frequencies of > 30.0%. In contrast, ≥ 26.0% of the isolates obtained from swine and chickens were resistant to these antibiotics and to tetracycline, and sulphamethoxazole/trimethoprim. Resistance to kanamycin, tetracycline, and sulphamethoxazole/trimethoprim were significantly higher in the isolates from pigs and chickens (12.0-40.0%) than in those from cattle (2.5-7.5%). Isolates from cattle were significantly more resistant to streptomycin (43.7%) than those from pigs (25.7%) and chickens (34.0%) ($p < 0.01$). Resistance to ampicillin was significantly higher in isolates from pigs (50.5%) than in isolates from cattle (38.7%) or chickens (32.0%). Low prevalence of resistances to gentamicin (1.0-2.0%) and chloramphenicol (2.0-9.5%) were observed among the isolates from pigs and chickens. Chloramphenicol resistance was significantly higher in the isolates from pigs (9.5%) than in those from chickens (2.0%). No resistances to gentamicin or chloramphenicol were detected in the *E. coli* isolates from cattle. There were no

significant differences in the prevalence of resistance between the *E. coli* isolates from faeces (30.0% in cattle and 29.7% in pigs) and carcass swabs (31.0% in cattle and 35.2% in pigs). The isolates from chickens were not compared statistically because of the small number of isolates from pharyngeal swabs.

Table 3.3: Resistance phenotypes of *E. coli* per animal species by disc diffusion method.

Resistance to x antimicrobial agents (x = 0 - ≥ 5)	Cattle (n = 80)	Pigs (n = 105)	Chickens (n = 50)
0	31 (38.7)	37 (35.2)	13 (26.0)
1	25 (31.2)	23 (21.9)	17 (34.0)
2	19 (23.8)	10 (9.5)	10 (20.0)
3	4 (5.0)	15 (14.3)	6 (12.0
4	1 (1.2)	15 (14.3)	3 (6.0)
≥5	0 (0.0)	5 (4.8)	1 (2.0)



Antimicrobial agents: Amp, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; SxT, sulphamethoxazole/trimethoprim; Tet, tetracycline.

Fig. 3.1: Prevalence of antimicrobial resistance among *E. coli* isolated from cattle (n =80), pigs (n =105) and chickens (n =50).

Forty different resistance patterns were recorded. The most prevalent resistance patterns of each species are shown in Table 3.4. Only five (12.5%) of these resistance patterns were found to be common in isolates from all three animal sources. The resistance spectra of the porcine isolates varied more than those from bovine or avian isolates. Single resistances to ampicillin, streptomycin and tetracycline were present in *E. coli* isolates from all three animal species. The resistance patterns most frequently observed in cattle were resistance to streptomycin and ampicillin in combination and streptomycin or ampicillin alone. Resistance to tetracycline, and sulphamethoxazole/trimethoprim was most frequently seen among the isolates from chickens. The most frequent multidrug resistance pattern (ampicillin/tetracycline/ streptomycin/ sulphamethoxazole/trimethoprim) shown by isolates from pigs was also found in two chicken isolates. No significant differences were observed between the patterns of resistance among the carcass swab and faecal sample isolates from either cattle or pigs. The disc diffusion results for the 154 isolates correlated well with the MIC for all the agents tested ($r = 0.949$).

The minimum inhibitory concentrations of each antimicrobial agent varied widely with the isolate tested (Table 3.5). Some isolates showed exceptionally high MICs for various antimicrobials. These included, ampicillin with thirty three (21.4%), streptomycin with twenty two (14.3%), chloramphenicol with eight (5.2%) and tetracycline with twenty (13.0%) of the isolates showing MICs $\geq$ 256 μg/ml. In addition, forty two (27.3%) of the isolates showed MICs $\geq$ 1216/64 μg/ml for the sulphamethoxazole/trimethoprim combination.

Table 3.4: The most prevalent antimicrobial resistance profiles of *E. coli* isolates from food-producing animals in Kenya

Resistance phenotype	Number of isolates		
	Cattle (n = 80)	Pigs (n = 105)	Chickens (n = 50)
Amp	10 (12.5%)	15 (14.3%)	6 (12.0%)
Sm	13 (16.5%)	1 (0.9%)	4 (8.0%)
Tet	2 (2.5%)	5 (4.8%)	4 (8.0%)
SxT	-	2 (1.9%)	3 (6.0%)
AmpSm	15 (18.8%)	2 (1.9%)	1 (2.0%)
AmpTet	-	1 (0.9%)	2 (4.0%)
KmSm	3 (3.8%)	-	2 (4.0%)
SxTTet	1 (1.3%)	1 (0.9%)	4 (8.0%)
AmpKmSm	4 (5.0%)	-	-
AmpKmTet	-	2 (1.9%)	-
AmpSxTTet	-	5 (4.8%)	-
TetSmSxT	-	2 (1.9%)	-
AmpKmSxTTet	-	3 (2.9%)	-
AmpSmSxTTet	-	7 (6.7%)	2 (4.0%)
AmpCmKmSmSxTTet	-	2 (1.9%)	-

Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; SxT, sulphamethoxazole/trimethoprim; Tet, tetracycline.

Table 3.5: MIC distribution of 7 antimicrobials tested against 154 *E. coli* isolates

Antimicrobial agent	No. of isolates for which MIC (µg/ml) is											MIC (µg/ml)	
	≤0.25	0.5	1	2	4	8	16	32	64	128	≥256	MIC_{50}	MIC_{90}
Ampicillin	0	0	0	1	21	35	41	8	12	3	33	16	≥256
Chloramphenicol	0	0	0	12	40	89	5	0	0	0	8	8	8
Gentamicin	0	2	12	85	47	8	0	0	0	0	0	2	4
Kanamycin	0	0	0	0	26	81	35	12	0	0	0	8	16
Streptomycin	0	0	0	0	15	30	57	17	5	8	22	16	≥256
Tetracycline	0	2	4	34	57	19	2	2	3	11	20	4	≥256
Sulphamethoxazole /trimethoprim (19:1)[a]	95	3	8	0	4	0	0	2	42			≤0.25	≥64

[a] The MICs of sulphamethoxazole/ trimethoprim (19:1) are expressed as MICs of trimethoprim. Dark grey shaded area shows the region with resistant isolates; the light grey shaded area shows the intermediate resistant isolates; while the clear region shows the susceptible isolates. Breakpoints are based on NCCLS document M31-S1, (2004), except for streptomycin (DANMAP, 2001).

3.3.3.2. Antimicrobial susceptibility and MIC of *Salmonella* isolates

Six (37.5%) of the 16 *Salmonella* isolates were resistant to at least one or more antimicrobials namely ampicillin, chloramphenicol, streptomycin, tetracycline and sulphamethoxazole/trimethoprim. Resistance to ampicillin or tetraycline was observed in three isolates while streptomycin or chloramphenicol resistance were observed in two isolates. Only one isolate showed resistance to sulphamethoxazole/trimethoprim. Two (12.5%) of the isolates were MDR and six different resistance patterns were recorded (Table 3.6). Ampicillin and chloramphenicol resistances were observed in isolates obtained from faecal samples while tetracycline and streptomycin resistance were found in both faecal and carcass swab sample isolates. MICs were determined for eight antimicrobials to 14 of the 16 *Salmonella* strains that were able to grow from the lyophilised cultures (Table 3.7). The majority of the *Salmonella* isolates had low MIC values for all commonly available antibiotics in the test panel. Resistance to ampicillin, chloramphenicol, streptomycin and tetracycline was confirmed in all the isolates studied.

All three *S.* Heidelberg isolates were fully susceptible to all the eight antimicrobials tested. In contrast one *S.* Braenderup isolate obtained from a faecal sample was multiply resistant to ampicillin, tetracycline, and streptomycin while the other was fully susceptible to all antimicrobials tested. Three *S.* Saintpaul isolates were solely resistant to either ampicillin, or chloramphenicol, or streptomycin. One *S.* Saintpaul isolate from a carcass swab sample was intermediately resistant to tetracycline. Resistance to ampicillin and chloramphenicol were only observed in the faecal isolates while resistances to streptomycin and tetracycline were observed in both faecal and carcass swab isolates. All isolates were fully susceptible to gentamicin, kanamycin, sulphamethoxazole/trimethoprim or nalidixic acid.

Table 3.6: Serotypes and antimicrobial resistance profiles of *Salmonella* isolates.

Isolate No.	Serotype	Antimicrobial resistance profile
24F_S	Nd	$Amp^{R}Tet^{R}SxT^{R}Cm^{I}$
64F-S	*S.* Braenderup	$Amp^{R}Sm^{R}Tet^{R}$
66C-S	*S.* Saintpaul	Sm^{R}
68F-S	*S.* Saintpaul	Amp^{R}
73C-S	*S.* Saintpaul	Tet^{I}
73F_S	*S.* Saintpaul	Cm^{R}

Abbreviations: nd, not determined; Amp, ampicillin; Cm, chloramphenicol; Sm, streptomycin; SxT, sulphamethoxazole/trimethoprim (19:1); Tet, tetracycline; ^{R}, resistant; ^{I}, intermediately resistant.

Table 3.7: MICs of 8 antimicrobial agents for 14 non-typhoid *Salmonella* isolates from pigs.

Antimicrobial agent	MIC ($\mu g\ ml^{-1}$)				% resistant
	Range	Mode	MIC_{50}	MIC_{90}	
Ampicillin	≤2 – 128	4	4	128	14.3
Chloramphenicol	4 – 128	8	8	8	7.1
Gentamicin	≤2 – 4	≤2	≤2	≤2	0.0
Kanamycin	≤2 - 16	8	8	16	0.0
Nalidixic acid	≤2 – 16	≤2	≤2	8	0.0
Streptomycin	4 – 64	16	16	64	14.3
Sulphamethoxazole/ trimethoprim (19:1)[a]	≤9.5/0.5 – 38/2	≤9.5/0.5	≤9.5/0.5	38/2	0.0
Tetracycline	≤2 - ≥256	4	4	16	7.1

Resistance (R) breakpoints (μg/ml) based on NCCLS standards (NCCLS, 2003; NCCLS, 2004) except for streptomycin, breaks for streptomycin were based on DANMAP 2001: ampicillin, ≥32; chloramphenicol, ≥32, gentamicin, ≥16; kanamycin, ≥64; nalidixic acid, ≥32; streptomycin, ≥32; tetracycline, ≥16 sulphamethoxazole/trimethoprim, ≥76/4. [a]The MICs of sulphamethoxazole/ trimethoprim (19:1) are expressed as MICs of trimethoprim.

3.4: DISCUSSION

The isolation rates of *E. coli* in this study were high (range 72.7-90.7%) in all the animal species. Similar results were reported by Bywater *et al.* (2004) and Kijima-Tanaka *et al.* (2003) for *E. coli* isolated from food-producing animals in Europe and Japan, respectively. *E. coli* was more frequently detected on pork (72.4%) than on beef carcasses (69.1%). This finding is in agreement with the observation by Hansson, (2001) that *E. coli* was more common on pork carcasses than on beef carcasses. The contamination of carcasses by *E. coli* occurs during carcass dressing (Gill and McGinnis, 2000) and may originate from either the animal faeces (Aslam *et al.*, 2003), or slaughterhouse environment or equipments (Gill and McGinnis, 2000). Slaughter hygiene is therefore a determinant factor in minimizing carcass contamination.

The present study detected a moderate prevalence (13.8%) of *Salmonella* spp. in pigs, but failed to isolate *Salmonella* from the cattle and chicken samples analysed. Even though there have been studies on *Salmonella* in pigs, cattle and chickens at slaughter in other countries, it is difficult to compare the findings from these studies because of considerable variations in sampling strategies and methods as well as in *Salmonella* culture techniques. The failure to find *Salmonella* in samples from cattle and chickens in this study may probably be the result of very low *Salmonella* carrier rates in the animals sampled and further studies involving larger sample sizes are necessary to confirm this status.

Low prevalence of *Salmonella* carriage in slaughter cattle has been reported in most of countries where such surveys have been conducted. Kariuki *et al.* (2002) reported a low frequency (1.5%) of *Salmonella* from beef carcass swab and abattoir effluent samples in Kenya. In Great Britain, Davies *et al.* (2004), detected a very low faecal carriage of *Salmonella* in cattle by testing 1 g of faeces, and even when a 25 g sample size was tested the prevalence was still low. A low frequency of *Salmonella* was found on beef carcasses in

Northern Ireland (Madden *et al.*, 2001) and even when calves were infected with *Salmonella* during rearing, most had shed the infection by the time of slaughter (Galland *et al.*, 2000).

Mikolajczk and Radkowski, (2002) reported low carcass contamination rates for slaughter birds in Poland. In contrast, other studies have shown a high prevalence of *Salmonella* in cattle (Beach *et al.*, 2002; Troutt *et al.*, 2001) and chickens (Uyttendaele, 1998). The failure to isolate *Salmonella* from the indengous chicken studied may have been due to the fact the main sources of *Salmonella* infection in poultry are contaminated feed, drinking water and litter, and under extensive systems of management as in indengous poultry production, the chances of chickens eating and drinking contaminated material are minimal (Mdegela *et al.*, 2000).

The prevalence of *Salmonella* on pig carcasses (19.0%) was higher than in faeces (8.6%). This observation is in line with previous studies in Belgium (Botteldoorn *et al.*, 2003) and Germany (Käsbohrer *et al.*, 2000) that reported observing higher frequencies of *Salmonella* on pig carcasses than in their faeces. Conversely, a study in the Netherlands reported a lower carcass contamination frequency of 1.4% and 25.6% faecal carriage (Swanenburg, 2000). The differences in the prevalence rates reported could be due to different sampling regimes and techniques used in the various studies, differences in the hygiene of dressing operations, the geographical variation in the incidence of *Salmonella* (McEvoy *et al.*, 2003) or samples involved in the studies (Ejeta *et al.*, 2004). A higher prevalence of *Salmonella* on carcasses than in faeces suggests the presence of severe contamination during slaughtering process as a result of poor hygienic conditions during subsequent dressing operations (Ejeta *et al.*, 2004). The major contamination sources of pig carcasses are pig faeces, stomach contents during evisceration, contaminated water used for washing carcasses, contact with contaminated slaughterhouse environment and *Salmonella*-carrying slaughterhouse personnel (Borch *et al.*, 1996; Molla *et al.*, 2003a). Additionally, highly

contaminated intestine or lymph nodes could be a primary source of carcass contamination during evisceration (Botteldoorn *et al.*, 2003). The fact that 78.6% of the pigs in this study had culture-positive carcass swab samples and culture negative faeces suggest that slaughterhouse contamination is an important food safety risk.

In the current study, *S.* Saintpaul (64.3%) was the most frequently isolated serotype followed by *S.* Heidelberg and *S.* Braenderup. These results are contrary to a previous study in Kenya (Kariuki *et al.*, 2002), which found *S.* Agona to be the main serotype in pigs. These differences may probably be due to the difference in the type of samples analysed, in this study, majority of the *Salmonella* isolates were isolated from carcass swabs while all the isolates in the study by Kariuki *et al.* (2002) were from faeces. Wonderling *et al.* (2003) found that 54% of the carcasses were contaminated with *Salmonella* types not found in the faeces of the same animal. The remaining contaminated carcasses (28%) were contaminated by strains originating from the slaughterhouse environment. Variations in *Salmonella* serotypes isolated from pigs between sampling days and between slaughterhouses has also been reported (Botteldoorn *et al.*, 2003).

In Kenya, *S.* Saintpaul has previously been isolated from humans and dairy cows (Kariuki *et al.*, 2002). *S.* Saintpaul is one of the most frequent serotypes in Japan since 1999 (Hata *et al.*, 2003) and is among the predominant serotypes isolated from food animals, slaughterhouse personnel and retail meat products in Ethiopia (Molla *et al.*, 2003a). *S.* Heidelberg accounted for approximately one fifth of the serotypes identified. To my knowledge this is the first report of *S.* Heidelberg among food animals in Kenya. In Kenya, *S.* Heidelberg has previously been reported in human outpatients with diarrhoea (Utsunomiya, 1983). Reports from countries including the United States, Canada, Italy and Ethiopia describe a high prevalence of *S.* Heidelberg in both human and non-human sources, mainly food and livestock (Schoeni *et al.*, 1995; Demczuk *et al.*, 2003; Mammina *et al.*, 2003; Molla

et al., 2003a). *S.* Braenderup has been reported in chickens in Kenya (Kariuki *et al.*, 2002) and as a dominant serotype from mutton and pork in Ethiopia (Ejeta *et al.*, 2004).

Resistance in bacteria isolated from food represents a potential source of resistance in human pathogens (Wise and Soulsby, 2002; Bywater *et al.*, 2004). Where resistance is present among zoonotic organisms, such as *Salmonella* species, then it is by definition possible for resistant bacteria from animals to be transmitted to a human subject (Bywater *et al.*, 2004). Contact with food animals or their excrements or consumption of foods contaminated by animal carcasses during slaughter or production of foods has been suggested to be the main route of dissemination of resistance from food-producing animals into human populations (Helmuth and Hensel, 2004). The majority (64.3%) of the *Salmonella* isolates studied were fully susceptible to all the eight antimicrobials tested with all isolates being sensitive to gentamicin, kanamycin, sulphamethoxazole/trimethoprim or nalidixic acid. Similar observations were made in analyses of non-typhoidal *Salmonella* serotypes from food animals in Kenya (Kariuki *et al.*, 2002). One *S.* Braenderup isolate exhibited multidrug resistance to ampicillin, tetracycline and streptomycin. Multidrug resistance in *S.* Braenderup has been reported in Ethiopia (Molla *et al.*, 2003b) and Canada (Poppe *et al.*, 1995). Three out of the four *S.* Saintpaul isolates showing resistance were only resistant to chloramphenicol, streptomycin or tetracycline whereas the fourth strain was intermediately resistant to tetracycline.

Among the *E. coli* strains, resistance was more commonly observed among chicken isolates while multidrug resistance was significantly higher in isolates from chickens and pigs than those from cattle. Pigs and chickens are usually housed under relatively intensive conditions. Intensive housing may be associated with greater disease potential and therefore, a greater tendency for antibiotic use to control disease (Bywater *et al.*, 2004). Furthermore, most antimicrobial preparations in pigs and chickens production are orally administered and

thus the intestinal flora is more exposed to antibiotics in these species. The *E. coli* isolates showed high prevalence of resistance to ampicillin. Isolates from pigs were significantly more often resistant to ampicillin and tetracycline than those from other animal species. Due to their relatively low cost and ready availability these drugs are widely used by farmers for therapeutic and prophylactic applications (Kariuki *et al.*, 997).

Penicillins and tetracyclines are the most widely used antibiotics in humans and food animals, respectively, in Kenya and extended-spectrum penicillins account for 67.5% of the penicillins used (Mitema and Kikuvi, 2004) while tetracyclines account for nearly 55% of the antimicrobial use in food animals (Mitema *et al.*, 2001). Broadspectrum penicillins disturb the colonization resistance of the intestinal tract profoundly (Vollaard and Clasener, 1994), facilitating overgrowth by and increasing excretion of resistant bacteria (van den Bogaard *et al.*, 2000). Moreover, ampicillin is one of the most widely available orally administered antibiotics in humans in Kenya (Gakuya, *et al.*, 2001). A high prevalence of antimicrobial drug-resistant *E. coli* could also occur if the animals received high doses of these isolates from the environment (Khachatryan *et al.*, 2004). Dissemination of ampicillin resistance by *E. coli* from humans may reach pigs through feeding contaminated swill, which is a common practice by small-scale farmers in Kenya. The majority of the pigs slaughtered at our sampling site came from small-scale farmers.

Isolates of *E. coli* from cattle had significantly lower rates of resistance to tetracycline, sulphamethoxazole/trimethoprim, or kanamycin than did isolates from pigs and chickens. Whereas this may reflect lower usage of these antimicrobials in cattle, it may also be explained by the greater maturity at slaughter, since adult cattle have been shown to harbour less resistant bacteria than calves (Khachatyran *et al.*, 2004). Resistance to streptomycin was significantly higher in isolates from cattle (43.7%) than in isolates from both pigs (25.7%) and chickens (34.0%). The selective pressure exerted by streptomycin in streptomycin-penicillin

combinations in intramammary and injectable preparations for the treatment of mastitis and other bacterial infections in cattle (Mitema *et al.*, 2001) might account for this finding. However, the relatively high incidence of streptomycin resistance among *E. coli* isolates from pigs and chickens was unexpected since oral formulations for mass administration are not available. This may be as a result of co-resistance with other unrelated compounds, horizontal transfer of resistance genes or transfer of streptomycin-resistant *E. coli* from cattle through cow dung as indegenous chicken are known to eat termites and other insects found in cow dung.

The levels of resistances to gentamicin observed among the *E. coli* and to chloramphenicol observed in both *E. coli* and *Salmonella* isolates of this study were comparable to the levels of resistance in other countries (Bywater *et al.*, 2004). Gentamicin, although a relatively old antimicrobial agent has had little use in animals (Bywater *et al.*, 2004) and in Kenya, no formulations are available for use in chickens. The resistance detected in *E. coli* isolates from chickens (2.0%) may have been caused by off-label use or the clonal spread of resistant isolates as suggested by Kijima-Tanaka *et al.* (2003).

Approximately five percent (4.7%) of the *E. coli* and 12.5% of the *Salmonella* isolates showed resistance to chloramphenicol. Chloramphenicol resistance was significantly higher in the isolates from pigs than those from chickens. In Kenya, as in the European Union or the USA chloramphenicol is not approved for use in food animals and its fluorinated analog, florfenicol has not been in use. Thus the observed resistance is unlikely to be mediated by a gene encoding resistance to florfenicol (White *et al.*, 2000). Other researchers have also reported chloramphenicol resistance among *E. coli* and *Salmonella* spp. isolates from chickens and pigs in the absence of chloraphenicol use in these animal species and suggested co-resistance with other unrelated compounds to be a possible explanation (Bywater *et al.*, 2004). Co-selection of chloramphenicol resistance during selective pressure imposed by the

use of sulphonamides and streptomycin due to linkage of genes has been reported (Kehrenberg and Schwarz, 2001; Bischoff *et al.*, 2005) and this may probably account for the resistance observed in the absence of chloramphenicol use. Sulphonamides are the second commonly used antimicrobial agents in food animals in Kenya while streptomycin accounts for more than 90% of the aminoglycoside use in these animals (Mitema *et al.*, 2001). Chloramphenicol resistance may also be acquired via horizontal transmission of genes from other sources, such as water contaminated with human sewage or due to illegal use of chloramphenicol (van Donkersgoed *et al.*, 2003). Chloramphenicol resistant organisms from humans can reach chicken or pigs via contact with animal attendants.

Differences in production systems and antimicrobial usage patterns in the various populations may account for the differences in the resistance patterns observed among the *E. coli* isolates from the three animal sources. In addition, these differences could also be related to the different antibiotic regimens used for the different antimicrobial agents and livestock species (Schwarz and Chaslus-Dancla, 2001; Guerra *et al.*, 2003). Taking into account that β-lactams, streptomycin and tetracycline are among the most commonly used drugs in food-producing animals in Kenya (Mitema *et al.*, 2001), the occurrence of resistances to these antimicrobial agents in *E. coli* isolates from all three species and *Salmonella* isolates from pigs was no surprise. Furthermore, these drugs are widely available for sale "over the counter" to farmers and thus play a major role in the small and large-scale food animal production in Kenya (Kariuki *et al.*, 1997).

Among the chicken *E. coli* isolates, resistance to tetracycline, and sulphamethoxazole/trimethoprim was the most common resistance phenotype. This may be explained by the fact that sulphonamide drugs are the commonly used oral preparations in chickens and pigs in Kenya (Mitema *et al.*, 2001). Moreover, great amounts of tetracycline are used in chickens rearing in Kenya (Kariuki *et al.*, 1997). Tetracyclines and

sulphonamides/trimethoprim accounted for nearly 78% of the antimicrobials commonly used for therapy in food-producing animals in Kenya (Mitema *et al.*, 2001). The resistance most frequently observed in *E. coli* from cattle was to streptomycin and ampicillin in combination and streptomycin or ampicillin alone. The selective pressure exerted by streptomycin-penicillin combinations in intramammary and injectable preparations for the treatment of mastitis and other bacterial infections in cattle (Mitema *et al.*, 2001) might account for this finding. The observation that there were no significant differences in the prevalence and patterns of resistance between the faecal and carcass swab isolates from either cattle or pigs may be due to the fact that slaughter is potentially the most important stage for bacterial contamination (Bywater *et al.*, 2004) and as a result resistant isolates from the gut may readily contaminate carcasses (Aslam *et al.*, 2003).

The results of this study give baseline information on the magnitude of the resistance problem in contemporary Kenyan *E. coli* isolates from food animals and can contribute to the development of a surveillance program in Kenya for antimicrobial resistant bacteria. Additionally, the results indicate that food animals may represent a reservoir of MDR *E. coli* and a considerable proportion of *E. coli* contaminants on fresh cattle and pig carcasses areresistant to a variety of antibiotics. Since the entry of these strains into the food-chain and the further exchange of these isolates among populations is possible, they pose a great risk in both selection and the spread of resistance. Secondly, this study demonstrated that *Salmonella* is present in pigs at slaughter and on pork carcasses and revealed the potential importance of pigs as source of single and multiple antimicrobial-resistant *Salmonella* isolates to commonly used antimicrobials including ampicillin, chloramphenicol, streptomycin and tetracycline. The data suggest that selection pressure imposed by the use of tetracycline derivatives, aminoglycosides, sulphonamides and penicillins in food animals is a key driving force in the selection of antimicrobial resistance in *E. coli* and non-typhoidal *Salmonella* serotypes.

It is recommended that prudent use of antimicrobial agents in food animals should be encouraged, slaughter hygiene improved and adequate heat processing of foods of animal origin in order to destroy bacteria and minimize the risk of transfer of antimicrobial resistant bacteria to humans. A national antimicrobial resistance surveillance program in food animals should be established to identify the emergence of resistant bacteria.

CHAPTER FOUR

4.0: GENETIC CHARACTERIZATION OF AMINOGLYCOSIDE AND CHLORAMPHENICOL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA ENTERICA* SUBSP. *ENTERICA* SEROTYPES BRAENDERUP AND SAINTPAUL FROM CATTLE, PIGS AND CHICKENS

ABSTRACT

Understanding the molecular mechanisms by which antimicrobial resistance emerges and spreads is crucial in designing future intervention strategies to reduce its progression. In this study, thirty *E. coli* and three *Salmonella* isolates were investigated for the molecular basis of aminoglycoside and chloramphenicol resistance. A total of 23 (76.7%) of 30 *E. coli* isolates harboured plasmids ranging in size from 2 to 106 kb while all three *Salmonella* isolates were plasmid-free. Twenty eight (96.6%) of the 29 *E. coli* and two of the *Salmonella* isolates resistant to streptomycin were positive for at least one of the two streptomycin resistance genes tested. All *Salmonella* isolates were positive for *strA* only. Among the *E. coli* isolates, 21 (72.4%) were positive for *strA* and *aadA1* while the remaining seven isolates (24.1%) were positive for *strA* only. The *catA1* gene was detected in all the chloramphenicol-resistant *E. coli* and *Salmonella* isolates. All the kanamycin- and/or gentamicin-resistant *E. coli* isolates were negative for the two genes analysed.

Sequence analysis of an unusual large *strA* amplicon of 1.2 kb from *E. coli* isolates revealed disruption of the *strA* gene by the insertion of a functionally active gene cassette *dfrA14* encoding trimethoprim resistance. Such amplicons were observed in 10 isolates, all of which harboured also the *aadA1* gene. Two small plasmids of 6 and 8 kb mediating resistance to streptomycin and sulphonamides, and to streptomycin, sulphonamides and tetracycline, respectively were identified. The *strA* gene was physically linked to the sulphonamide

resistance gene *sul2* in both plasmids. The *strA* and *catA1* genes were conjugally co-transferred with resistances to ampicillin, tetracycline, sulphamethoxazole/trimethoprim and sulphonamides. The *aadA1* gene was found as cassette-borne gene within class 1 integrons and was linked to trimethoprim resistance gene cassette *dfrA1*.

The results of this study indicate that *E. coli* and *Salmonella* serotypes Braenderup and Saintpaul from food animals in Kenya may represent a reservoir of streptomycin and chloramphenicol resistance genes, which may be transferable to other bacteria. The physical linkage of streptomycin resistance gene *strA* to sulphonamides resistance gene *sul2* offers the possibility of co-selection of this gene during selective pressure imposed by the use of sulphonamides and highlights the need for their prudent use in animal husbandry. The location of *strA* and *catA1* on conjugative plasmids and the *aadA1* gene within class 1 integrons constitutes an effective way to spread streptomycin and chloramphenicol resistance among bacteria from different ecosystems. This underlines the need for detailed epidemiological and molecular studies on acquisition of resistance genes and distribution of antimicrobial-resistant *E. coli* and *Salmonella* isolates among food animals, food products and humans in Kenya.

4.1: INTRODUCTION

Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the anti-infective armamentarium (Kotra *et al.*, 2000) to treat a broad range of life-threating infections in humans and animals (Prescott *et al.*, 2000; Gonzalez-Zorn *et al.*, 2005). Chloramphenicol is a broad-spectrum antibiotic that has been used extensively to treat bacterial infections in many countries for many years and in less developed settings, it retains a major role in the treatment of bacterial meningitis in humans (Shultz *et al.*, 2003).

Resistance against aminoglycosides and chloramphenicol in *E. coli* and *Salmonella* serotypes of food animal origin have been reported in various parts of the world (Lanz *et al.*, 2003; Guerra *et al.*, 2003; Bywater *et al.*, 2004; Bischoff *et al.*, 2005). One of the most common resistance mechanisms against aminoglycosides is the production of aminoglycoside acetyltransferases (AACs), aminoglycoside phosphorylases (APHs), and aminoglycoside adenyltransferases (ANTs) (Shaw *et al.*, 1993), which are mainly mediated by transferable large plasmids (Yamane *et al.*, 2005). The phosphotransferase *aph(3'')-Ib* and *aph(6)-Id* genes (also known as *strA* and *strB*, respectively) and the adenyltransferase gene *ant(3'')-Ia* (also designated *aadA1*) are the most frequently encountered streptomycin resistance genes in *E. coli* (Guerra *et al.*, 2003; Reyes *et al.*, 2003), *Salmonella spp.* and other Gram-negative bacteria (Gebreyes and Altier, 2002; Frech *et al.*, 2003). The genes *aph(3')-Ia* and *ant(2'')-Ia* are among the commonly reported genes mediating resistances to kanamycin and neomycin, and to kanamycin, tobramycin and gentamicin in *E. coli* (Sandvang and Aarestrup, 2000; Guerra *et al.*, 2003, Saenz *et al.*, 2004) and diverse *Salmonella* serotypes (Frech *et al.*, 2003).

Resistance to chloramphenicol is known to be mediated enzymatically by the plasmid-located chloramphenicol acetyltransferases (CATs) (Cannon *et al.*, 1990) or by the nonenzymatic chloramphenicol resistance genes *cmlA* (Dorman and Foster, 1982), or *floR* that encode efflux pumps (Cloeckaert *et al.*, 2001).The *floR* gene is similar in primary structure to

cmlA and confers resistance to both chloramphenicol and florfenicol (Bischoff *et al.*, 2005). Resistance phenotypes may arise from many different genetic determinants and each determinant may present specific epidemiological features (Lanz *et al.*, 2003). Therefore, the assessment of the resistance situation at the genetic level is an important aspect in the understanding and control of antimicrobial resistance (Lanz *et al.*, 2003).

Transfer of resistance genes by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons (Schwarz and Chaslus-Dancla, 2001; Carattoli, 2001) is an important factor that can contribute to the increase in multiresistant bacteria (Saenz *et al.*, 2004). Integrons are genetic units containing elements for site-specific recombination, capture and mobilization of gene cassettes (Hall and Stokes, 1993). The class 1 integrons are the best characterized (Reyes *et al.*, 2003) and the most frequently detected integrons among *Enterobacteriaceae* (Guerra *et al.*, 2003), and are important in the proliferation of bacterial multidrug resistance in these species (Chen *et al.*, 2004). Gene cassettes consist of a gene coding region (or opening reading frame) and a recombination site known as a 59-base element which is located downstream of the gene in the linear integrated form (Hall *et al.*, 1991; Hall and Stokes, 1993). Integron-borne gene cassettes conferring resistance to aminoglycosides and chloramphenicol are much diffused in MDR *E. coli* (Sandvang and Aarestrup, 2000; Bunny *et al.*, 1995, Bischoff *et al.*, 2005) and *Salmonella* (Gebreyes and Altier, 2002; Nogrady *et al.*, 2005) isolates.

Previous studies in Kenya have reported aminoglycoside and/or chloramphenicol resistance in bacteria of animal origin (Gakuya *et al.*, 2001; Kariuki *et al.*, 2002), but none of them have analysed in depth the mechanisms of resistance underlying these resistance phenotypes. Therefore the genetic background and the mechanisms responsible for aminoglycoside or chloramphenicol resistance in *E. coli* and *Salmonella* serotypes of animal origin in Kenya are still largely unknown. Understanding the molecular mechanisms by which

antimicrobial resistance emerges and spreads is crucial in designing future intervention strategies to reduce its progression (Chen *et al.*, 2004). The identification and determination of the genetic location and the potential linkage of the genes responsible for aminoglycoside and chloramphenicol resistance with other antimicrobial resistance genes among multidrug resistant *E. coli* and *Salmonella* isolates are important in predicting the risk of further spread or persistence of the resistance.

4.1.1. Objectives

The objectives of this study were:

1. To investigate the presence and distribution of streptomycin resistance genes *strA* and *aadA1*, kanamycin resistance gene *aph(3')-Ia*, kanamycin/gentamicin resistance gene *ant(2'')-Ia* and chloramphenicol resistance genes *catA1, catA3* and *cmlA* among *E. coli and Salmonella* isolates from cattle, pigs and chickens.
2. To determine the location of these genes and whether they are transferable by conjugation and/ or transformation.

4.2: MATERIALS AND METHODS

4.2.1. Bacterial isolates and antimicrobial susceptibility testing

The thirty *E. coli* and three *Salmonella* isolates included in this study were selected according to their resistance to aminoglycosides and/or chloramphenicol. The *E. coli* isolates were from cattle (5), pigs (19) and chickens (6) while all three *Salmonella* isolates were from pigs and belonged to serotypes Braenderup (one isolate) and Saintpaul (two isolates). The intial susceptibility testing was performed by disc diffusion and broth macrodilution as described in chapter 3 sections 3.2.5.1 and 3.2.5.2, respectively. Susceptibility testing of the transformants and transconjugants was also performed by disc diffusion.

4.2.2. Plasmid profile analysis

4.2.2.1. Isolation of plasmid DNA

Three different methods were used to isolate plasmid DNA from the resistant isolates and their transformants or transconjugants. The choice of the method for plasmid extraction was based on the purpose for which DNA had to be used. To screen the resistant isolates or their transformants or transconjugants for the presence of plasmids the Kado and Liu (1981) method was used. Plasmid DNA for transformation and PCR amplification was prepared by the minilysate method, which is a modification of alkaline lysis by Birnboim and Doly (1979). The affinity chromatography method was used to prepare plasmid DNA for restriction analysis and sequencing. The affinity chromatography method involves plasmid DNA extraction by alkaline denaturation and subsequent purification by affinity chromatography and produces plasmid DNA of high concentration and purity.

4.2.2.1.1. Kado and Liu method for plasmid isolation

A modification of the method of Kado and Liu (1981), which is particularly suitable for the recovery of large enterobacterial plasmids was followed as previously described (Frech and Schwarz, 2000). Bacterial cells were grown overnight in 3 ml of LB broth (Oxoid, Wesel, Germany) at 37 °C in a shaking incubator. The bacterial cells were harvested by centrifuging 1.5 ml of the overnight culture in eppendorf tubes for 7 min at 4 °C and 13000 rpm. The supernatant was discarded and the pellet resuspended in 20 µl Tris/EDTA [50mM Tris acetate: 1mM EDTA pH (7.9)] by vortexing. The cells were then lysed by the addition 100 µl of freshly prepared lysis solution [1 ml of 250 mM Tris, 75 µl of 5 mM NaOH (Roth, Karlsrule, Germany), 1 ml of 15% sodium deodecyl sulphate (SDS) (Roth) and 2.88 ml distilled water], incubated for 25 minutes at 58 °C, and extracted with 100 µl of phenol-chloroform (1:1 [vol/vol]). After centrifugation, for 20 min at 13000 rpm the supernatant was carefully pipetted into new eppendorf tubes and incubated on ice for 30 min after which 30 µl were analysed by electrophoresis as described below in section 4.2.3.

4.2.2.1.2. Minilysate method for plasmid isolation

The minilysate method was carried out as described by Kehrenberg *et al.* (2003). A single bacterial colony was inoculated into 3 ml of Luria Bertani (LB) broth (Oxoid) supplemented with 30 µg/ml streptomycin or 20 µg/ml chloramphenicol and then incubated overnight in a shaking incubator at 37 °C. The bacterial cells were harvested by centrifuging 1.5 ml of the overnight culture in eppendorf tubes for 10 min at 4 °C and 7000 rpm. The supernatant was discarded and the pellet resuspended in 100 µl of buffer 1 with RNAse (50 mM Tris, Sigma)/HCl, Oxoid), pH 8.0, 10 mM EDTA, Roth) and 100 µl/ml RNAse) followed by 200 µl of lysis buffer 2, (200 mM NaOH, Roth) and 1% sodium deodecyl sulphate (SDS,

Roth) and then incubated on ice for 5 min. One hundred and fifty microlitres of minilysate solution 3 (3 M sodium acetate, pH 4.8, Roth) was added and mixed by vortexing. After 10 min incubation on ice, the suspension was centrifuged for 30 min at 13000 rpm.

The supernatant was carefully decanted into new eppendorf tubes containing 1 ml of cold absolute (100%) ethanol (Roth) kept at –24 °C. After 60 min incubation at room temperature, the suspension was centrifuged at 4 °C for 30 min at 13000 rpm. After discarding the supernatant, the pellet was resuspended in 500 µl of cold 80% ethanol (Roth) kept at -24 °C for washing and then centrifuged at 4 °C 10 min at 13000 rpm. The supernatant was removed with a pasteur pipette and the pellet air dried in an exsiccator for 20 min. The DNA was resuspended in 30 µl of sterile double distilled water.

4.2.2.1.3. Affinity chromatography method for plasmid isolation

Commercially available plasmid preparation kits (Midi; Qiagen, Hilden, Germany) were used as recommended by the manufacturers to prepare plasmid DNA for restriction endonuclease analysis or sequencing. A single bacterial colony was inoculated into 100 ml of LB medium supplemented with 30 µg/ml streptomycin and incubated overnight in a shaking incubator at 37 °C. The overnight culture was transferred into two 50 ml sterile Falcon tubes and centrifuged for 10 min at 4 °C and 6000 rpm. The supernatant was discarded and the bacterial pellets in both tubes resuspended in 4 ml of buffer P1 plus RNAse (Tris/HCl pH 8.0 50 mM, EDTA 10 mM and RNAse 100 µl/ml) and the suspension transferred to corex tubes. Cell wall lyses and alkaline denaturation were done by adding 4 ml of buffer P2 (NaOH 200 mM and SDS 1%) and after 5 min incubation at room temperature, 4 ml of chilled buffer P3 (potassium acetate, pH 4.8, 2.55 M) were added. Following gentle mixing and incubation on ice for 10 min, the suspension was centrifuged at 10000 rpm for 30 min.

The column was prepared and 4 ml of buffer QBT (NaCl, 750 mM, morpholinopropansulfonic acid (MOPS), ethanol 15% (v/v) pH 7.0) added and allowed to empty by gravity flow. The supernatant from the centrifugation was applied and allowed to enter the resin by gravity flow. The column was then washed twice with 10 ml of buffer QC (NaCl, 1M MOPS 50 mM, ethanol 15% (v/v) pH 7.0). Plasmid DNA was eluted with 5 ml of buffer QF (NaCl 1.2 M, MOPS 50 mM, ethanol 15% (v/v) pH 8.0) in a corex tube. The DNA was precipitated by adding 3.5 ml of isopropanol and then pelleted by centrifuging at 10000 for 30 min. The supernatant was discarded and the pellet resuspended in cold 80% ethanol for washing then centrifuged for 10 min at 10000. After discarding the supernatant the pellet was air-dried in an exsiccator for 20 min. The dried plasmid DNA pellet was resuspended in 100 µl of double distilled sterile water.

4.2.3. Agarose gel electrophoresis

Plasmid and whole cell DNA were analysed by electrophoresis on 0.8% and 1% agarose gels, respectively, whereas PCR amplification and restriction analysis products were analysed in 1.5% gel on horizontal tanks containing 1x TAE (Tris Acetate-EDTA) as running buffer. The 0.8% and 1% agarose gels were prepared by dissolving, 0.4 g of "multipurpose"-agarose (Roth, Karlsrule, Germany) in 50 and 40 ml of 1x TAE (0.4 M Tris-HCL, 0.02 M $Na_2EDTA.2H_2O$, 0.2 M sodium acetate, 1.02 M acetic acid), respectively, while the 1.5% gel was prepared by dissolving 0.6 g of the agarose in 40 ml of 1x TAE. Agarose was heated until it dissolved completely, cooled to 55 °C and poured onto horizontal gel tanks mounted with combs for making the wells. The gel was allowed to set for 30 min and then placed in an electrophoresis chamber (10 cm x 5 cm x 0.7 cm) (Sub-Cell GT Agarose Gel Electrophoresis System, BioRad, Munich, Germany) filled with 1x TAE buffer.

Ten microlitres of DNA was mixed with 4 µl of 'blue maker' (50% v/v glycerine, 50 mM EDTA and 0.25% Bromophenol blue) and loaded onto the wells. The blue marker served to make the migration of the DNA to be visible. Electrophoresis was performed at 40 V for about 15 min until the samples had migrated from the wells and then 80 V (Power supply: Power Pac 300, BioRad, Munich, Germany) for 2 h. Molecular size standards were included in the gel every time electrophoresis was done. The plasmids of *E. coli* V517 which has eight plasmids of known size ranging from 2.1 to 54.0 kb (Macrina *et al.*, 1978), and the *Klebsiella pneumoniae* plasmid R55 (150 kb), and the 90 kb plasmid of *Salmonella* Typhimurium LT2 served as the molecular size standards (Blickwede and Schwarz, 2004) for the plasmids to ensure identification of the presence of large plasmids. The gel was stained in ethidium bromide (Boehringer, Mannheim, Germany) (10 µg/ml) for 1 min and destained in water on a shaker (Reax 3, Jürgens, Gehrden, Germany) for 15 min.

4.2.4. Documentation of gels and estimation of the plasmid molecular sizes

After destaining, the gels were visually inspected after illumination by ultraviolet light of 312 nm wavelength on a transilluminator (Herolab UVT-20 M, Herolab, Wiesloch, Germany). The results were photographically documented using a camera documentation system (Herolab E.A.S.Y, RH 429K, Herolab, Wiesloch, Germany) and printed on a special photography paper (High Density Printing Paper UPP-110 HD, Digital Graphic Printer UP D 860 E, Sony Tokyo, Japan) using a digital graphic printer (Sony Digital Graphic Printer UP-D860E, SONY Tokyo, Japan). The migration distances of DNA bands were measured directly from photographs of the gels. The plasmid sizes were estimated by standard polynomial curves generated with the logarithm of the relative migration of DNA on the *X* axis and the logarithm of the molecular size of standard plasmids on the *Y* axis with the Microsoft Excel program as previously described by Rochelle *et al.* (1985) and Wand *et al.* (2003). The

plasmid profiles from various isolates were compared for any plasmid that was common for the isolates.

4.2.5. Determination of plasmid location of resistance genes

4.2.5.1. Plasmid transfer by transformation

A modification of the calcium chloride ($CaCl_2$) method by Dagert and Ehrlich (1979) as previously described (Kehrenberg *et al.*, 2003) was used to prepare competent cells for transformation experiments. Two millilitres of LB broth were inoculated with a single colony of *E. coli* JM 109 cells (Stratagene, Amsterdam, The Netherlands) and the culture grown to saturation in a shaking incubator overnight at 37 °C. After overnight incubation, the 2 ml of overnight culture were added into 100 ml LB medium and the culture grown to an optical density (OD) of between 0.2 - 0.3 at 600 nm (OD_{600}) and then incubated on ice for 10 minutes to stop further growth. The culture was centrifuged for 10 min at 4 °C and 7000 rpm, the supernatant discarded and the pellet incubated on ice for 20 min. After incubation the pellet was resuspended in 20 ml of ice cold sterile 0.1 M $CaCl_2$ solution (Merck, Darmstadt, Germany) and incubated on ice for 30 min after which the suspension was centrifuged for 7 min at 4 °C and 7000 rpm.

After discarding the supernatant, the pellet was resuspended in 500 µl of the ice cold sterile 0.1 M $CaCl_2$ and incubated on ice for 1 h to yield the final competent cells suspension. Ten microlitres of plasmid DNA prepared as described in section 4.2.2.1.2 were added to aliquots of 100 µl of the *E. coli* JM 109 competent cells in an eppendorf cup and carefully mixed. The mixture was incubated on ice for 15 min and at 37 °C for 5 min in a thermomixer (Eppendorf thermomixer 5436, Engeldorf, Germany). After repeating these incubations twice, the suspension was further incubated on ice for 15 min. Four hundred microlitres of sterile LB medium were added to each transformation solution and then incubated at 37 °C for 1 h.

Following the incubation, aliquots of 100 µl were transferred to five LB agar plates supplemented with either 30 µg/ml streptomycin or 20 µg/ml chloramphenicol or 50 µg/ml kanamycin and then spread with sterile, bent metal streaking rod, ('hockey stick'). The plates were incubated for 16 to 20 h at 37 °C. Any colonies on the selective plates (transformants) were analysed for their antimicrobial susceptibility by disc diffusion and plasmid content as described earlier in chapter 3 section 3.2.5.1 and above in section 4.2.2.1.1, respectively to investigate their resistance properties and the plasmids transferred.

4.2.5.2. Plasmid transfer by conjugation

Conjugation experiments were carried out by plate-mating procedure as described by Petroni *et al.* (2002) with nalidixic acid resistant (Nal^R) mutant of *E. coli* K12 strain 7118N Lac^- as the recipient strain. The recipient and the donor strains were grown overnight at 37 °C in 10 ml LB-medium containing 60 µg/ml nalidixic acid and 30 µg/ml streptomycin or 20 µg/ml chloramphenicol or 50 µg/ml kanamycin. The overnight cultures were centrifuged at room temperature at 10000 rpm for 15 min. After decanting the supernatants, pellets were allowed to dry at room temperature. The pellets were subsequently resuspended in 1 ml of LB medium without antibiotics and the optical density measured at 578 nm for a value of 2.5 for both the donor and the recipient cell suspensions. Seven hundred and fifty microlitres of the recipient cells suspension were mixed with 150 µl of the donor cells suspension (5:1 ratio). The mixture was centrifuged for 15 min at 10000 rpm and after decanting the supernatant the pellet was partially dried at room temperature for 2 h. The dried pellet was subsequently abraded with the aid of the wire loop and placed carefully in the centre of dried surface of LB agar without antibiotic supplement.

Following incubation at 37 °C for 18 h the bacteria films were abraded and resuspended in 1 ml LB-medium. A serial dilution step of this suspension was carried out starting with 10

µl of the original suspension into 990 µl of LB broth to have a dilution of 10^{-1}. Further dilution up to 10^{-8} was performed and 100 µl of each dilution was spread on the following agar plates; LB without antibiotics, LB supplemented with 30 µg/ml streptomycin, LB supplemented with 60 µg/ml nalidixic acid, LB supplemented with 20 µg/ml chloramphenicol, and LB supplemented with both 30 µg/ml streptomycin and 60 µg/ml nalidixic acid or 20 µg/ml chloramphenicol and 60 µg/ml nalidixic acid or 50 µg/ml kanamycin and 60 µg/ml nalidixic in order to determine the transfer frequencies of the plasmid DNA. The conjugation frequency was determined after counting and analysing the number of colonies obtained at the end of the overnight incubation of the plates and the transconjugants analysed as described above for transformants in section 4.2.5.1.

4.2.6. Plasmid restriction endonuclease profile analysis

The plasmid DNA prepared as described in section 4.2.2.1.3 were digested/restricted with a battery of enzymes according the manufacturers' recommendations (Roche Applied Science, Mannheim, Germany and Fermentas Life Sciences, Opesstrasse, St-Leon-Rot, Germany). The enzymes and buffers used are listed in Table 4.1.

Table 4.1: Restriction endonucleases, buffers and incubation temperatures.

Enzyme	Buffer	Incubation temperature (°C)	Recognition sequence	Manufacturer (s)
*Bam*HI	B	37	G↓GATCC	Roche
*Bcl*I	M	50	T↓GATCA	Roche
*Bgl*II	M	37	AGATCT	Roche
*Dra*I	M	37	TTT↓AAA	Roche
*Eco*RI	H	37	G↓AATTC	Roche, Fermentas
*Eco*RV	B	37	GAT↓ATC	Roche
*Hin*dIII	B	37	A↓AGCTT	Roche
*Hpa*I	B+	37	C↓CGG	Fermentas
*Kpn*I	L	37	GGTAC↓C	Roche
*Ksp*I	L	37	CCGC↓GG	Roche
*Pst*I	H	37	CTGCA↓G	Roche
*Pvu*II	M	37	CAG↓CTG	Roche
*Sac*I	A	37	GAGCT↓C	Roche
*Sal*I	H	37	G↓TCGAC	Roche
*Sma*I	A	25	CCC↓GGG	Roche
*Xba*I	H	37	T↓CTAGA	Roche
*Xho*I	H	37	C↓TCGAG	Roche

NB: Arrrow indicates the point at which the enzyme cuts the recognition sequence

4.2.6.1. Single digests

For single digests, restriction was carried out in 15 µl reaction volume comprising 1 µl bovine serum albumin (BSA) (Boehringer, Mannheim, Germany), 1.5 µl (10x reaction buffer), 1.0 -11.5 µl DNA (~ 300 ng), 1 µl enzyme (5-10 U) (Boehringer, Mannheim, Germany) and double distilled sterile water added up to make a final volume of 15 µl. The mixture was shortly centrifuged at 10000 rpm for 7 seconds and incubated in eppendorf thermomixer at the optimum temperature for the particular enzyme as recommended by the manufacturers (Roche or Fermentas) for 1.5 h.

4.2.6.2. Double digests

Double restriction digests were carried out according to (Danna, 1980). Plasmid DNA was digested with the first enzyme for 75 min after which for enzymes requiring no change of buffer the second enzyme was added and the mixture incubated for 75 min at the appropriate temperature according to the manufacturer's instructions. For the enzymes requiring a change in buffer concentration, the appropriate amounts of NaCl and Tris-HCL in microlitres required, were calculated using the following formula:

$$\text{Required volume (µl)} = \frac{\text{Difference in concentration (mmol/L)}}{\text{Concentration of the stock solution (mmol/L)}} \times \text{Reaction volume (µl)}$$

The enzyme combinations used are shown in appendices 10, 12 and 14. For double digests with enzymes requiring different optimum temperatures, digestion was carried out first with the enzyme with the low temperature requirement followed by the enzyme with the high temperature requirement.

4.2.6.3. Analysis of the restriction digests and construction of restriction maps

Restriction fragments were separated by agarose gel electrophoresis as earlier described in section 4.2.3 and the fragment lengths in single and double digestions estimated against the fragments of the 1 kb ladder (Gibco BRL, Eggestein, Germany) used as a marker. The distances between restriction enzyme cleavage sites were determined by the patterns of fragments produced by the restriction enzyme digestions. Restriction maps showing the positions at which the endonucleases cut the plasmid DNA were constructed by ordering the fragments obtained from the plasmids DNA as previously described by Kehrenberg and Schwarz (2000). The restriction maps were compared with one another and with maps of other plasmids from *E. coli* known to mediate these resistance properties.

4.2.7. Determination of the presence of resistance genes by PCR analysis

4.2.7.1. Isolation of DNA templates

4.2.7.1.1. Isolation of whole-cell DNA

A modification of the phenol-chloroform extraction method previously described by Kehrenberg and Schwarz (2000) was followed in preparing whole-cell DNA from the *E. coli* isolates. The *E. coli* isolates were subcultured from glycerine cultures stored at -80 °C onto LB agar supplemented with 30 µg/ml streptomycin or 20 µg/ml chloramphenicol or 50 µg/ml kanamycin. After overnight incubation, a single colony was inoculated into 5 ml of LB broth supplemented with 30 µg/ml streptomycin or 20 µg/ml chloramphenicol or 50 µg/ml kanamycin and incubated overnight at 37 °C in a shaking incubator at 120 rpm. The bacterial cells were harvested by centrifuging the broth culture in a cooled centrifuge (3K 30, Sigma, Deisenhofen, Germany) for 10 min, at 4 °C and 6000 rpm. The supernatants were discarded, the bacterial pellets suspended in 1 ml of Tris-EDTA-sodium chloride (TES) buffer and

vortexed. Following centrifugation, the supernatants were discarded and the pellets resuspended in 0.5 ml of TES buffer and the suspensions transferred into eppendorf tubes.

After adding 10 µl of the lysis buffer, 20% sodium deodecyl sulphate (SDS) (Roth) the suspensions were mixed by gently rotating the cups until the mixture became slimy in appearance. These suspensions were then incubated at room temperature for 10 min. Equal volumes of 540 µl phenol/chloroform (1:1) (Roth) were added, the solution vortexed for 2 min and then centrifuged for 5 min at 13000 rpm. Approximately 500 µl of the upper clear phase were carefully transferred to new eppendorf tubes using pipette tips with cut ends.

The extraction was repeated twice with phenol/chloroform (1:1) and once with 500 µl of (24:1) chloroform/isoamyl alcohol (Merck, Darmstadt, Germany). The upper clear phase was transferred into new eppendorf tubes and 500 µl isopropanol (Roth) added. The solutions were mixed by rotating the eppendorf tubes gently until the formation of a white pellet was observed. The suspensions were then centrifuged at 4 °C and 13000 rpm for 20 min. After carefully removing the supernatants with a pasteur pipette, the pellets were air dried in an exsiccator for 25 min. The dry DNA pellets were resuspended in 30 µl of double distilled sterile water, then stored overnight at 4 °C before electrophoresis was done as described in section 4.2.6 to assess the quality of the DNA isolated.

4.2.7.1.2. Isolation of plasmid DNA

Plasmid DNA was isolated as earlier described earlier in section 4.2.2.1.2.

4.2.8. PCR assays

Specific PCR assays were used for the detection of the resistance genes *strA*, *aadA1* (streptomycin resistance), *aph(3')-Ia* (kanamycin resistance), *catA1*, *catA*, *cmlA* (chloramphenicol resistance), and *ant(2'')-Ia* (kanamycin and gentamicin resistance). In

addition, the physical linkage of the streptomycin resistance gene *strA*, with the sulphonamide resistance gene *sul2* and location of the streptomycin resistance gene *aadA1* in the class 1 integron were investigated. PCRs followed previously described protocols (Frech and Schwarz, 2000; Kehrenberg and Schwarz, 2001; Waturangi *et al.*, 2003). Following conjugation the genes responsible for the co-transferred resistances were also analysed by PCR. Table 4.2 shows the primers and annealing temperatures used in this study while the rest of the amplification conditions are shown in appendix 19.

All PCR assays were performed in a top heated T3 Thermocycler (Trio Thermoblock, Biometra, Göttingen, Germany) in 50 µl reaction mixtures containing 3 µl (20 ng/µl) of template DNA, 1 µl (20 pmol) of each primer, 3 µl dNTP- Mix (2 mM dATP, dCTP, dGTP, dTTP); 5 µl buffer for polymerase (10x concentration), 0.5 µl DNA-Polymerase (*Taq*– 4 U/µl; Carl Roth, Karlsruhe, Germany) and 36.5 µl double distilled sterile water. *Pwo* polymerase (Peglab, Erlangen, Germany) with high proof-reading activity was used to prepare blunt end PCR products for cloning. The template consisted of whole cell DNA and/or plasmid DNA prepared as previously described in sections 4.2.7.1.1 and 4.2.2.1.2, respectively. A negative control (double distilled sterile water) and positive control (DNA known to contain the gene(s) being investigated) were included as control for the PCR reactions. PCR amplifications were carried out using program conditions consisting of an initial denaturation, followed by cycles of denaturation, annealing and extension and final extension at 72 °C for 7 min except for *catA1* gene. The program for *catA1*, consisted of one cycle of denaturation, annealing and extension, followed by cycles of denaturation, annealing and extension, and further extension at 72 °C for 10 min.

PCR analysis to confirm the linkage of *strA* and *sul2* resistance genes was performed with primer pairs *sul2* forward primer – *strA* reverse primer as well as *strA* forward primer – *sul2* reverse primer on plasmid DNA from transformants and transconjugants. For this, the

PCR conditions followed exactly those described for *sul2* and *strA*. The PCR products were detected by electrophoresis in 1.5% agarose gels. The PCR products obtained with primers *sul2* forward and *strA* reverse, 5'CS and 3'CS, and the unusual large 1.2 kb *strA* primer amplicons were cloned into pCR® BluntII TOPO® (Invitrogen™ Groningen, The Netherlands) and sequenced (MWG-Biotech, Ebersberg, Germany) as described below in sections 4.2.9 and 4.2.9.4, respectively.

Table 4.2: Sequences of oligonucleotides used as primers and annealing temperature for the detection of resistance determinants and class 1 integrons

Target gene/Primer	Oligonucleotide sequence (5′→ 3′)	Amplicon size (bp)	Annealing temp (°C)	Reference sequence {Genbank Accession number (s)}
intI1	f:- (5′CS) – GGCATCCAAGCAGCAAG r:- (3′CS) – AAGGAGACTTGACCTGA	variable	56	*Pseudomonas aeruginosa* (AY460181)
aadA1	f:- GTGGATGGCGGCCTGAAGCC r:- ATTGCCCAGTCGGCAGCG	527	56	*E. coli* (M10241, X02340)
strA	f:- GACTGGTTGCCTGTCAGAGG r:- CAGTTGTCTTCGGCGTTAGCA	646	64	Plasmid RSF 1010 M28829
aph(3')-Ia	f:- AACGTATTGCTCGAGGCCGCG r:- GGCAAGATCATGGATTCGGTCTCG	669	60	*Enterococcus faecalis* V01547
ant(2'')-Ia	f:- GGGCGCGTCATGGAGTT r:- TATCGCGACCTGAAAGCGGC	328	56	Unidentified bacterium X04555
bla_{PSE}	f:- CGCTTCCCGTTAACAAGTAC r:- CTGGTTCATTTCAGATAGCG	465	58	*Salmonella Typhimurium* AF153200
bla_{TEM}	f:- CCGTGTCGCCCTTATTCCC r:- GCCTGACTCCCCGTCGTGT	780	51	*Enterobacter cloacae* AY302260
catA1	f:- GGCATTTCAGTCAGTTG r:- CATTAAGCATTCTGCCG	551	50	Tn9 *E. coli* (V00622)
catA3	f:- ACCATGTGGTTTTAGCTTAACA r:- GCAATAACAGTCTATCCCCTTC	473	56	Uncultured eubacterium (AJ271879)
cmlA	f:- CCGCCACGGTGTTGTTGTTATC r:- CACCTTGCCTGCCCATCATTAG	698	40	*Pseudomonas aeruginosa* (M64556)
sul1	f:- ATGGTGACGGTGTTCGGCATTCTG r:- CTAGGGATGATCTAACCCTCGGTC	418	64	Plasmid R388 (X12869)
sul2	f:- ACAGTTTCTCCGATGGAGGCCG r:- CTCGTGTGTGCGGATGAAGTCA	704	64	*E. coli* plasmid p9123 (AY360321)
tet(A)	f:- GTAATTCTGAGCACTGT r:- CCTGGACAACATTGCTT	954	45	RP1 from *E. coli* (X00006)
tet(B)	f:- ACGTTACTCGATGCCAT r:- AGCACTTGTCTCCTGTT	1170	48	Tn*10* from *Shigella flexneri* (J01830)
tet(C)	f:- AACAATGCGCTCATCGT r:- GGAGGCAGACAAGGTAT	1138	50	pSC101 from *Salmonella Typhimurium* (X01654)
tet(H)	f:- ATACTGCTGACACCGT r:- TCCCAATAAGCGACGCT	1076	50	pVM111 from *Pasteurella multocida* (Y15510).

f, forward primer; r, reverse primer

4.2.9. TOPO cloning and sequencing of PCR products

4.2.9.1. Cloning of PCR products to pCR®-Blunt II-TOPO® vector

Amplicons representing the *sul2-strA*, variable regions of the class 1 integron and ~1.2 kb *strA* were prepared using a high fidelity *Pwo* polymerase (Peqlab, Erlangen, Germany) and ligated into the pCR®-Blunt II-TOPO® (Invitrogen™ Groningen, The Netherlands) vector. The ligation reaction was carried out in 10 µl reaction mixture consisting, 3 µl of blunt PCR product, 1 µl of 10x ligation buffer (with ATP), 1 µl (25 ng) pCR®-Blunt vector, 1 µl T4 DNA Ligase (4 U/µl) and 4 µl sterile double distilled water. The reaction mixture was incubated at 16 °C for 1 h after which it was used to transform competent *E. coli* TOP10 cells.

4.2.9.2. Transformation of competent TOP10 *E. coli* cells

Two microlitres of the ligation reaction mixtures were pipetted directly into eppendorf tubes containing 100 µl aliquots of competent *E. coli* TOP10 cells (Invitroagen, Groningen, The Netherlands) and mixed by stirring gently with a pipette tip. The eppendorf tubes were incubated on ice for 30 min and then the cells were heat shocked for 30 seconds in waterbath (Water bath, SW-20C, Julabo, Seelbach, Germany) at 42 °C without shaking. The eppendorf tubes were then immediately placed on ice for 2 min and thereafter 250 µl of room temperature LB medium added to each eppendorf tube. The cups were capped tightly and shaken horizontally at 37 °C for 1 h at 200 rpm in a shaking incubator. Fifty microlitres from each cloning cup were pipetted to the centre of prewarmed LB agar plates containing 50 µg/ml kanamycin (Sigma, Deisenhofen, Germany) and immediately spread. The plates were then incubated overnight at 37 °C.

4.2.9.3. Screening for transformed TOP10 *E. coli* cells

The pCR®-Blunt II-TOPO® vector contains the lethal *E. coli* gene *ccdB* and a kanamycin resistance gene. Without the insert the vector expresses the toxic CcdB protein which kills *E. coli* cells while ligation of an insert from PCR disrupts expression of the CcdB protein allowing the cells to grow. Colonies growing on selective media with kanamycin exhibit succeful transformation and presence of insert. To confirm the presence of plasmids, plasmids from the transformants were isolated as described in section 4.2.2.1.3 and then screened for the desired inserts by restriction with *Eco*RI since the TOPO® cloning site in the pCR®-Blunt II-TOPO® vector is found between two *Eco*RI restriction sites.

4.2.9.4. Sequencing of cloned DNA and homology searches

Plasmid DNA was prepared from clones (transformed TOP10 *E. coli* cells) by affinity chromatography as described in section 4.2.2.1.3 and the amount of DNA yield determined by UV spectrophotometry at an absorbance of 260 nm. Sequencing was carried out at MWG-Biotech, Ebersberg, Germany. Homology searches for comparative analysis of nucleotide sequences were performed with the BLAST program at the National Centre for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences were deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers, AJ884723, AJ884724 and AJ884725.

4.3: RESULTS

4.3.1. Resistance phenotypes and plasmid profiles of *E. coli* isolates

Table 4.2 shows the resistance phenotypes and plasmid profiles of the 30 MDR isolates used in this study. Aminoglycoside resistances were for streptomycin (29 isolates, 96.7%), kanamycin (6, 20.0%), and gentamicin (2, 6.7%). Resistance to chloramphenicol was found in seven (23.3%) of the isolates. Other resistances found in these isolates were to ampicillin (72.4%), sulphamethoxazole/trimethoprim (55.2%) and tetracycline (51.7%). Twenty three (76.7%) of the 30 *E. coli* strains investigated carried plasmids. Of these, 17 were obtained from pigs, 4 from chickens and 2 from cattle. The number of plasmids per isolate ranged between one and four while the size of the plasmids ranged from 2 to 106 kb. A variety of both small and large plasmids were detected in the isolates showing resistance to aminoglycosides while seven of the strains were plasmid-free. All the chloramphenicol resistant isolates carried plasmids and 5 (71.4%) of the 7 isolates carried large plasmids ranging in size from 62 to 106 kb (Table 4.3). Overall, a 106 kb plasmid was most frequently (30.4%) detected and was harboured by isolates with diverse resistance phenotypes. Figure 4.1 shows the plasmid profiles for selected isolates.

Table 4.3: Resistance phenotypes and plasmid profiles for the *E. coli* isolates.

Animal species	Strain No.	Resistance phenotype	No. of plasmids	Approximate size in kb
Pig	12C	$Amp^RSxT^RSm^R$	1	90
	35F	SxT^RSm^R	1	31
	3C	$Amp^RTet^RSxT^RSm^R$	1	72
	3F	$Amp^RTet^RSxT^RSm^RGm^RKm^RCm^R$	1	95
	70C	$Amp^RSxT^RSm^RCm^R$	1	2
	72C	$Amp^RTet^RSxT^RSm^RCm^R$	1	106
	33F	$Amp^RTet^RSxT^RSm^R$	1	9
	7F	Amp^ISm^R	1	6
	60C	$Amp^RTet^RSxT^RCm^R$	1	5
	27F	$Amp^RTet^RSxT^RSm^RKm^I$	2	85, 62
	65F	$Tet^RSxT^RSm^RKm^RCm^R$	2	100, 58
	20C	$Amp^RSxT^RSm^R$	2	10, 3
	4F	$Amp^RTet^RSxT^RSm^R$	3	18, 6, 4
	80F	$Amp^RTet^RSxT^RSm^RKm^RCm^R$	3	106, 10, 5
	8C	Tet^RSm^R	3	8, 6, 4
	66C	Amp^ISm^R	4	106, 16, 6, 4
	74C	Tet^RSm^R	4	106, 6, 5, 4
	21C	$Tet^RSxT^RSm^R$	-	-
	29F	$Amp^RTet^RSxT^RSm^R$	-	-
Chicken	51a	$Sm^RGm^IKm^I$	1	100
	70P	Tet^RSm^R	1	3
	45D	$Amp^RTet^RSxT^RSm^RCm^R$	3	62, 9, 4
	85P	SxT^RSm^R	3	106, 10, 4
	83K	$Amp^RTet^RSxT^RSm^R$	-	-
	87K	Sm^R	-	-
Cattle	12C1	$Amp^RTet^RSm^RKm^I$	1	106
	2F1	$Amp^RSm^R Km^I$	1	106
	6F1	Amp^RSm^R	-	-
	16C1	Amp^RSm^R	-	-
	19C1	$Amp^RSm^RSxT^R$	-	-

Key: Amp, ampicillin; Cm, chloramphenicol; Gm; gentamicin; Km, kanamycin; Sm, streptomycin; SxT, sulphamethoxazole/trimethoprim; Tet, tetracycline; ^{R} resistant; ^{I} intermediately resistant, -, plasmid-free.

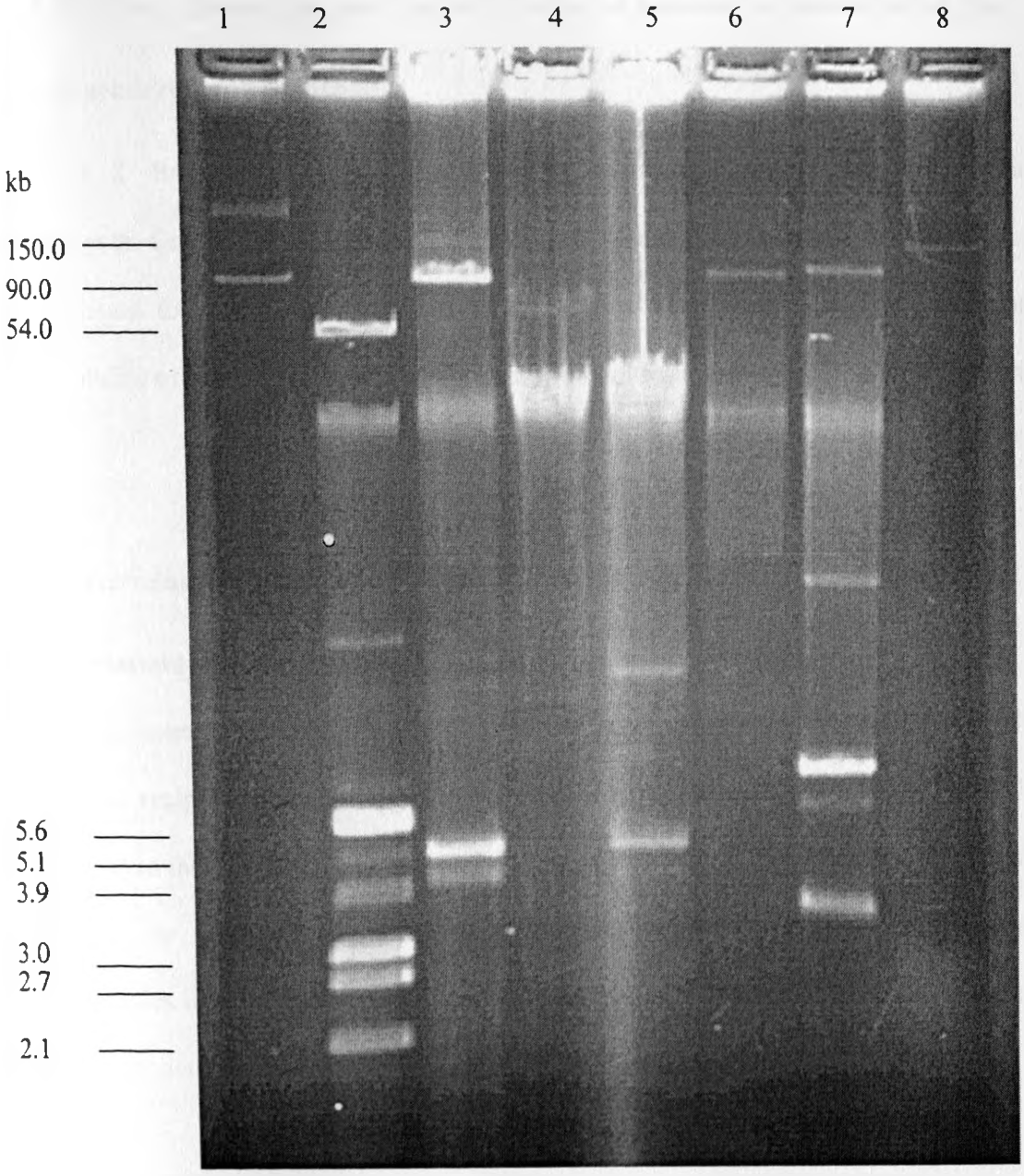


Fig. 4.1: Plasmid profiles of selected *E. coli* isolates: Lane 1, *Salmonella Typhimurium* LT2 (90 kb); lane 2, *E. coli* V517 with plasmids 2.1 to 54.0 kb; lanes, 3-7 *E. coli* isolates, 3F, 3C, 12C1, 51a, 66C and lane 8 *Klebsiella pneumoniae* R55 (150 kb).

4.3.2. Resistance phenotypes and plasmid profiles of *Salmonella enterica* serotypes Braenderup and Saintpaul

The *S.* Braenderup isolate was multiply resistant to ampicillin, tetracycline and streptomycin. One of the *S.* Saintpaul isolates was resistant to streptomycin while the other was resistant to chloramphenicol. All three isolates were plamid-free and therefore the transferability of aminoglycoside and chloramphenicol resistances was not examined in these strains.

4.3.3. Determination of plasmid location of resistance genes in *E. coli* isolates

4.3.3.1. Plasmid transfer by transformation

Only plasmids from 4 of the 23 plasmid-bearing isolates were successfully transformed into *E. coli* recipient strain JM 109. Two different types of small plasmids of about 6 kb in size (found in three isolates, 4F, 66C and 74C) and 8 kb (found in one isolate, 8C) mediating resistances to streptomycin (Sm) and sulphonamides (Sul), and to streptomycin, sulphonamides and tetracycline (Tet), respectively, were identified. Gentamicin, kanamycin and chloramphenicol resistances were not transferable via transformation.

4.3.3.2. Plasmid transfer by conjugation

Conjugation experiments were carried out with the aminoglycoside and chloramphenicol-resistant isolates with plasmids of size >30 kb. Streptomycin resistance was conjugally transferable to a recipient *E. coli* strain K12 7118 Lac$^-$ in seven (46.7%) of 15 isolates while chloramphenicol resistance was transferable in three (60.0%) of five isolates studied. The conjugational resistance transfer frequency ranged from 4.2×10^{-9} to 2.1×10^{-6} and 1.8×10^{-8} to 5.8×10^{-7} for streptomycin and chloramphenicol, respectively. Three of the isolates co-transferred resistances to ampicillin, tetracycline and

ulphamethoxazole/trimethoprim with both streptomycin and chloramphenicol resistance. Additionally, streptomycin resistance was also co-transferred with resistances to sulphamethoxazole/trimethoprim, ampicillin, and tetracycline in one isolate and to tetracycline and sulphamethoxazole/trimethoprim in another isolate. Plasmid profiles of each donor *E. coli* strain and its transconjugant showed that the transconjugants acquired plasmids in the range of 62 – 106 kb. Three of the isolates with transferable streptomycin and chloramphenicol resistance also possessed smaller plasmids (4 – 16 kb). None of these plasmids conferred kanamycin or gentamicin resistance.

4.3.3.3. Restriction profiles of plasmids with resistance genes

Comparative restriction analysis of the three sulphonamide/streptomycin (Sul^RSm^R) resistance plasmids using the endonucleases *Dra*I, *Eco*RI, *Eco*RV, *Hpa*I, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I and *Sma*I showed indistiguishable fragment patterns consisting of 1 to 2 fragments. Therefore, a common designation, pSSGK1, was chosen for these plasmids while the sulphonamide/streptomycin/tetracycline ($Sul^RSm^RTet^R$) resistance plasmid was designated, pSSTGK1. The restriction profiles of the plasmids pSSGK1 and pSSTGK1 are shown in figures 4.2 and 4.3, respectively. The restriction maps revealed that these plasmids were different from each other, but were similar to the previously described sulphonamide/streptomycin and sulphonamide/streptomycin/tetracycline resistance plasmids pSSOJO1 and pTOJO1, respectively (Fig. 4.4). Restriction analysis of the 106 kb plasmids transferred via conjugation in two of the *E. coli* isolates namely, 72C and 2F1 with *Hin*dIII, *Eco*RI, *Eco*RV, *Dra*I, *Kpn*I, *Xba*I and *Sac*I restriction enzymes showed different fragment patterns suggesting that these plasmids were distinctly different.

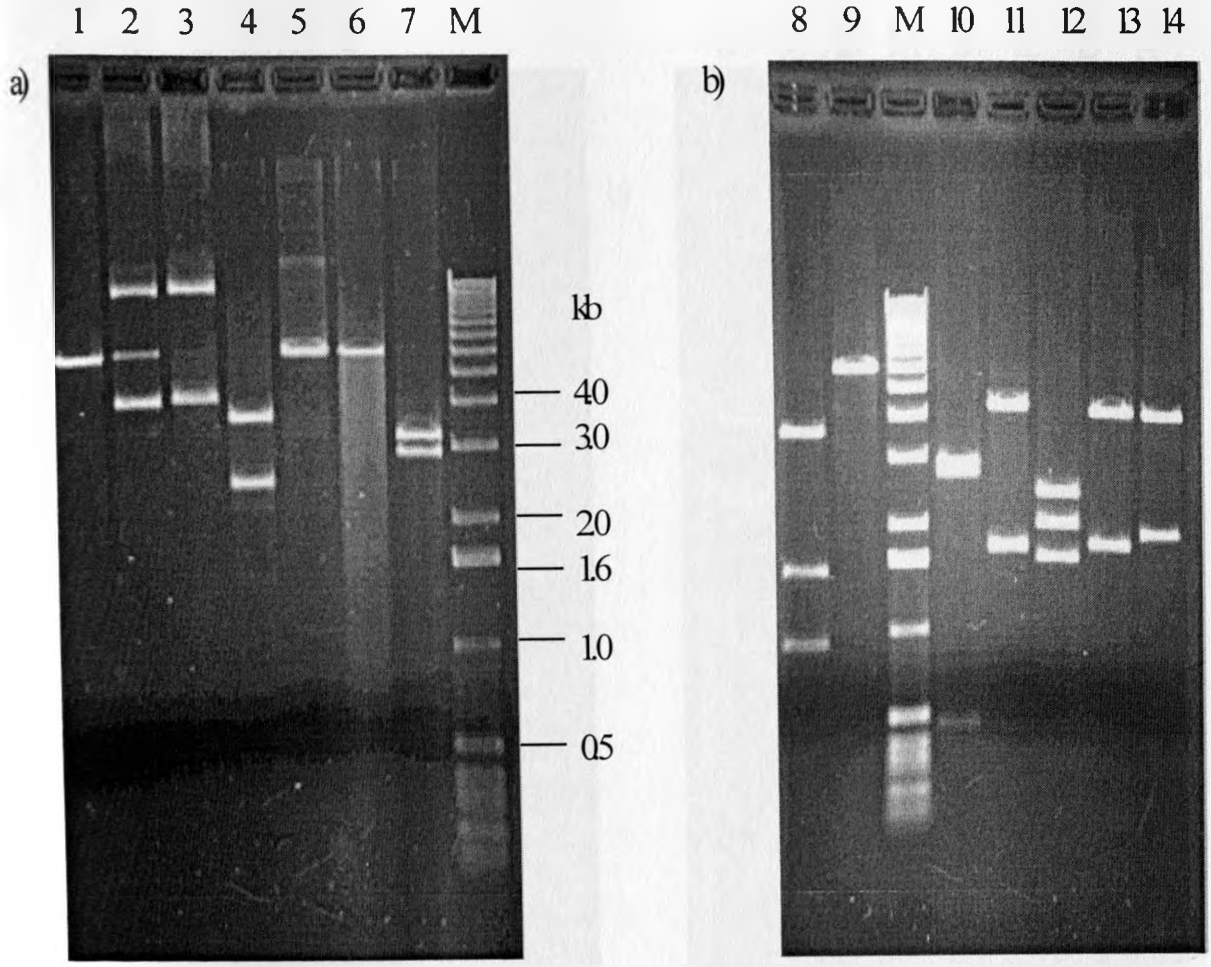


Fig. 4.2: Agarose gel electrophoresis of some restriction digests of pSSGK1 plasmid DNA after a) Single digests: lanes 1 *Pvu*II; 2 *Bam*HI; 3 *Hin*dIII, 4 *Eco*RV, 5 *Sac*I, 6 *Dra*I and 7 *Pst*I. b) Double digests: lanes 8 *DraI/Eco*RV, 9 *Eco*RI/*Hpa*I, 10 *DraI/Pst*I, 11 *KpnI/Dra*I, 12 *KspI/Dra*I, 13 *PvuII/Eco*RI, and 14 *SacI/Kpn*I. Lane M contains the DNA size standard (1 kb ladder, Gibco-BRL) of which the sizes of some fragments are given on the right hand side of figure (a).

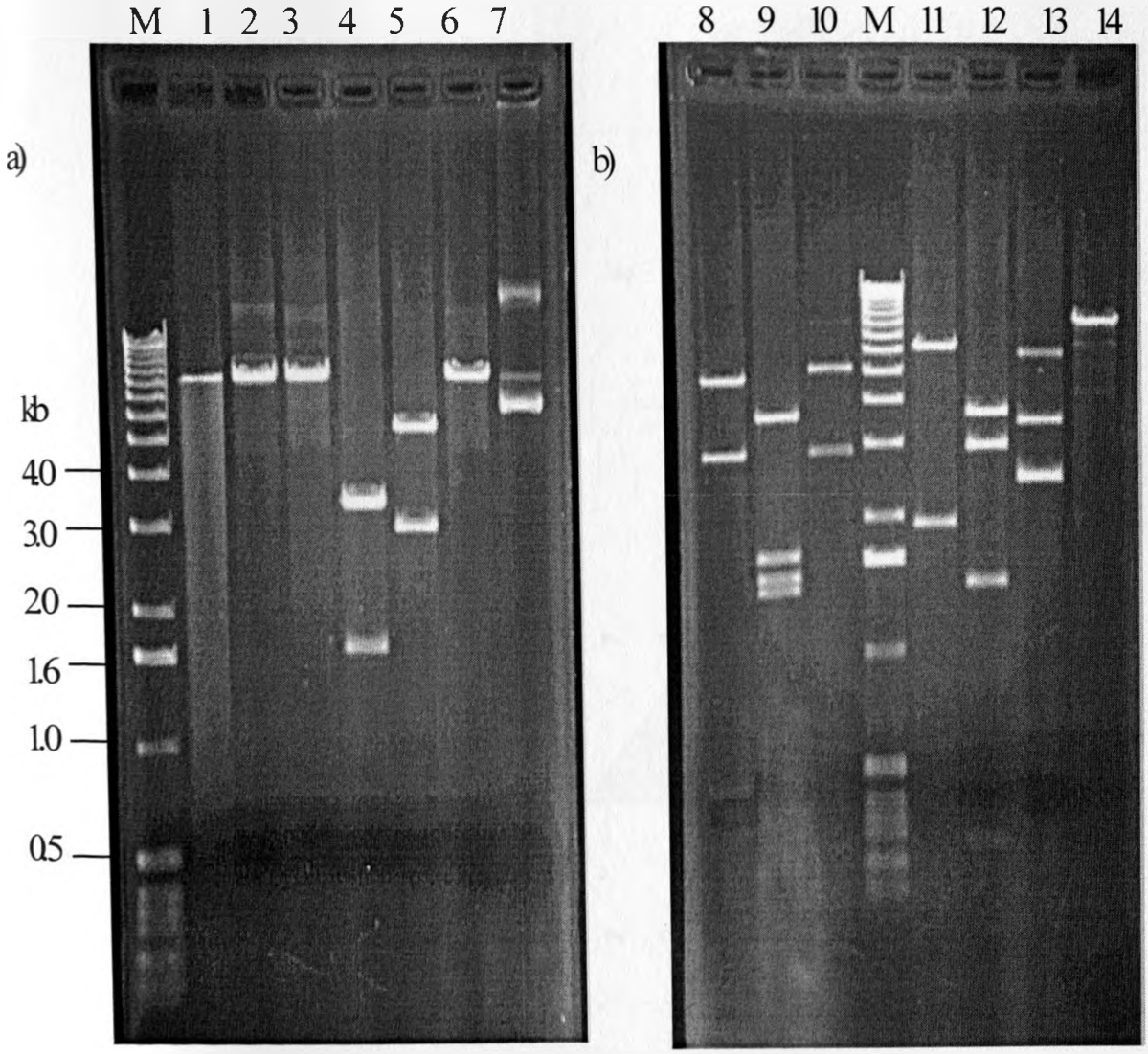


Fig. 4.3: Agarose gel electrophoresis of pSSTGK1 plasmid DNA after digestion with restriction endonucleases. a) Single digests; lanes, 2 *DraI*, 3 *SacI*, 4 *KpnI*, 5 *KspI*, 6 *PstI*, 7 *EcoRI* and 8 *BclI*. b) Double digests; lanes 1 *DraI/PstI*, 2 *DraI/EcoRV*, 3 *PstI/EcoRI*, 5 *SacI/KpnI*, 6 *EcoRV/BglII*, 7 *SmaI/SacI*, 8 *EcoRI/HpaI*. Lane M contains the DNA size standard (1 kb ladder, Gibco-BRL) of which the sizes of some fragments are given on the left hand side of figure (a).

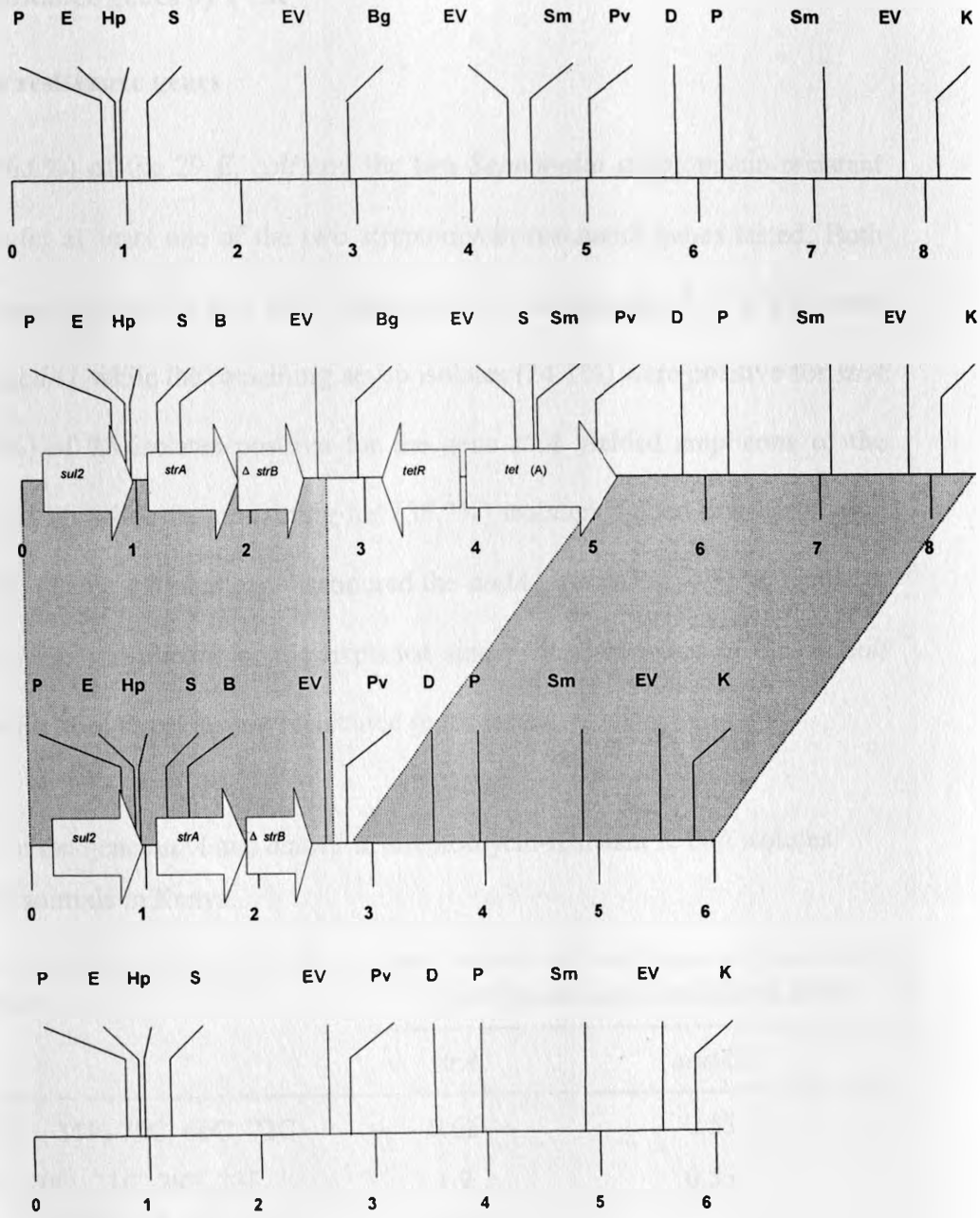


Fig. 4.4: Comparative analysis of the restriction maps of resistance plasmids pSSGK1 and STGK1 (this study) as well as pTOJO1 and pSSOJO1 (Ojo *et al.*, 2003). Restriction endonucleases: B (*Bcl*I), Bg (*Bgl*II); D (*Dra*I); E (*Eco*RI); EV (*Eco*RV); Hp (*Hpa*I); K (*Kpn*I); P (*Pst*I); Pv (*Pvu*II); S (*Sac*I) and Sm (*Sma*I).

4.3.4. Detection of resistance genes by PCR

4.3.4.1. Streptomycin resistance genes

Twenty eight (96.6%) of the 29 *E. coli* and the two *Salmonella* streptomycin-resistant isolates were positive for at least one of the two streptomycin resistance genes tested. Both *Salmonella* isolates were positive for *strA* only. Among the *E. coli* isolates, 21 (72.4%) were positive for *strA* and *aadA1* while the remaining seven isolates (24.1%) were positive for *strA* only. Eighteen (64.3%) of 28 isolates positive for the gene *strA* yielded amplicons of the expected size of ~ 0.65 kb while the remaining ten (35.7%) isolates yielded unusually large amplicons of ~ 1.2 kb (Table 4.4) and also harboured the *aadA1* gene (Fig. 4.5). All isolates positive for *aadA1* yielded amplicons of the expected size, ~ 0.53 kb. One of the *E. coli* isolates was negative for both streptomycin resistance genes tested.

Table 4.4: Presence of the genes *strA* and *aadA1* in streptomycin-resistant *E. coli* isolates from food animals in Kenya.

Animal species	Strains	Approximate amplicon size(s) in kb	
		strA	*aadA1*
Pig	3C, 4F, 35F, 70C, 66C, 72C	0.65	0.53
	12C, 20C, 21C, 29F, 33F	1.2	0.53
	3F, 8C, 27F, 65F, 74C, 80F	0.65	-
	7F	-	-
Chicken	70P, 85P, 51a	1.2	0.53
	83K, 87K	0.65	0.53
	45D	0.65	-
Cattle	19C1, 2F1, 6F1	0.65	0.53
	16C2, 12C1	1.2	0.53

Key: – Negative for the gene

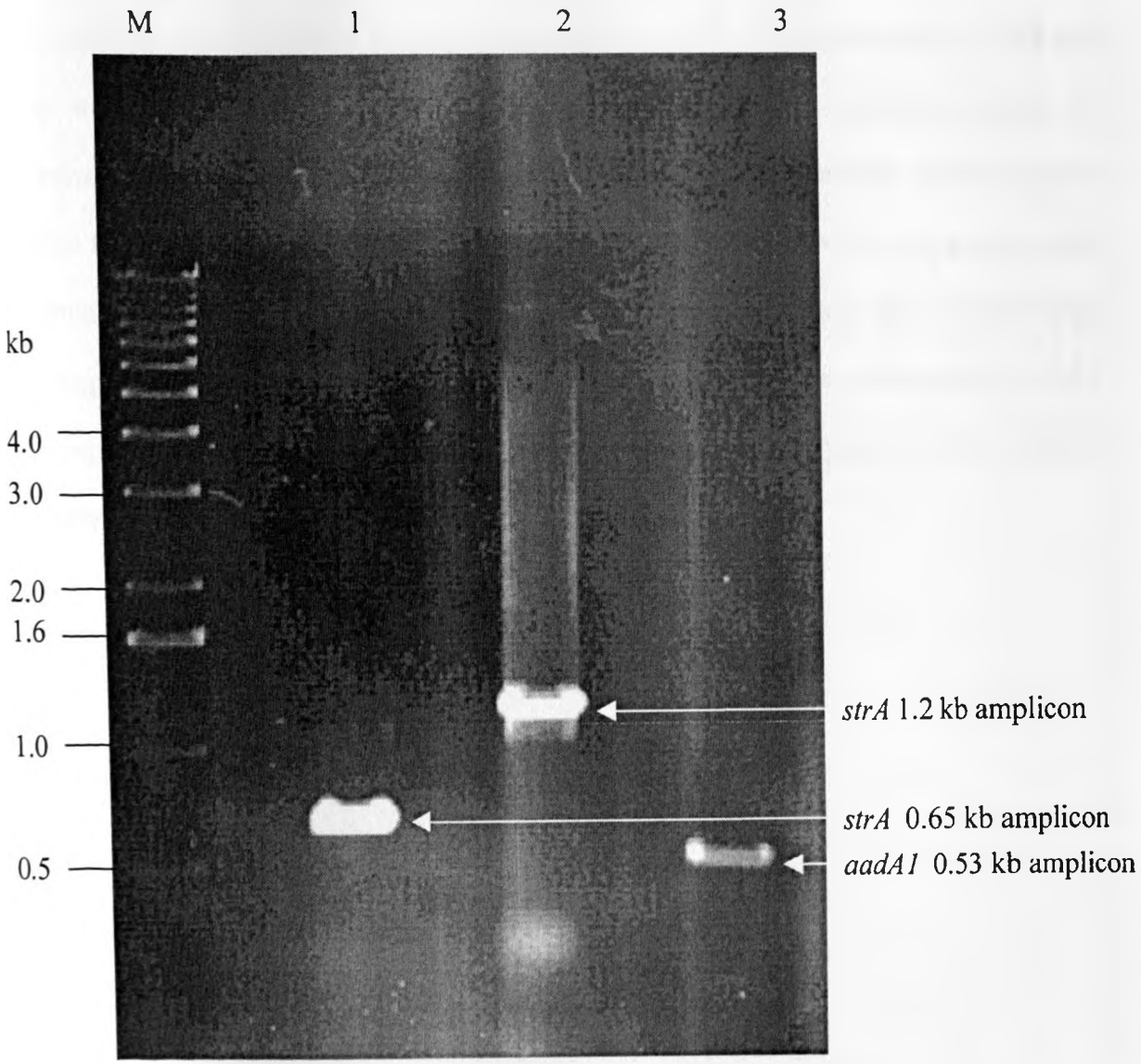


Fig. 4.5: PCR amplicons obtained with *strA* (lanes 1 –2) and *aadA1* (lane 3) primers. Lane M contains the DNA size standard (1 kb ladder, Gibco-BRL) of which the sizes of some fragments are given on the left hand side.

.3.4.2. PCR test for the transfer of *strA* and *aadA1* genes

In order to determine whether one or both of the streptomycin resistance genes were ransferable via transformation or conjugation, specific PCR for the detection of *strA* and *aadA1* were carried on plasmid DNA isolated from the transformants or transconjugants. All he transformant and transconjugant strains that transferred streptomycin resistance were ositive for the specific *strA* gene (~ 0.65 kb amplicon), but negative for the *aadA1* gene, suggesting that only the *strA* gene was transferred in the isolates studied. The unusually large *strA* amplicons were not detected in plasmid DNA of either the transformants or the transconjugants. The results of the PCR analysis for the streptomycin resistance gene(s) transferred via conjugation are summarized in Table 4.5.

Table 4.5: Resistance transfer profiles, frequency of transfer and the streptomycin resistance gene transferred by conjugation.

Donor strain	Resistance phenotype of donor strains	Resistance pattern(s) transferred	No.of plasmids transferred	Transfer frequency	PCR product amplicon size (s) kb with primer for		Streptomycin gene transferred
					strA	*aadA1*	
2F1	$Amp^{R}Sm^{R}Km^{I}$	Sm^{R}	1	2.9×10^{-7}	0.65	0.53	*strA*
72C	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	1	2.0×10^{-7}	0.65	0.53	*strA*
27F	$Amp^{R}Tet^{R}SxT^{R}Km^{I}Sm^{R}$	$Amp^{R}T^{R}SxT^{R}Sm^{R}$	2	2.1×10^{-6}	0.65	-	*strA*
45D	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	2	4.2×10^{-9}	0.65	-	*strA*
65F	$Tet^{R}SxT^{R}K^{R}Sm^{R}Cm^{R}$	$Tet^{R}SxT^{R}Sm^{R}$	2	2.1×10^{-8}	0.65	-	*strA*
80F	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	$Amp^{R}Tet^{R}SxT^{R}Sm^{R}Cm^{R}$	2	4.3×10^{-8}	0.65	-	*strA*
66C	$Amp^{I}Sm^{R}$	Sm^{R}	3	2.9×10^{-6}	0.65	0.53	*strA*

Key: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; SxT, sulphamethoxazole/trimethoprim (19:1); Tet, tetracycline.

4.3.4.3. Physical linkage of streptomycin resistance gene *strA* gene and sulphonamide resistance gene *sul2*

The carriage of the *sul2* gene in the plasmid DNA extracted from transformants or transconjugants showing resistance to at least streptomycin and sulphonamides was confirmed by PCR. Only DNA from the transformants (plasmids pSSGK1 and pSSTGK1) yielded amplicons of ~ 1.5 kb with the primer pair *sul2-strA* (Fig. 4.6) suggesting that physically linked *sul2* and *strA* genes were present in these plasmids and were arranged in the orientation *sul2-strA*. The primer pair *strA* forward-*sul2* reverse yielded no amplicons. In repeated attempts with plasmid DNA from the transconjugants the primer pairs *sul2*-forward - *strA*-reverse or *strA*-forward - *sul2*-reverse yielded no amplicons while the primer pairs *sul2*-forward - *sul2* reverse and *strA*-forward - *strA*-reverse resulted in amplicons characteristic of genes *sul2* and *strA*. Tetracycline resistance in the plasmid pSSTGK1 was encoded by the *tet*(A) gene.

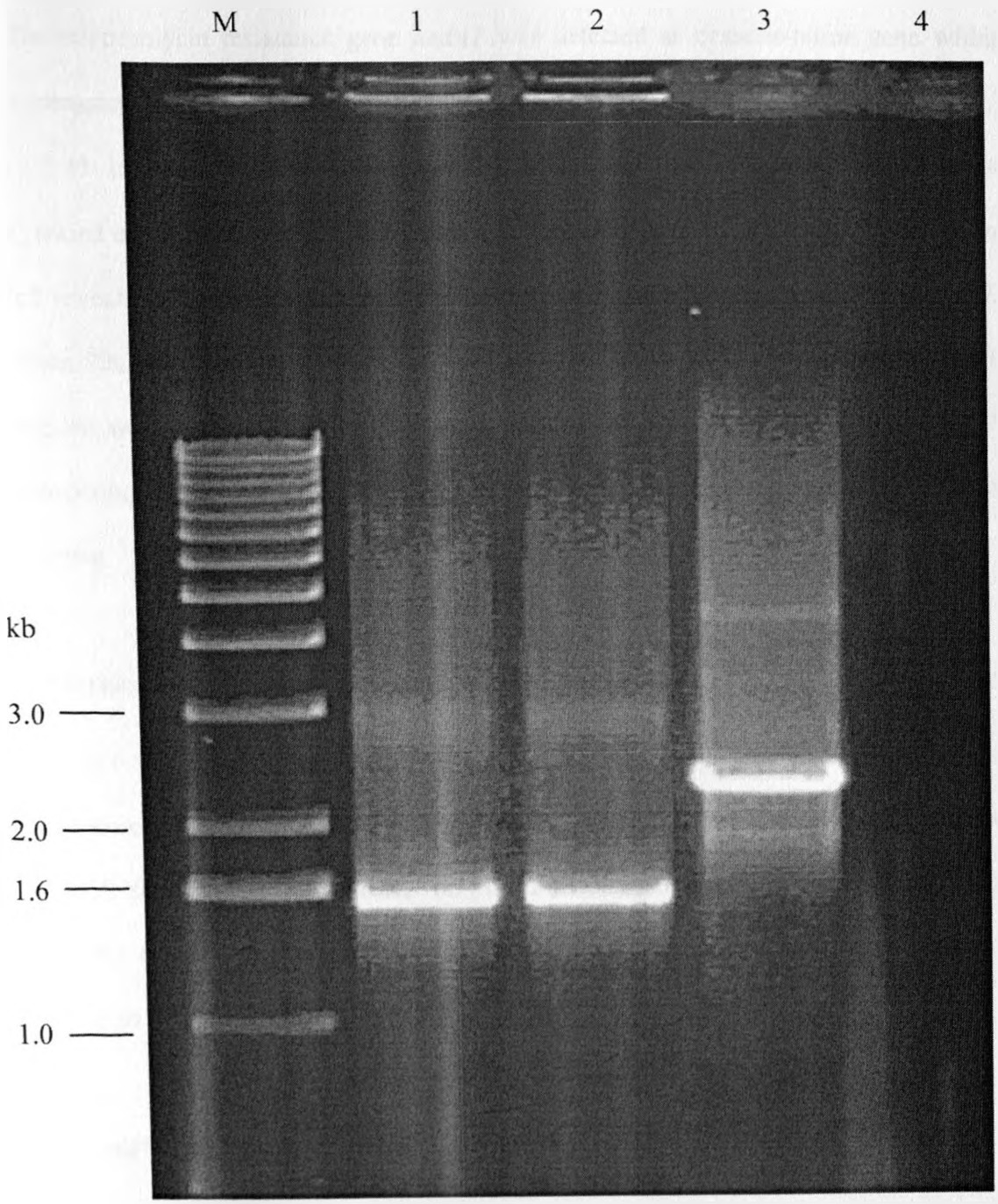


Fig. 4.6: Amplicons obtained with primer pair *sul2-strA* (lanes 1-2), positive control; the plasmid pMHSCS1 carrying physically linked *sul2-strA* in a 2231 bp resistance gene cluster *sul2-catA3-strA* (Kehrenberg and Schwarz, 2001) (lane 3), negative control (H_2O) (lane 4) and molecular size marker (lane M).

3.4.4. Class 1 integron location of streptomycin resistance gene *aadA1*

The streptomycin resistance gene *aadA1* was detected as cassette-borne gene within lass 1 integrons in only 2 (9.5%) of the 21 isolates harbouring the gene. Amplicons of ~0.7 nd ~ 1.5 kb in size were detected in one (72C) of the isolates whereas the second isolate 83K) yielded one amplicon of 1.5 kb. Restriction analysis of the 1.5 kb amplicons with *Cla*I nd *Bcl*I revealed same fragment patterns. Therefore one of the amplicons namely that of *E. oli* isolate 72C, was chosen for sequence analysis. The isolate 83K was plasmid-free while he integrons were not detectable by PCR using plasmid DNA from *E. coli* K12 strain 7118N Lac⁻ transconjugants for strain 72C. These two strains carrying class 1 integron also carried the *sul1* gene

4.3.5. Chloramphenicol resistance genes

The chloramphenicol resistance gene *catA1* was the only gene detected in all the chloramphenicol-resistant *E. coli* (n = 7) and *Salmonella* Saintpaul isolate (n = 1) isolates. Specific PCR for the detection of *catA1* on plasmid DNA isolated from the transconjugants confirmed the location of the *catA1* gene on conjugative plasmids in the chloramphenicol-resistant *E. coli* isolates.

4.3.6. Kanamycin and gentamicin resistance genes

All the kanamycin-resistant (n = 6) and gentamicin-resistant (n =2) isolates analysed in this study were negative for the genes *aph(3'')-Ia* (kanamycin resistance) and *ant(2)-Ia* (kanamycin and gentamicin resistance), respectively.

3.7. Sequence analysis

Analysis of the nucleotide sequence from the ~1.2 kb *strA* gene primer amplicons revealed disruption of the *strA* gene by the insertion of a 568-bp (492 to 1059) gene cassette carrying a functionally active trimethoprim resistance gene *dfrA14* (formerly known as *dfrIb*). The upstream part of the truncated *strA* gene consisted of 491 bp while the downstream part consisted of 155 bp. No base pairs were lost or gained at the integration site suggesting precise integration of the cassette. The 59-base element (59 be) of the *dfrA14* gene cassette consisted of 87 bp. The core site for the site-specific insertion, GTTAACC, was found at position 491-497 while the inverse core GGTTAAC was at positions 979-985 and included the translational stop codon (Fig. 4.7). Comparison of the nucleotide sequence of this 1214 bp amplicon with other sequences in the database showed 99% identity with a part of a plasmid pSTOJO1 (accession no AJ13522). The base pairs were the same from 1263 to 2576 except a substitution of a G for an A at position 1385 and a C for a G at position 2570.

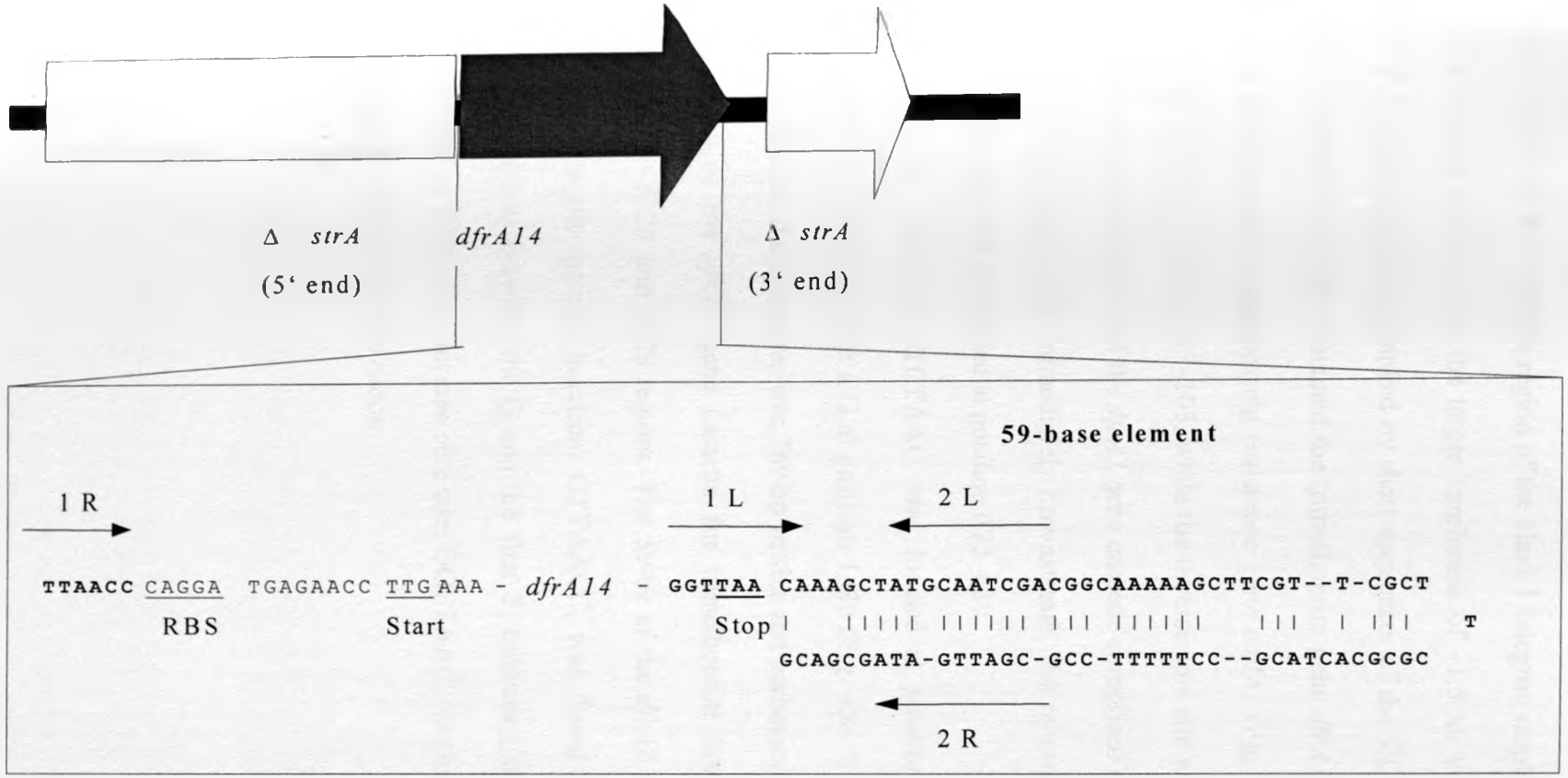


Fig. 4.7: Schematic representation of a *dfrA14* gene cassette integrated at a secondary site within the *strA* gene resulting in *strA* amplicons of 1214 bp. In the 59-base element, the putative *IntI1* integrase binding domains 1L, 2L, 2R, and 1R are indicated by arrows. The translational start and stop codons, and ribosomal binding site (RBS) are underlined.

Analysis of the nucleotide sequence of the *sul2-strA* amplicons revealed that the nplicons consisted of 770 bp of the *sul2* gene, 684 bp of the *strA* gene and a spacer of 60 bp. equence analysis of the variable region of the class 1 integron amplicons obtained with 5'CS nd 3'CS primers showed that the larger amplicons of ~1.5 kb were 1586 bp in size and omprised two gene cassettes flanked by short sequences of the 5'CS and 3'CS regions. The rst gene cassette of 577 bp contained the trimethoprim gene *dfrA1*, and the second cassette f 856 bp harboured the streptomycin resistance gene *aadA1* (Fig. 4.8). The recombination ore site was found at position 97–103, while the inverse core site was found at position 586-92. The 59 base element (be) of the *dfrA1* gene cassette comprised of 95 bp and ended with a G at position 674 (Fig. 4.8). Immediately downstream, the recombination core site of the *aadA1* gene cassette was identified at position 675–680.

The inverse core site, GTCTAAC, was located at position 1477-1483. The 59-be comprised 60 bp and ended with a G at position 1530 (Fig. 4.8). The smaller amplicon of ~ 0.7 kb found in one of the isolates was 769 bp in size and harboured one gene cassette of 616 bp which carried the *dfrA7* gene cassette for trimethoprim resistance flanked by short sequences of the 5'CS and 3'CS regions. The 59-be of the *dfrA7* consisted of 133 bp. The core site for the site-specific insertion, GTTAACC, was found in position 97-103. The recombination site is between the G and the first T, indicating the beginning of the gene cassette at position 98 bp. The inverse core site, GGCTAAC, was located at position 587-593 and included a translational stop codon.

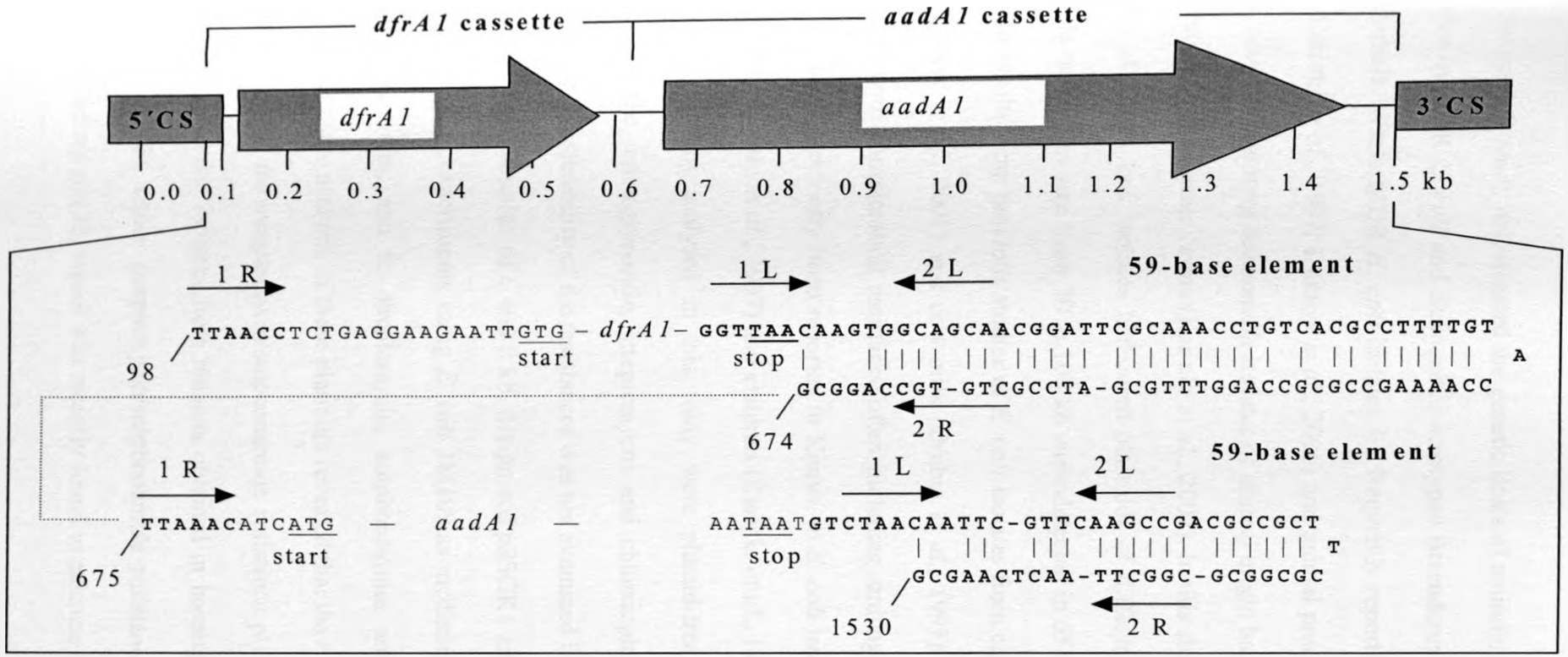


Fig.4.8: Schematic representation of *dfrA1* and *aadA1* gene cassettes detected in this study. The *dfrA1* and *aadA1* reading frames are shown as arrows and the 5′and 3′ conserved segments (5′CS and 3′CS) of the class 1 integron are shown as boxes. The beginning and the end of the gene cassettes are shown in detail below. The translational start (GTG, ATG) and stop (TAA) codons are underlined. The 59-base element of the gene cassettes is shown in bold type. In the 59- base elements, the putative Int1 intergrase binding domains 1L, 2L, 2R and 1R are indicated by arrows. Numbers indicating important positions of the base in the 59-base element refer to the corresponding database entries. The dotted line indicates the connection between the two gene cassettes.

I: DISCUSSION

The present study investigated the genetic basis of aminoglycoside and chloramphenicol sistance in MDR *E. coli* and *Salmonella* serotypes Braenderup and Saintpaul isolates from od animals. While MDR *E. coli* isolates are frequently reported in animals (Bebora *et al.*, 994; Kariuki *et al.*, 1997; Gakuya *et al.*, 2001) and animal products (Ombui *et al.*, 1995) in enya, resistance among *Salmonella* isolates of animal origin has so far only been reported in ıickens from large-scale farms (Kariuki *et al.*, 2002). In this study, more than three quarters 76.7%) of the *E. coli* isolates harboured plasmids of different sizes and numbers. Large lasmids ranging in size from 30 to 106 kb were detected in 65.2% of the isolates. This is in ine with results from previous studies of *E. coli* isolates from chickens (Bebora *et al.*, 1994), ·ats (Gakuya *et al.*, 2001) and cow milk (Ombui *et al.*, 1995) in Kenya. Diverse plasmids ıssociated with antimicrobial resistance often including aminoglycoside or chloramphenicol resistance have previously been reported in Kenya, in *E. coli* isolates from chickens (Bebora *et al.*, 1994; Kariuki *et al.*, 1997) and children (Kariuki *et al.*, 1997). The finding that all the *Salmonella* isolates analysed in this study were plamid-free suggested a chromosomal location of the aminoglycoside, streptomycin and chloramphenicol resistance genes and therefore the transferability of the resistances was not examined in these strains.

Two small plasmids of 6 and 8 kb, designated pSSGK1 and pSSTGK1 were identified by transformation experiments using *E. coli* JM109 as mediating resistance to streptomycin and sulphonamides, and to streptomycin, sulphonamides and tetracycline, respectively. Restriction enzyme analysis of these plasmids revealed that the 6 kb plasmid pSSGK1 is very closely related to the streptomycin/sulphonamide resistance plasmid, pSSOJO1 detected in uropathogenic *E. coli* isolates from humans obtained in hospitals in South-western Nigeria (Ojo *et al.*, 2003). Other streptomycin/sulphonamide resistance plasmids have also been reported including, p9123, which was recently found to enhance host fitness in the absence of

ɔecific antimicrobial selective pressure (Enne *et al.*, 2004) and pBP1, which was prevalent in ıe *E. coli* population in the 1970s and 1980s (Korfmann *et al.*, 1983). The 8 kb plasmid SSTGK1 is similar to pTOJO1, found in uropathogenic *E. coli* from humans in Nigeria (Ojo *t al.*, 2003).

In the present study, resistance was transferable via conjugation to *E. coli* K12 strain '118N Lac⁻ in seven (24.1%) of aminoglycoside- and three (42.8%) of the chloramphenicol-esistant isolates. Among the aminoglycosides, only resistance to streptomycin was ransferable. This suggests that conjugation of plasmids encoding streptomycin and/or chloramphenicol resistance may be one mechanism for the wide dissemination of these resistances among food animal *E. coli*. Co-transfer of resistance to at least sulphamethoxazole/trimethoprim, ampicillin and tetracycline was observed. The ability of MDR *E. coli* isolates to transfer resistance to *E. coli* K12 strain 7118N Lac⁻ has been reported by Niljesten *et al.* (1996) to range from 26% to 50% in isolates from humans and about 50% to 76% in isolates from pigs and by O'Brien *et al.* (1993) to be 24% in isolates from poultry. A plasmid of about 106 kb was most frequently transferred in both the aminoglycoside and chloramphenicol resistant isolates. The diversity of restriction endonuclease fragment patterns observed in two representatives of this plasmid indicates that the R-plasmids were – despite of their similar size – distinctly different in their structure. Similarly, Kariuki *et al.* (1997) detected a plasmid of about 100 - 110 kb with diverse restriction fragments in antimicrobial-resistant *E. coli* isolates from poultry and children in Kenya. The finding that kanamycin and gentamicin resistance were not transferable either by transformation to *E. coli* JM 109 or by conjugation to *E. coli* K12 strain 7118N Lac⁻ suggests chromosomal location of the resistance genes. Nonconjugative plasmid or chromosomal gentamicin (Casetta *et al.*, 1998; Ahmed *et al.*, 2004) and kanamycin (Terakado *et al.*, 1981) resistance determinants have been reported.

The phosphotransferase *aph(3'')-Ib* and *aph(6)-Id* genes (also named *strA* and *strB*, respectively) and the adenyltransferase gene *ant(3'')-Ia* (also named *aadA1*) are the most

requently encountered streptomycin resistance genes in *E. coli* (Guerra *et al.*, 2003; Reyes *et al.*, 2003), *Salmonella spp.* and other Gram-negative bacteria (Gebreyes and Altier, 2002; Frech *et al.*, 2003). In this study, the *strA* gene was detected in all but one (*E. coli*) of the *E. coli* and *Salmonella* isolates analysed while the *aadA1* gene was detected in the *E. coli* isolates only and was found in 72.4% of the strains. The gene *strA* has been found frequently among streptomycin-resistant *Salmonella* isolates, such as *Salmonella* Typhimurium (Gebreyes and Altier, 2002; Frech *et al.*, 2003). It was interesting to note that 21 (72.4%) of the *E. coli* strains harboured both the *aadA1* and *strA* genes, of which ten (47.6%) yielded unsually large amplicons of ~1.2 kb with the *strA* gene primers. The occurrence of both the *aadA1* and *strA* genes in the same *E. coli* strain has also been documented previously (Guerra *et al.*, 2003). Independent acquisition of resistance genes on mobile genetic elements, such as transposons, before the development/acquisition of the chromosomal resistance genes has been suggested as a possible explanation for the presence of more than one gene coding for the same resistance property in the same bacterial isolate (Frech *et al.*, 2003). The *aadA1* gene has been reported to occur frequently among streptomycin-resistant *E. coli* of normal intestinal flora of healthy domestic animals (Sunde and Norstrom, 2005). The one strain not possessing any of these two genes may harbour other genes mediating resistance to streptomycin or the resistance may be conferred via chromosomal mutations that alter the ribosomal binding site of streptomycin (Sunde and Norstrom, 2005).

Analysis of the nucleotide sequence of the unusually large *strA* gene PCR amplicons of 1214 bp revealed disruption of the *strA* gene by the insertion of a functionally active trimethoprim resistance gene cassette *dfrA14* (formerly known as *dhfrIb*). No base pairs were lost or gained at the integration site suggesting precise integration of the cassette. Comparison of the nucleotide sequence of this 1214 bp amplicon with other sequences in the data bank showed 99% identity with a part of a plasmid pSTOJO1 (accession no AJ13522). The integration of a gene cassette at a secondary site was assumed to be an *IntI*-catalysed

recombination event, which involves a secondary recombination site (Recchia and Hall, 1995). The *strA* sequence at the integration site, GATAT, corresponded to the consensus sequences for secondary sites: Gt/aT (Recchia *et al.*, 1994) or GatTa/ca/t (Francia *et al.*, 1993). This is the first report of the disruption of the *strA* gene by the insertion of the cassette-borne trimethoprim resistance gene, *dfrA14* among *E. coli* isolates from food animals.

The disruption of the *strA* gene by the insertion of the cassette-borne trimethoprim resistance gene, *dfrA14* has only previously been reported in uropathogenic *E. coli* from humans (Ojo *et al.*, 2002). The finding that *E. coli* from food animals carried a *dfrA14* gene cassette at a secondary site within the *strA* gene as those described in pathogenic *E. coli* isolates underlines the presence of links between bacteria of humans and animals. Truncation of an RSF1010-like *strA* gene by the insertion of the non-cassette-borne trimethoprim resistance gene, *dfrA9*, has been reported (Sköld., 2001) and is believed to have occurred as a consequence of high selective pressure imposed by frequent use of trimethoprim (Jansson *et al.*, 1992). A similar condition can be assumed for the integration of the *dfrA14* gene cassette at a secondary site within the *strA* gene in this study since sulphonamides/trimethoprim combinations are among the most frequently used antimicrobial drugs in food animals in Kenya (Mitema *et al.*, 2001).

PCR analysis of the plasmid DNA isolated from the *E. coli* transformants and transconjugants for the streptomycin resistance genes *aadA1* and *strA* indicated that only the *strA* gene was transferred. Failure to detect the *aadA1* gene on plasmids following either transformation or conjugation experiments suggests chromosomal localization of the gene, although location on large non-conjugative plasmids, cannot be excluded. The *strA* gene was originally described in the small, non-conjugative, broad-host-range IncQ plasmid RSF1010 (Scholz *et al.*, 1989). It has been subsequently found as part of transposon Tn*5393* and related elements in phytopathogenic *Erwinia amylovora*, *Pseudomonas syringae* pathovar papulans, and *Xanthomonas campestris* pathovar vesicatoria (Chiou and Jones, 1993). Tn*5393* carrying

strA is typically plasmid encoded but may also be chromosomally inserted (Sundin, 2000). Additionally, the *strA* with a truncated Tn*5393 tnpR* gene was detected on a transferable streptomycin resistance plasmid in a clinical isolate of the *Yersinia pestis* (Guiyoule *et al.*, 2001). The detection the *strA* gene in plasmid-free resistant *Salmonella* serotypes Braenderup and Saintpaul suggests a chromosomal localization of the gene in these strains. Chromosomally located *strA* has been reported in *Salmonella* Typhimurium (Madsen *et al.*, 2000). The genes coding for resistance to tetracycline [*tet*(A) and *tet*(B)] and ampicillin (bla_{TEM}), were detected in plasmid DNA of transconjugants of strains which co-transferred these resistance together streptomycin and chloramphenicol resistances.

Physically linked *sul2* and *strA* genes transferable via transformation were present only in the small plasmids pSSGK1 and pSSTGK1 and were arranged in the orientation *sul2-strA*. Plasmids carrying physically linked *sul2* and *strA* genes in the same oreintation have previously been reported in bacteria of the genera *Pasteurella* and *Mannheimia* (Kehrenberg and Schwarz, 2001). In repeated attempts with plasmid DNA from the transconjugants and the primer pairs, *sul2*-forward - *strA*-reverse or *strA*-forward-*sul2*-reverse yielded no amplicons while *sul2*-forward - *sul2* reverse and *strA*-forward – *strA*-reverse resulted in amplicons characteristic of genes *sul2* and *strA*. This observation strongly suggested that the plasmids in the transconjugants carried independent copies of the gene *sul2* and *strA*. DNA sequence analysis revealed that the *sul2-strA*, 1514 bp amplicon consisted of 770 bp of the *sul2* gene, 684 bp of the *strA* gene and a spacer of 60 bp. The 60 bp spacer region between the genes *sul2* and *strA* identified in this study was within the range of 25 to 152 bp reported by Kehrenberg and Schwarz (2001) for linked *sul2-strA* genes in bacteria of the genera *Pasteurella* and *Mannheimia*.

Resistance to tetracycline in the 8 kb, pSSTGK1 plasmid was encoded by the *tet*(A) gene. Similarly, an 8.4 kb plasmid carrying *tet*(A), *sul2,* and *strA* has been described in uropathogenic *E. coli* from humans (Ojo *et al.*, 2003). The *aadA1* gene cassette is most

frequently found in the variable region of class 1 integrons, as the only cassette present (Rosser and Young, 1999) or in combination with one or more other resistance gene cassettes (Saenz *et al.*, 2004). In the present study, the *aadA1* gene was detected as cassette-borne gene within class 1 integrons in only 2 (9.5%) of *E. coli* isolates harbouring the gene and was linked with trimethoprim resistance gene cassette *dfrA1* in both isolates. As in the case of the results from this study, most class 1 integrons published are composed of two or more gene cassettes (Guerra *et al.*, 2003; Maynard *et al.*, 2003). The occurrence of gene cassettes *aadA1* and *dfrA1* on the same class 1 integron has been reported previously (Guerra *et al.*, 2003; Saenz *et al.*, 2004).

The reasons for the low prevalence of *aadA1* on a class 1 integron in this study was not clear; however, it may have been due in part to the inserted gene cassette regions of the class 1 integrons being too large to be amplified by conventional PCR techniques or such integrons lack the 3'CS generally associated with this class of integrons (Barlow *et al.*, 2004). Nass *et al.* (1998) described a class 1 integron identified in the *Pseudomonas aeruginosa* clinical isolate which contained a 5'CS, two resistance gene cassettes (*aacA4* and bla_{OXA-20}) but no 3'-conserved segment. The *aadA1* has recently also been found in a class 2 integron (Saenz *et al.*, 2004). These alternative possibilities are worthy of further investigation. A second class 1 integron carrying the trimethoprim resistance gene cassette *dfrA7* was also detected in one of the isolates. Carriage of two different class 1 integrons in the same *E. coli* isolate has been reported (Yu *et al.*, 2003). These two strains carrying class 1 integron also carried the *sul1* gene. The finding that one of these isolates (83K) was plasmid-free and the integrons were not detectable by PCR using plasmid DNA from *E. coli* K12 strain 7118N Lac$^-$ transconjugants for strain 72C suggests that all integrons were located in the chromosomal DNA in these isolates. Chromosomally located class 1 integrons carrying the *aadA1* and *dfrA1* gene have been recently described in *Salmonella* serotype Typhimurium DT 104b (Daly *et al.*, 2004). It has been reported that cassettes preferentially recombine into the *attI* site of an integron, rather

that the *attC* site at the 3'-end of cassettes already present in the integron (Collis *et al.*, 1993; Hall and Collis, 1995). The observation that the *dfrA1* gene cassette was found upstream of the *aadA1* resistance gene suggests that the *aadA1* gene may be either the first cassette to be acquired by an integron and/or may be more stably integrated into the integron than the *dfrA1* gene cassette (Hall and Collis, 1995).

Chloramphenicol resistance was encoded by the *catA1* gene in all the chloramphenicol-resistant *E. coli* and *Salmonella* isolates in this study. For the *E. coli* isolates, the *catA1* gene was confirmed to be plasmid-borne and transferable via conjugation by PCR analysis. The *catA1* gene has previously been detected in *E. coli* (Guerra *et al.*, 2003) and *Salmonella* (Frech *et al.*, 2003; Nogrady *et al.*, 2005) isolates and is known to be commonly located on transposon Tn*9* which is widespread among Gram-negative bacteria (Frech *et al.*, 2003). The detection of *catA1* gene in plasmid-free resistant *Salmonella* serotypes Braenderup and Saintapaul isolates suggests its chromosomal localization in these isolates. This presumed location is a cause of concern since chromosomal resistance gene location has proved to be very efficient in acquiring and establishing efficient traits and in supporting spread of *S.* Typhimurium DT104 through the food chain in Western countries (Casin *et al.*, 1999). The *aph(3'')-Ia* and *ant (2)-Ia* genes were not detected in the kanamycin- and gentamicin-resistant strains studied, respectively. This may suggest that either, resistance genes other than *aph(3'')-Ia* and/or *ant (2)-Ia* or other mechanisms of resistance were responsible for the kanamycin and gentamicin resistances in these strains.

The results of this study indicate that *E. coli* and *Salmonella* serotypes from food animals in Kenya may represent a reservoir of streptomycin and chloramphenicol resistance genes which may be transferable to other bacteria. Physical linkage of *strA* and *sul2* genes offers the possibility of co-selection of this gene during selective pressure imposed by the use sulphonamides and highlights the need for their prudent use in animal husbandry. The location of the *strA* and *catA1* genes on conjugative plasmids and the *aadA1* gene within class

1 integrons constitutes an effective means to spread streptomycin and chlorampenicol resistances among bacteria from different ecosystems. This underlines the need for detailed epidemiological and molecular studies on acquisition of resistance genes and distribution of antimicrobial-resistant *E. coli* and *Salmonella* isolates among food animals, food products and humans in Kenya.

CHAPTER FIVE

5.0: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1: GENERAL DISCUSSION

E. coli and *Salmonella* colonize the gastrointestinal tracts of a wide range of wild and domestic animals, especially food animals (Schoeder *et al.*, 2002; van Duijkeren *et al.*, 2002). While *Salmonella* species are pathogenic *E. coli* occurs as a commensal although lateral gene transfer events have allowed the transition of some *E. coli* strains from commensals to pathogens (Sooka *et al.*, 2004). In this study, the isolation rates were high for *E. coli* in all the animal species and moderate for *Salmonella* in pigs. *E. coli* was more frequently isolated from faeces (84.1%) than from carcasses (70.8%) and was detected more commonly on pork carcasses than on beef carcasses while the prevalence of *Salmonella* on pig carcasses was higher than in faeces. These findings suggest the presence of severe cross-contamination during slaughtering process and as a result of poor hygienic conditions during subsequent dressing operations. This is the first report of *S.* Heidelberg among food animals in Kenya. *S.* Heidelberg is a major cause of human infections in Canada and the USA (Demczuk *et al.*, 2003) and has been associated with severe disease symptoms, including extra-intestinal infections (Wilmshurst and Sutcliffe, 1995), septicaemia and myocarditis (Burt *et al.*, 1990).

For both the *E. coli* and *Salmonella* isolates, the most common resistance was to ampicillin, streptomycin, sulphamethoxazole/trimethoprim and tetracycline. The resistance patterns most frequently observed in *E. coli* isolates from cattle were resistance to streptomycin and ampicillin in combination and streptomycin or ampicillin alone. Resistance to tetracycline and sulphamethoxazole/trimethoprim was most frequently seen among the isolates from chicken. The most frequent multidrug pattern (ampicillin/tetracycline/streptomycin/sulphamethoxazole/trimethoprim) observed in the isolates from pigs was also in two chicken isolates. The differences in resistance patterns

observed among the *E. coli* isolates in this study are a reflection of the differences in production systems and antimicrobial usage patterns in the three animal sources.

Resistance to streptomycin was significantly higher in isolates from cattle (43.7%) than in isolates from both pigs (25.7%) and chickens (34.0%). Resistance to kanamycin, sulphamethoxazole/trimethoprim and tetracycline was significantly lower in isolates from cattle than in isolates from the other species ($p<0.01$). The streptomycin resistance gene *strA* was detected in all but one of the streptomycin resistant *E. coli* and in all the streptomycin resistant *Salmonella* isolates while the *aadA1* gene was only detected in 72.4% of the *E. coli* isolates. The streptomycin resistance gene *strA* was found to be physically linked to the sulphonamide resistance gene *sul2* in two small plasmids of 6 and 8 kb, designated pSSGK1 and pSSTGK1 which were identified by transformation experiments into *E. coli* JM109 to mediate resistance to streptomycin and sulphonamides, and to streptomycin, sulphonamides and tetracycline, respectively.

Since sulphonamide drugs are the second most frequently used antimicrobials in food animals (Mitema *et al.*, 2001), it may be concluded that selection pressure imposed by use of sulphonamides in chickens and pigs in Kenya may be a driving force in the selection for streptomycin resistance in these species. Streptomycin resistance gene *strA* was transferable via conjugation to *E. coli* K12 strain 7118N Lac$^-$ and was co-transferred with the tetracycline resistance genes [*tet*(A) and *tet*(B)] and ampicillin resistance gene (bla_{TEM}). The detection the *strA* gene in plasmid-free resistant *Salmonella* serovars Braenderup and Saintpaul suggests a chromosomal localization of the gene in these strains.

The finding that *E. coli* from food animals carried a *dfrA14* gene cassette at a secondary site within the *strA* gene as those described in uropathogenic *E. coli* isolates from humans underlines the presence of links between bacteria of humans and animals. The *aadA1* gene was detected as cassette-borne gene within class 1 integron and was linked with trimethoprim resistance gene cassette *dfrA1*. The finding that one of the isolates (83K) harbouring the class

1 integron was plasmid-free and the integrons were not detectable by PCR using plasmid DNA from *E. coli* K12 strain 7118N Lac$^-$ transconjugants for strain 72C suggests that all the integrons in these isolates were located in the chromosomal DNA. The *dfrA1* gene cassette was found upstream of the *aadA1* resistance gene suggesting that the *aadA1* gene may be either the first cassette to be acquired by an integron and/or may be more stably integrated into the integron than the *dfrA1* gene cassette (Hall and Collis, 1995).

Although chloramphenicol is not approved for use in food animals in Kenya, resistance to it was observed in 4.7% of the *E. coli* and 12.5% of the *Salmonella* isolates. In all the chloramphenicol-resistant *E. coli* and *Salmonella* isolates resistance was encoded by the *catA1* gene. In the *E. coli* isolates the chloramphenicol resistance gene *catA1* was confirmed to be plasmid-borne and transferable via conjugation by PCR. The chloramphenicol resistance was co-transferred with resistance to sulphamethoxazole/trimethoprim, ampicillin and tetracycline. The detection of the *catA1* gene in plasmid-free chloramphenicol-resistant *Salmonella* isolates suggests its chromosomal localization. In Kenya, penicillins and tetracyclines are the most widely used antibiotics in humans (Mitema and Kikuvi, 2004) and food-producing animals (Mitema *et al.*, 2001), respectively. Therefore, selective pressure imposed by use of ampicillin, sulphamethoxazole/trimethoprim or tetracycline may select for chloramphenicol resistance. Chloramphenicol resistant organisms from humans can reach chicken or pigs through contact with animal attendants. The *strA* and *catA1* genes were transferred by plasmids ranging in size 62–106 kb from representative *E. coli* strains to *E. coli* K12 strain 7118N Lac$^-$ recipient via conjugation. This emphasizes the significance of conjugative R-plasmids in the spread and persistence of streptomycin and chloramphenicol resistance genes.

Gentamicin, although a relatively old antimicrobial agent has had little use in animals (Bywater *et al.*, 2004) and in Kenya, no formulations are available for use in chickens. Thus the resistance observed in *E. coli* isolates from pigs and chicken (1.0-2.0%) may have been

caused by off-label use or the clonal spread of resistant isolates as suggested by Kijima-Tanaka *et al.* (2003). Kanamycin and gentamicin resistances were not transferable by either transformation to *E. coli* JM 109 or by conjugation to *E. coli* K12 strain 7118N Lac$^-$ suggesting chromosomal location of the resistance genes. The kanamycin resistance gene *aph(3'')-Ia* and gentamicin/kanamycin resistance *ant (2)-Ia* gene were not detected in strains studied, this suggests that either, other resistance genes or other mechanisms of resistance were responsible for the kanamycin and/or gentamicin resistances in these strains.

5.2: CONCLUSIONS

1. Multidrug resistant *E. coli* is commonly found in cattle, pigs and chicken and on fresh cattle and pig carcasses in Kenya.
2. *Salmonella* is present in pigs at slaughter and on pork carcasses and pigs are a potential source of single and multiple antimicrobial-resistant *Salmonella* isolates to commonly used antimicrobials including ampicillin, chloramphenicol, streptomycin and tetracycline.
3. *E. coli* from cattle, pigs and chicken and *Salmonella* serotypes Braenderup and Saintpaul from pigs in Kenya represent a reservoir of streptomycin and chloramphenicol resistance genes which may be transferable to other bacteria.
4. Physical linkage of *strA* and *sul2* genes offers the possibility of co-selection of these genes during selective pressure imposed by the use of sulphonamides.
5. The location of the streptomycin resistance gene, *strA* and the chloramphenicol resistance gene *catA1* on conjugative plasmids and the streptomycin resistance *aadA1* gene within class 1 integrons in *E. coli* from apparently healthy food animals constitutes an effective means to spread streptomycin and chloramphenicol resistances among bacteria from different ecosystems.

5.3: RECOMMENDATIONS

1. The microbial contamination of carcasses during the slaughter process should be reduced to minimize the risk of transfer of antimicrobial resistant bacteria to humans.
2. Slaughterhouse personnel should adhere to adequate abattoir hygiene, take care during evisceration and decontaminate of carcasses by use of chlorinated water to minimize the risk of transfer of resistant bacteria to humans.
3. Adequate cooking of meat must be ensured to destroy resistant bacteria that can infect human consumers.
4. There is need for the establishment of antimicrobial prudent use guidelines in food animals in order to minimize the selection and spread of resistant bacteria.
5. A national antimicrobial resistance surveillance program in food animals should be established.
6. There is need for detailed epidemiological and molecular studies on acquisition of resistance genes and distribution of antimicrobial-resistant *E. coli* and *Salmonella* isolates among food animals, food products and humans in Kenya.

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APPENDICES

Appendix 1: Diameter (mm) of zones of inhibition for *E. coli* isolates from cattle.

Sampling date	Sample ID	Amp	Tet	SxT	Sm	Km	Gm	Cm
06.11.01	2F1	12	21	22	6	17	21	26
06.11.01	4F1	21	22	20	13	18	20	27
06.11.01	4C1	16	22	27	20	20	22	26
06.11.01	5C1	10	20	24	14	19	20	26
06.11.01	6F1	10	20	20	12	19	22	27
06.11.01	7F1	16	22	27	19	20	20	26
06.11.01	8F1	20	22	21	19	22	22	26
06.11.01	8C1	6	20	27	23	18	19	26
06.11.01	9F1	16	19	20	14	21	24	24
06.11.01	10C1	21	22	20	13	18	20	27
06.11.01	10F1	18	20	24	20	24	20	28
06.11.01	11C1	10	20	24	12	18	20	26
06.11.01	12C1	10	20	24	19	16	20	28
06.11.01	13F1	16	19	21	14	21	24	27
06.11.01	14F1	18	19	28	18	20	20	26
06.11.01	15C1	22	19	22	20	22	22	24
06.11.01	15F1	18	20	26	20	24	22	24
06.11.01	16C1	6	20	24	6	18	20	28
06.11.01	16F1	18	19	22	13	20	22	28
06.11.01	17F1	19	16	26	21	24	20	28
06.11.01	18C1	6	24	26	14	21	18	27
06.11.01	18F1	12	22	27	14	20	19	24
06.11.01	19C1	12	20	6	10	16	19	26
06.11.01	19F1	13	19	22	18	20	18	26
06.11.01	20F1	22	19	22	20	22	22	24
06.11.01	23C1	22	19	22	20	22	22	24
06.11.01	27C1	6	19	22	13	20	18	26

06.11.01	27F1	18	19	28	18	20	20	26
06.11.01	28F1	19	19	21	14	21	24	27
06.11.01	28C1	20	25	27	6	22	20	27
04.12.01	30F1	24	20	26	18	22	21	28
04.12.01	30C1	20	22	21	19	22	22	26
04.12.01	31F1	15	6	24	14	18	19	27
04.12.01	31C1	18	20	22	12	19	22	27
04.12.01	32C1	14	16	19	18	22	20	28
04.12.01	33F1	16	24	24	13	20	21	24
04.12.01	33C1	16	22	26	14	16	19	28
04.12.01	34F1	12	21	22	18	20	21	27
04.12.01	34C1	6	20	24	19	22	22	26
04.12.01	35F1	16	22	27	20	20	20	26
04.12.01	35C1	18	24	27	14	24	20	26
04.12.01	36F1	6	20	20	12	24	19	27
04.12.01	36C1	6	20	24	10	18	20	26
04.12.01	37F1	6	21	22	18	19	22	26
04.12.01	37C1	20	22	24	21	18	19	30
04.12.01	38F1	20	22	24	20	18	24	27
04.12.01	38C1	20	22	22	19	21	20	27
04.12.01	39F1	16	16	22	21	21	19	28
04.12.01	39C1	16	16	6	17	20	18	26
04.12.01	40F1	17	22	24	14	18	18	27
04.12.01	40C1	19	19	21	18	21	24	27
04.12.01	41F1	18	19	19	18	22	20	28
04.12.01	41C1	20	24	26	24	24	24	26
04.12.01	42F1	21	22	20	19	18	20	27
04.12.01	42C1	20	25	27	14	22	20	27
04.12.01	43F1	24	20	26	18	22	21	28
04.12.01	43C1	10	20	24	12	18	20	26
04.12.01	44F1	20	22	21	19	22	22	26
04.12.01	44C1	6	18	24	13	18	19	27
04.12.01	45F1	18	20	22	12	19	22	27
04.12.01	45C1	15	16	24	11	16	20	28

04.12.01	46F1	18	19	28	18	20	20	26
04.12.01	46C1	24	20	26	18	22	21	28
04.12.01	47F1	18	19	19	18	22	20	28
04.12.01	47C1	22	19	22	20	22	22	24
04.12.01	48C1	22	24	24	13	20	21	24
04.12.01	48F1	22	19	22	20	22	22	24
04.12.01	49C1	13	20	24	14	18	20	28
04.12.01	49F1	18	19	19	18	22	20	28
04.12.01	50F1	16	22	26	24	16	19	28
04.12.01	50C1	20	22	27	14	20	19	24
04.12.01	51F1	12	21	22	18	20	21	27
04.12.01	51C1	19	19	22	13	20	22	28
04.12.01	52F1	18	19	19	18	22	20	28
04.12.01	52C1	22	19	22	20	22	22	24
04.12.01	53F1	19	16	26	21	24	20	28
04.12.01	53C1	10	24	26	18	21	18	27
04.12.01	54C1	20	22	27	14	20	19	24
04.12.01	54F1	6	20	27	12	16	19	26
04.12.01	55C1	6	19	22	18	20	18	26
E. coli	25922	20	24	26	24	24	24	26
E. coli	25922	21	22	30	18	22	24	26

Key: Amp, ampicillin (≤ 13); Cm, chloramphenicol (≤ 12); Gm, gentamicin (≤ 12); Km, kanamycin (≤ 13); Sm, streptomycin (≤11); SxT, sulphamethoxazole/trimethoprim (≤10); Tet, tetracycline (≤14). The figures in brackets represent the breakpoint diameter in mm for defining resistance.

Appendix 2: Diameter (mm) of zones of inhibition diameter for *E. coli* isolates from pigs.

Sampling date	Sample ID	Amp	Tet	SxT	Sm	Km	Gm	Cm
05.07.02	1F	16	12	6	17	19	20	21
05.07.02	2C	16	10	6	18	20	16	12
05.07.02	3C	6	12	6	10	17	20	25
05.07.02	3F	6	10	6	11	16	12	6
05.07.02	4F	6	6	6	6	19	20	30
05.07.02	5C	16	11	6	16	17	19	24
05.07.02	5F	14	6	6	20	18	24	24
05.07.02	6C	16	17	20	14	19	19	20
05.07.02	6F	15	12	21	14	20	21	23
05.07.02	7F	6	7	8	6	20	19	26
05.07.02	8C	18	12	23	11	19	17	27
05.07.02	8F	20	16	30	26	24	28	26
05.07.02	9C	6	6	6	21	11	20	24
05.07.02	10C	17	25	6	22	22	19	22
05.07.02	10F	19	26	26	18	21	26	30
05.07.02	12C	6	12	6	11	18	17	24
05.07.02	12F	18	17	24	16	18	17	21
05.07.02	14F	21	25	28	26	24	26	30
05.07.02	16C	14	6	6	19	20	21	24
05.07.02	17C	20	21	25	19	20	20	24
05.07.02	18C	6	6	6	6	22	24	28
05.07.02	19C	6	6	6	7	19	24	28
05.07.02	20C	6	6	6	6	21	21	26
05.07.02	21C	21	6	6	6	24	22	25
05.07.02	23C	15	23	22	22	26	23	24
05.07.02	23F	16	19	22	16	21	18	23
05.07.02	24C	15	15	22	17	17	22	22
05.07.02	25F	21	18	6	24	21	18	28
11.09.02	26C	16	19	21	23	20	19	22
11.09.02	26F	18	19	18	19	20	21	26
11.09.02	27C	20	22	24	18	24	22	26

11.09.02	27F	6	15	6	10	17	18	25
11.09.02	28F	17	19	23	17	19	18	26
11.09.02	29C	18	18	20	20	18	20	23
11.09.02	29F	6	6	7	7	19	20	28
11.09.02	30C	18	20	26	17	20	16	24
11.09.02	30F	17	20	25	18	18	18	20
11.09.02	31F	6	15	17	22	11	24	15
11.09.02	32C	20	24	26	20	22	20	24
11.09.02	32F	18	20	24	19	20	19	25
11.09.02	33C	20	23	28	16	20	20	30
11.09.02	33F	6	8	6	6	24	19	26
11.09.02	34C	20	21	30	20	19	18	30
11.09.02	34F	16	20	27	17	18	20	26
11.09.02	35C	12	17	18	20	17	16	21
11.09.02	35F	18	16	6	12	19	18	21
11.09.02	36F	15	23	27	20	21	21	26
11.09.02	39C	18	26	28	18	18	20	26
11.09.02	39F	26	26	30	15	20	18	26
11.09.02	40C	6	14	15	20	11	24	24
11.09.02	40F	18	22	25	26	22	20	22
11.09.02	41C	18	22	27	18	18	16	26
11.09.02	41F	20	22	24	20	19	22	28
11.09.02	42C	18	20	6	14	22	20	26
11.09.02	42F	20	22	25	25	22	20	24
11.09.02	43C	20	21	27	21	18	25	26
11.09.02	44F	16	19	22	16	22	20	26
11.09.02	45F	16	8	15	20	20	19	26
11.09.02	46C	19	20	6	18	22	20	26
11.09.02	46F	20	22	26	18	18	20	25
11.09.02	48C	19	23	28	20	22	20	24
11.09.02	49F	18	22	25	17	19	19	26
11.09.02	51F	20	24	29	20	20	20	26
11.09.02	52C	16	22	29	18	22	22	24
11.09.02	52F	15	22	27	15	20	19	24

11.09.02	54F	20	24	30	26	24	20	30
11.09.02	55C	16	21	22	22	24	28	28
11.09.02	60C	9	12	6	10	20	20	6
05.07.02	61C	16	19	18	19	19	20	20
05.07.02	61F	14	21	14	18	16	18	23
05.07.02	62C	18	12	28	20	16	16	24
05.07.02	62F	16	20	16	16	14	20	26
05.07.02	63C	14	20	22	16	18	22	23
05.07.02	64C	18	24	20	16	22	26	28
05.07.02	64F	17	22	28	18	18	20	27
05.07.02	65C	12	28	30	18	22	18	20
05.07.02	65F	6	8	6	13	10	18	6
05.07.02	66C	16	20	22	6	20	18	19
05.07.02	67F	12	22	26	16	18	18	26
05.07.02	68C	23	25	22	22	22	18	22
05.07.02	68F	20	26	24	20	24	20	30
11.09.02	69C	20	18	18	22	25	24	26
05.07.02	69F	6	21	26	26	20	19	17
11.09.02	70C	6	24	6	9	22	20	6
05.07.02	70F	6	22	24	20	20	20	28
11.09.02	72C	6	12	6	6	18	19	6
11.09.02	72F	20	22	26	18	18	18	20
11.09.02	74C	23	14	23	6	25	19	24
11.09.02	74F	10	20	24	22	20	18	20
11.09.02	75C	19	25	26	6	20	23	25
11.09.02	75F	6	14	17	20	20	21	14
11.09.02	76C	15	11	6	18	19	18	22
11.09.02	76F	18	20	22	20	18	19	24
11.09.02	77C	23	18	20	18	22	23	26
11.09.02	77F	19	20	28	18	20	20	22
11.09.02	78C	18	20	30	20	20	17	20
11.09.02	78F	21	26	30	24	26	26	31
11.09.02	79F	18	22	26	20	18	19	28
11.09.02	80C	18	22	26	16	20	18	22

11.09.02	80F	6	10	6	6	10	16	6
11.09.02	81C	20	29	26	20	22	22	32
11.09.02	81F	18	26	24	16	22	20	28
11.09.02	82F	16	20	21	16	17	18	26
11.09.02	84F	11	24	25	18	20	18	28
05.07.02	100C	16	18	21	24	26	27	25
E. coli	25922	19	28	26	28	26	25	30
E. coli	25922	17	25	23	22	24	25	21

Key: Amp, ampicillin (≤ 13 mm); Cm, chloramphenicol (≤ 12); Gm, gentamicin (≤ 12); Km, kanamycin (≤13); Sm, streptomycin (≤11); SxT, sulphamethoxazole/trimethoprim (≤10); Tet, tetracycline (≤14). The figures in brackets represent the breakpoint diameter in mm for defining resistance.

Appendix 3: Diameter (mm) of zones of inhibition for *E. coli* isolated from chickens.

Sampling date	Sample ID	Amp	Tet	SxT	Sm	Km	Gm	Cm
11.09.02	1P	20	26	26	18	20	23	26
11.09.02	1K	18	24	24	26	26	22	23
11.09.02	2	20	23	27	20	24	24	28
11.09.02	14	6	26	28	20	18	26	30
01.10.02	16c	20	27	26	20	20	22	23
11.09.02	16b	19	16	28	12	18	20	26
11.09.02	19	20	8	6	20	22	18	29
11.09.02	20	20	16	6	20	22	22	32
11.09.02	21	20	21	6	22	22	18	24
11.09.02	22	16	23	25	19	20	23	27
11.09.02	23	20	16	6	24	18	26	27
11.09.02	40D	26	16	28	21	24	26	26
11.09.02	42	16	23	27	16	19	20	28
11.09.02	45D	6	9	6	6	20	23	6
11.09.02	49	23	28	6	18	24	20	26
11.09.02	51a	20	22	26	12	14	14	26
11.09.02	52	6	14	26	16	20	22	25
11.09.02	57	18	19	22	18	20	20	24
11.09.02	57b	16	26	26	13	24	24	28
11.09.02	58	20	23	22	17	24	21	24
11.09.02	60	18	22	26	13	15	18	28
11.09.02	61a	20	23	27	12	16	18	26
11.09.02	61b	20	24	28	22	18	18	30
11.09.02	63	16	23	26	22	24	18	28
11.09.02	64K	18	24	20	18	22	18	24
11.09.02	64P	16	25	26	12	16	18	26
01.10.02	68P	20	26	24	22	18	19	26
11.09.02	70K	18	18	26	24	20	26	28
11.09.02	70P	6	8	6	6	19	19	26
11.09.02	71	6	22	26	18	20	18	26

11.09.02	72D	20	12	6	12	16	18	26
11.09.02	73D	20	21	24	19	18	19	25
11.09.02	74D	19	20	24	24	26	21	24
11.09.02	77K	18	9	20	18	21	26	26
11.09.02	77P	16	16	28	18	20	18	30
01.10.02	82K	6	26	6	16	22	19	20
01.10.02	82P	17	23	6	20	20	21	22
01.10.02	83K	6	6	6	6	20	16	28
01.10.02	83P	16	20	30	18	25	18	26
01.10.02	84P	18	20	30	14	20	25	32
01.10.02	84K	18	20	28	18	20	26	28
01.10.02	85P	18	29	6	6	16	18	20
01.10.02	86K	21	18	6	23	21	18	28
01.10.02	87K	20	20	26	10	20	16	26
01.10.02	87P	16	20	20	11	18	18	24
01.10.02	89K	20	22	21	28	19	19	29
01.10.02	89P	6	16	26	10	20	20	20
01.10.02	90K	20	22	27	20	20	23	25
01.10.02	90P	20	20	26	20	21	30	28
01.10.02	91K	16	15	28	22	22	20	24
E. coli	25922	20	24	26	24	24	24	26

Key: Amp, ampicillin (≤ 13 mm); Cm, chloramphenicol (≤ 12); Gm, gentamicin (≤ 12); Km, kanamycin (≤ 13); Sm, streptomycin (≤11); SxT, sulphamethoxazole/trimethoprim (≤10); Tet, tetracycline (≤14). The figures in brackets represent the breakpoint diamater in mm for defining resistance.

Appendix 4: Diameter (mm) of zones of inhibition for *Salmonella* isolates from pigs (n =16).

Sample ID	Serotype	Amp	Tet	SxT	Sm	Km	Gm	Cm	Nal
17C_S	S. Heidelberg	20	21	26	14	18	22	22	28
23C_S	S. Heidelberg	23	26	24	17	22	16	28	24
24F_S	nd	6	12	6	19	19	22	13	Nd
29F_S	nd	14	24	24	16	22	22	26	Nd
30C_S	S. Heidelberg	20	22	20	14	24	24	24	24
64C_S	S. Braenderup	22	25	26	16	26	22	24	30
64F_S	S. Braenderup	6	12	26	6	18	20	14	30
66C_S	S. Saintpaul	20	22	26	12	16	18	20	28
67C_S	S. Saintpaul	20	22	26	16	16	22	24	28
68F_S	S. Saintpaul	16	26	28	14	22	20	26	20
71C_S	S. Saintpaul	18	20	24	14	22	20	20	21
73C_S	S. Saintpaul	16	22	28	16	18	20	18	29
73F_S	S. Saintpaul	26	24	20	24	22	16	10	30
74C_S	S. Saintpaul	20	24	26	24	24	24	28	16
76C_S	S. Saintpaul	16	18	20	20	20	18	18	24
79C_S	S. Saintpaul	20	24	30	15	24	24	28	28
E. coli ATCC 25922		28	28	26	24	24	24	26	31

Key: nd, not done; Amp, ampicillin (≤ 13); Cm, chloramphenicol (≤ 12); Gm, gentamicin (≤ 12); Km, kanamycin (≤ 13); Sm, streptomycin (≤11); SxT, sulphamethoxazole/trimethoprim (≤10); Tet, tetracycline (≤14). The figures in brackets represent the breakpoint diamater in mm for defining resistance.

Appendix 5: MICs for *E. coli* isolated from chickens (n =37).

Sample ID	Amp	Tet	SxT	Sm	Km	Gm	Cm
1P	8	0.5	≤4.75/0.25	16	16	4	8
14	128	2	≤4.75/0.25	8	8	4	8
16b	8	2	≤4.75/0.25	8	8	1	16
16c	4	8	≤4.75/0.25	16	16	2	8
19	8	≥256	≥1216/64	8	16	4	4
20	8	8	≥1216/64	32	8	2	8
21	8	4	≥1216/64	8	16	2	8
22	16	2	≤4.75/0.25	16	16	4	8
23	4	64	≥1216/64	16	8	2	4
40D	4	8	38/2	16	16	4	8
42	32	4	≤4.75/0.25	8	8	2	4
45D	≥256	≥256	≥1216/64	≥256	8	2	≥256
49	8	2	608/32	8	16	2	4
51a	8	2	≤4.75/0.25	64	32	8	8
52	≥256	≥256	19/1	8	4	2	4
57b	16	4	≤4.75/0.25	4	8	2	4
60	8	4	≤4.75/0.25	4	8	2	4
61a	4	4	≤4.75/0.25	4	8	2	4
63	16	4	≤4.75/0.25	8	8	2	8
64P	16	2	≤4.75/0.25	16	8	2	8
70K	4	8	19/1	4	4	1	4
70P	8	16	≥1216/64	64	8	2	8
71	128	4	≤4.75/0.25	16	16	2	8
72D	4	≥256	≥1216/64	32	32	2	4
77P	16	≥256	≤4.75/0.25	16	16	2	8
77K	8	≥256	≤4.75/0.25	16	16	2	4
82K	≥256	4	≥1216/64	4	16	4	8
82P	8	2	≥1216/64	16	16	4	4
83P	16	2	≤4.75/0.25	16	16	4	4
83K	≥256	128	≥1216/64	≥256	8	4	8
84P	8	4	≤4.75/0.25	32	8	4	8

85P	8	4	≥1216/64	≥256	32	4	8
86K	4	8	≥1216/64	16	8	4	8
87P	4	2	≤4.75/0.25	≥256	8	2	8
87K	8	4	≤4.75/0.25	≥256	16	4	8
89P	≥256	8	≤4.75/0.25	128	8	4	2
91K	16	8	≤4.75/0.25	16	8	2	8

:y: Amp, ampicillin (≥32); Cm, chloramphenicol (≥32); Gm, gentamicin (≥16); Km, kanamycin (≥64); Sm, streptomycin (≥32); SxT, sulphamethoxazole/trimethoprim (≥4); Tet, tetracycline (≥16). The figures in brackets represent the breakpoints for defining resistance.

Appendix 6: MICs for *E. coli* isolated from cattle (n = 49).

Sample ID	Amp	Tet	SxT	Sm	Km	Gm	Cm
2F1	64	2	9.5/0.5	128	32	4	8
4F1	4	2	≤4.75/0.25	32	4	0.5	8
5C1	64	2	≤4.75/0.25	16	8	1	4
6F1	128	2	≤4.75/0.25	≥256	16	4	4
8C1	≥256	2	≤4.75/0.25	16	4	2	4
9F1	16	2	≤4.75/0.25	32	8	2	2
10C1	8	2	≤4.75/0.25	8	8	4	8
11C1	64	4	≤4.75/0.25	≥256	4	8	8
12C1	64	4	≤4.75/0.25	≥256	32	2	4
13F1	16	4	≤4.75/0.25	8	8	4	8
16F1	16	1	≤4.75/0.25	16	16	4	8
16C1	≥256	4	19/1	128	8	4	16
17F1	4	8	≤4.75/0.25	8	8	4	4
18C1	≥256	2	≤4.75/0.25	16	8	2	8
18F1	32	2	≤4.75/0.25	8	16	2	16
19F1	32	4	9.5/0.5	16	8	2	4
19C1	64	2	≥1216/64	128	8	4	8
27C1	≥256	4	≤4.75/0.25	16	8	4	4
28F1	8	4	≤4.75/0.25	32	8	4	8
28C1	8	4	≤4.75/0.25	≥256	4	4	4
31F1	16	≥256	≤4.75/0.25	16	16	2	8
31C1	8	0.5	≤4.75/0.25	32	16	1	4
32C1	16	8	≤4.75/0.25	4	8	2	2
33F1	16	4	≤4.75/0.25	32	32	2	2
33C1	4	4	≤4.75/0.25	16	8	4	4
34C1	≥256	4	≤4.75/0.25	8	4	2	8
34F1	≥256	1	≤4.75/0.25	16	8	4	8
35C1	8	4	9.5/0.5	16	16	1	4
36F1	≥256	4	≤4.75/0.25	16	16	4	4
36C1	≥256	4	19/1	≥256	8	4	4
37F1	≥256	4	≤4.75/0.25	16	16	4	8

39C1	16	2	≥1216/64	8	4	4	8
39F1	16	8	≤4.75/0.25	8	4	4	8
40F1	8	2	≤4.75/0.25	8	4	4	8
42C1	8	1	≤4.75/0.25	32	8	4	8
43C1	64	4	≤4.75/0.25	16	8	2	8
44C1	≥256	4	≤4.75/0.25	16	8	4	8
45C1	16	2	≤4.75/0.25	≥256	32	2	2
45F1	8	4	≤4.75/0.25	16	8	4	8
48C1	4	4	≤4.75/0.25	16	8	2	8
49C1	32	4	≤4.75/0.25	16	8	2	4
50F1	16	4	≤4.75/0.25	4	16	2	2
50C1	4	2	≤4.75/0.25	16	8	2	8
51F1	16	4	≤4.75/0.25	16	8	2	8
51C1	8	4	≤4.75/0.25	32	8	2	8
53C1	64	4	≤4.75/0.25	16	8	2	4
54F1	≥256	4	≤4.75/0.25	16	32	2	8
54C1	4	4	≤4.75/0.25	8	8	2	8
55C1	≥256	4	19/1	16	8	2	8

Key: Amp, ampicillin (≥32); Cm, chloramphenicol (≥32); Gm, gentamicin (≥16); Km, kanamycin (≥64); Sm, streptomycin (≥32); SxT, sulphamethoxazole/trimethoprim (≥4); Tet, tetracycline (≥16). The figures in brackets represent the breakpoints for defining resistance.

Appendix 7: MICs for *E. coli* isolated from swine (n = 68).

Sample Identity	Amp	Tet	SxT	Sm	Km	Gm	Cm
1F	16	128	≥1216/64	4	4	1	8
2F	16	4	≤4.75/0.25	16	4	4	8
3C	≥256	128	≥1216/64	≥256	8	1	8
3F	≥256	≥256	≥1216/64	≥256	8	8	≥256
4F	≥256	≥256	≥1216/64	≥256	8	2	8
5F	16	128	≥1216/64	16	8	2	8
5C	8	≥256	76/4	16	8	4	8
6C	16	2	≤4.75/0.25	32	4	2	8
6F	16	≥256	≤4.75/0.25	32	8	8	8
7F	16	2	≤4.75/0.25	32	8	2	8
8F	4	2	≤4.75/0.25	8	8	2	2
8C	8	≥256	≤4.75/0.25	128	8	2	8
9C	32	32	≥1216/64	16	32	2	2
10C	8	2	≥1216/64	4	16	1	2
12C	64	2	≥1216/64	64	8	2	8
12F	8	8	≤4.75/0.25	4	4	4	8
16C	16	128	≥1216/64	8	8	2	8
18C	≥256	≥256	≥1216/64	≥256	4	4	4
19C	64	≥256	≥1216/64	≥256	16	2	4
20C	32	2	≥1216/64	32	8	1	8
21C	8	32	≥1216/64	32	8	4	4
23C	16	4	≤4.75/0.25	16	16	4	8
23F	16	4	19/1	4	8	0.5	8
24C	16	8	≤4.75/0.25	4	4	4	8
25F	4	8	≥1216/64	16	4	2	8
26C	16	4	19/1	16	8	2	4
27F	≥256	≥256	≥1216/64	≥256	32	4	8
29F	32	≥256	≥1216/64	32	8	2	8
29C	4	8	≤4.75/0.25	8	8	2	8
31F	≥256	8	≤4.75/0.25	16	32	8	4

33F	≥256	64	≥1216/64	128	8	2	8
34F	16	2	≤4.75/0.25	4	4	2	8
35C	≥256	8	≤4.75/0.25	4	4	4	8
35F	2	4	≥1216/64	128	4	2	2
36F	16	2	≤4.75/0.25	16	4	2	4
40C	64	≥256	≥1216/64	16	16	2	8
42C	4	1	76/4	16	8	2	8
44C	8	4	≤4.75/0.25	16	8	2	8
45F	16	≥256	≥1216/64	16	16	2	8
46C	4	16	≥1216/64	8	16	2	8
52C	16	2	≤4.75/0.25	16	16	2	8
52F	16	2	≤4.75/0.25	≥256	8	2	8
55C	16	4	≤4.75/0.25	8	4	1	8
60C	≥256	128	≥1216/64	≥256	4	4	≥256
61C2	16	4	≥1216/64	8	8	1	2
61C	16	4	≥1216/64	4	8	2	≥256
62F	4	4	76/4	16	8	2	8
62C	4	128	≤4.75/0.25	8	16	2	4
63C	64	4	≤4.75/0.25	8	8	2	8
65C	≥256	4	≤4.75/0.25	8	16	2	4
65 F	8	128	≥1216/64	32	16	2	≥256
66C	16	4	19/1	≥256	8	2	4
67F	16	2	≤4.75/0.25	16	16	2	4
69F	≥256	4	≤4.75/0.25	16	8	2	16
69C	8	4	≤4.75/0.25	16	8	2	8
70C2	≥256	4	≤4.75/0.25	8	8	1	8
70C	≥256	4	76/4	≥256	8	2	≥256
72C	≥256	128	≥1216/64	≥256	8	8	≥256
74F	≥256	4	≤4.75/0.25	8	8	2	8
74C	8	128	≤4.75/0.25	≥256	4	2	4
75F	32	≥256	≤4.75/0.25	8	8	8	16
75C	8	8	≤4.75/0.25	64	8	2	2
76C	16	128	≥1216/64	16	8	2	8
77C	8	8	≤4.75/0.25	16	4	2	8

80F	≥256	≥256	≥1216/64	≥256	8	4	≥256
82F	16	2	≤4.75/0.25	16	32	2	8
84F	64	4	≤4.75/0.25	8	4	2	8
100C	8	8	≤4.75/0.25	16	16	1	8

Key: Amp, ampicillin (≥32); Cm, chloramphenicol (≥32); Gm, gentamicin (≥16); Km, kanamycin (≥64); Sm, streptomycin (≥32); SxT, sulphamethoxazole/trimethoprim (≥4); Tet, tetracycline (≥16). The figures in brackets represent the breakpoints for defining resistance.

Appendix 8: Comparison of MIC data for *E. coli* isolates from cattle (n = 49), chickens (n = 37) and pigs (n = 68).

Antimicrobial agent	No. of isolates for which MIC (µg/ml) is											MIC (µg/ml)	
	≤0.25	0.5	1	2	4	8	16	32	64	128	≥256	MIC_{50}	MIC_{90}
Ampicillin													
Cattle	0	0	0	0	6	9	11	3	7	1	12	16	≥256
Chickens	0	0	0	0	8	14	7	1	0	2	5	8	≥256
Pigs	0	0	0	1	7	12	23	4	5	0	16	16	≥256
Total	0	0	0	1	21	35	41	8	12	3	33	16	≥256
Chloramphenicol													
Cattle	0	0	0	4	15	28	2	0	0	0	0	8	8
Chickens	0	0	0	1	13	21	1	0	0	0	1	8	8
Pigs	0	0	0	6	11	42	2	0	0	0	7	8	≥256
Total	0	0	0	11	39	91	5	0	0	0	8	8	8
Gentamicin													
Cattle	0	1	3	22	22	1	0	0	0	0	0	2	4
Chickens	0	0	2	20	14	5	0	0	0	0	0	2	4
Pigs	0	1	8	43	11	5	0	0	0	0	0	2	4
Total	0	2	13	86	47	10	0	0	0	0	0	2	4
Kanamycin													
Cattle	0	0	0	0	8	25	8	8	0	0	0	8	32
Chickens	0	0	0	0	2	15	14	4	0	0	0	8	16
Pigs	0	0	0	0	16	32	11	9	0	0	0	8	16
Total	0	0	0	0	26	72	33	21	0	0	0	8	16
Streptomycin													
Cattle	0	0	0	0	2	9	22	7	0	3	6	16	128
Chickens	0	0	0	0	5	7	13	3	2	1	5	16	≥256
Pigs	0	0	0	0	8	13	22	7	2	3	14	16	≥256
Total	0	0	0	0	15	29	57	17	4	7	25	16	≥256
Tetracycline													
Cattle	0	1	3	14	27	3	0	0	0	0	1	4	4
Chickens	0	1	0	9	11	7	1	0	1	1	6	4	≥256
Pigs	0	0	1	12	19	9	1	2	1	10	13	8	≥256
Total	0	2	4	35	57	19	2	2	2	11	20	4	≥256
Trimethoprim/sulphamethoxazole (1:19)[a]	41	3	3	0	0	0	0	0	2			≤0.25	1
Cattle	21	0	2	0	0	0	0	2	12			≤0.25	≥64
Chickens	33	0	3	0	4	0	0	0	28			≤0.25	≥64
Pigs	95	3	8	0	4	0	0	2	42			≤0.25	≥64
Total													

Appendix 9: Number and size(s) of fragments obtained from single digests for the 74C, 6 kb (Sm^RSul^R) plasmid.

Enzyme	No. of fragments	Size(s) of the fragment(s) in kb
*Pvu*II	1	6.0
*Eco*RI	1	6.0
*Hpa*I	1	6.0
*Sac*I	1	6.0
*Dra*I	1	6.0
*Pst*I	2	2.9, 3.1
*Eco*RV	2	3.5, 2.5
*Ksp*I	2	1.8, 4.2

Appendix 10: Number and size(s) of fragments obtained from double digests for the 74C, 6 kb (Sm^RSul^R) plasmid.

Enzyme	Buffer	Temperature (°C)	No. of fragments	Size(s) of the fragment(s) in kb
*Dra*I/*Pst*I	M-H	37	1	6.0
*Sac*I/*Eco*RI	A	37	1	6.0
*Pst*I/*Eco*RI	H	37	2	3.0, 3.0
DraI/*Eco*RI	M-H	37	2	2.8, 3.2
DraI/*Pvu*II	M	37	2	0.8, 5.2
SacI/*Ksp*I	L	37	2	1.6, 4.4
SacI/*Pvu*II	L-M	37	2	1.6, 4.4
SacI/*Dra*I	M-H	37	2	2.5, 3.5
PvuII/*Eco*RI	M-H	37	2	1.8, 4.2
DraI/*Eco*RV	B	37	3	1.0, 1.5, 3.5
KspI/*Pvu*II	L-M	37	3	1.6, 1.8, 2.6
EcoRV/*Eco*RI	B	37	3	1.7, 1.8, 2.5

Appendix 11: Number and size(s) of fragments obtained from single digests for the 4F and 66C (Sm^RSul^R) plasmids.

Enzyme	Number of fragments	Approximate size (s) in kb
*Eco*RI	1	6.0
*Pvu*II	1	6.0
*Sac*I	1	6.0
*Dra*I	1	6.0
*Hpa*I	1	6.0
*Kpn*I	1	6.0
*Sma*I	1	6.0
*Ksp*I	2	1.7, 4.3
*Eco*RV	2	2.5, 3.5
*Pst*I	2	2.8, 3.2

Appendix 12: Number and size(s) of fragments obtained from double digests for 4F and 66C (Sm^RSul^R) plasmids.

Enzyme	Buffer	Temperature (°C)	Number of fragments	Approximate size (s) in kb
SacI/EcoRI	A	37	1	6.0
EcoRI/HpaI	Y^+ x1	37	1	6.0
SacI/PvuII	L, M	37	2	1.6, 4.4
PvuII/EcoRI	M, H	37	2	1.8, 4.2
DraI/EcoRI	A	37	2	2.8, 3.2
DraI/PvuII	B	37	2	0.8, 5.2
SacI/KspI	L	37	2	1.6, 4.4
SacI/DraI	A	37	2	2.4, 3.6
SmaI/ EcoRI	A	25, 37	2	2.3, 3.7
KspI/SacI	L	37	2	1.7, 4.3
SacI/KpnI	L	37	2	1.9, 4.1
KpnI/DraI	L	37	2	1.7, 4.3
KpnI/PvuII	L, M	37	2	2.5, 3.5
SmaI/SacI	A	37	2	2.5, 3.5
EcoRI/SmaI	A	37	2	2.3, 3.7
KspI/PvuII	L, M	37	3	1.6, 1.8, 2.6
EcoRV/EcoRI	B	37	3	1.7, 1.8, 2.5
DraI/PstI	M, H	37	3	2.8, 2.8, 0.4
DraI/EcoRV	B	37	3	0.9, 1.6, 3.5
PstI/EcoRI	H	37	3	3.0, 2.8, 0.2
KspI/DraI	L, M	37	3	1.7, 1.9, 2.4

Appendix 13: Number and sizes of fragments obtained from 8C plasmid ($Sm^RSul^RTet^R$) after single digests.

Enzyme	Number of fragments	Approximate size (s) in kb
*Eco*RI	1	8.0
*Pvu*II	1	8.0
*Sac*I	1	8.0
*Dra*I	1	8.0
*Hpa*I	1	8.0
*Kpn*I	1	8.0
*Bgl*II	1	8.0
*Pst*I	2	2.9, 5.1
*Sma*I	2	2.4, 5.6
*Ksp*I	3	1.7, 3.4, 3.5
*Eco*RV	3	1.7, 2.8, 3.5

Appendix 14: Number and size of fragments obtained from 8C plasmid ($Sm^RSul^RTet^R$) after double digests.

Enzyme	Buffer(s)	Temperature (°C)	Number of fragments	Approximate size (s) in kb
*Eco*RI/*Hpa*I	Y^+ x1	37	1	8.0
*Sac*I/*Eco*RI	A	37	1	8.0
*Pst*I/*Eco*RI	H	37	2	2.9, 5.1
*Sac*I/*Kpn*I	L	37	2	1.9, 6.1
*Sac*I/*Pvu*II	L, M	37	2	3.6, 4.4
*Kpn*I/*Pvu*II	L, M	37	2	2.3, 5.7
*Sac*I/*Dra*I	A	37	2	3.5, 4.5
*Dra*I/*Eco*RI	A	37	2	3.2, 4.8
*Pvu*II/*Eco*RI	M, H	37	2	3.8, 4.2
*Pvu*II/*Bgl*II	L, M	37	2	2.0, 6.0
*Bgl*II/*Kpn*I	L, M	37	2	3.5, 4.5
*Bgl*II/*Sac*I	A	37	2	1.7, 6.3
*Dra*I/*Pst*I	M, H	37	3	2.9, 4.7, 0.4
*Sma*I/*Sac*I	A	25, 37	3	2.4, 2.4, 3.4
*Pst*I/*Sac*I	L	37	3	2.9, 4.9, 0.2
*Dra*I/*Eco*RV	B	37	4	1.4, 1.4, 1.7, 3.5
*Eco*RV/*Bgl*II	B	37	4	1.4, 2.8, 3.5, 0.3
*Eco*RV/*Eco*RI	B	37	4	1.7, 1.7, 2.8, 1.8

Appendix 15: Conjugational transfer of streptomycin resistance.

Strains	Selection agar plates and bacterial inoculun dilutions																			
	LB without antibiotic(s)				LB+Sm 30 µg/ml				LB+Nal 60 µg/ml				LB+Sm 30 µg/ml + Nal 60 µg/ml							
	-5	-6	-7	-8	-5	-6	-7	-8	-5	-6	-7	-8	-1	-2	-3	-4	-5	-6	-7	-8
2F2	++	++	++	++	++	++	++	++	++	++	++	75	154	22	-	-	-	-	-	-
27F	++	++	++	++	++	++	++	++	++	++	++	++	120	100	70	-	-	-	-	-
45D	++	++	++	++	++	++	++	200	++	++	++	++	1	-	-	-	-	-	-	-
65F	++	++	++	200	++	++	130	100	++	++	++	++	140	6	-	-	-	-	-	-
66C	++	++	++	++	++	++	++	++	++	++	81	17	107	11	5	-	-	-	-	-
72C	++	++	++	79	++	++	142	42	++	110	26	1	2	-	-	-	-	-	-	-
80F	++	++	++	++	++	++	++	++	++	++	300	115	63	5	-	-	-	-	-	-

Key: ++ Growth with colonies too many to count; - No growth

Appendix 16: Co-transfer of ampicillin and/or tetracycline resistance with resistance to streptomycin.

Strains	Selection agar plates and bacterial inoculun dilutions																			
	LB without antibiotic				LB+Tet 20 µg/ml *LB+Amp50 µg/ml				LB+Nal 60 µg/ml				LB+Tet 20 µg/ml + Nal 60 µg/ml *LB+Tet 20 µg/ml + Amp 50 µg/ml							
	-5	-6	-7	-8	-5	-6	-7	-8	-5	-6	-7	-8	-1	-2	-3	-4	-5	-6	-7	-8
27F	++	++	++	++	++	++	++	++	++	++	++	++	118	8	7	-	-	-	-	-
					*++	++	++	++					*100	15	2	-	-	-	-	-
45D	++	++	++	++	++	++	++	++	++	++	++	++	20	2	-	-	-	-	-	-
					*++	++	++	++					*100	60	-	-	-	-	-	-
65F	++	++	++	++	++	++	200	122	++	++	++	120	24	16	1	-	-	-	-	-

Key: ++ Growth with colonies with too many to count; - No growth

Appendix 17: Conjugal transfer of chloramphenicol resistance.

Strains	Selection agar plates and bacterial inoculun dilutions																			
	LB without antibiotics				LB+Cm 20 µg/ml				LB+Nal 60 µg/ml				LB+Cm 20 µg/ml + Nal 60 µg/ml							
	-5	-6	-7	-8	-5	-6	-7	-8	-5	-6	-7	-8	-1	-2	-3	-4	-5	-6	-7	-8
45D	++	++	++	++	++	++	++	++	++	++	++	++	39	5	-	-	-	-	-	-
72C	++	++	++	++	++	++	++	++	++	++	++	80	3	2	-	-	-	-	-	-

Key: ++ Growth with colonies too many to count; - No growth

Appendix 18: Antibiograms of TOPO clones, transformants and transconjugants.

Strain ID	Optical density	Amp	Cm	Gm	Km	Sm	Sul	Tet	Trim
20C_TP1[a]	0.080	S	S	S	S	S	S	S	R
4F_TF10[b]	0.085	S	S	S	S	R	R	S	S
74C_TF8[b]	0.068	S	S	S	S	R	R	S	S
66C_TF4[b]	0.084	S	S	S	S	R	R	S	S
8C_TF12[b]	0.079	S	S	S	S	R	R	R	S
3C_TC3[b]	0.078	R	S	S	S	R	R	R	R
27F_TC5[b]	0.069	R	S	S	S	R	R	R	R
65F_TC3[b]	0.076	S	S	S	S	R	R	R	R
2F1_TC5[b]	0.091	S	S	S	S	R	S	S	S
72C_TC1[b]	0.068	R	R	S	S	R	R	R	R
45D_TC3[b]	0.084	R	R	S	S	R	R	R	R
80F_TC2[b]	0.082	R	R	S	S	R	R	R	R
72C_TP6[c]	0.060	S	S	S	S	R	S	S	R
72C_TP11[c]	0.077	S	S	S	S	S	S	S	R
45D_TC4[d]	0.083	R	R	S	S	R	R	R	R
72C_TC1[d]	0.089	R	R	S	S	R	R	R	R

Key: Amp, Ampicillin; Cm, chloramphenicol; Gm, gentamicin, Km, kanamycin; Sm, streptomycin; Sul, Sulphonamides; Trim, Trimethoprim; TP, TOPO clones; TF, transformants; TC, transconjugants. [a]TOPO clones from streptomycin resistance gene *strA* unusual large 1.2 kb PCR amplicon. [b]TOPO clone from 5′and 3′ conserved segment primers (5′CS and 3′CS) PCR amplicons. [c]Transconjugants for streptomycin resistance transfer. [d]Transconjugants for chloramphenicol resistance transfer.

Appendix 19: PCR assays amplification conditions.

1. 5'CS – 3'CS:
   - 5 min 94 °C
   - 1 min 94 °C
   - 1 min 56 °C
   - 3 min 72 °C
   - } 30 cycles
   - 7 min 72 °C

2. *aadA1*:
   - 3 min 94 °C
   - 1 min 94 °C
   - 1 min 56 °C
   - 30 sec 72 °C
   - } 35 cycles
   - 7 min 72 °C

3. *aph(3')-Ia*:
   - 1 min 94 °C
   - 1 min 94 °C
   - 2 min 60 °C
   - 3 min 72 °C
   - } 34 cycles
   - 7 min 72 °C

4. *ant(2)-Ia* :
   - 3 min 94 °C
   - 45 sec 94 °C
   - 45 sec 56 °C
   - 1 min 72 °C
   - } 35 cycles
   - 7 min 72 °C

5. *catA1*:
   - 2 min 94 °C
   - 1 min 55 °C
   - 3 min 72°C
   - 30 sec 94 °C
   - 30 sec 50 °C
   - 30 sec 72 °C
   - } 34 cycles
   - 10 min 72 °C

6. *catA3*:
   - 7 min 94 °C
   - 1 min 94 °C
   - 2 min 56 °C
   - 3 min 72 °C
   - } 34 cycles
   - 7 min 72 °C

7. *strA*, *sul1* and *sul2*:
   - 2 min 94 °C
   - 1 min 94 °C
   - 1 min 64 °C
   - 1 min 72 °C
   - } 35 cycles
   - 7 min 72 °C

8. *tet (A)*:
   - 1 min 72 °C
   - 1 min 94 °C
   - 2 min 45 °C
   - 3 min 72 °C
   - } 34 cycles
   - 7 min 72 °C

9. bla_{TEM}:
   - 2 min 94 °C
   - 1 min 58 °C
   - 3 min 72 °C
   - } 2 cycles
   - 30 sec 94 °C
   - 58 min 30 °C
   - 30 sec 72 °C
   - } 34 cycles
   - 10 min 72 °C

10. bla_{PSE}:
    - 2 min 94 °C
    - 1 min 94 °C
    - 1 min 58 °C
    - 1 min 72 °C
    - } 34 cycles
    - 7 min 72 °C

11. *tet (B, tet(C)* and *tet (H)*:
    - 7 min 72 °C
    - 1 min 94 °C
    - 2 min 50 °C
    - 3 min 72 °C
    - } 34 cycles
    - 7 min 72 °C

12. *cmlA*:
    - 2 min 94 °C
    - 1 min 94 °C
    - 1 min 40 °C
    - 2 min 72 °C
    - } 30 cycles
    - 7 min 72 °C

Appendix 20: Nucleotide sequence for the physically linked streptomycin gene *strA* and suphonamides resistance gene *sul2*.

1 ACAGTTTCTCCGATGGAGGCCGGTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGCGTA
61 AGCTGATGGCCGAGGGGGCAGATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGACG
121 CCGCGCCTGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGGACGCGCTCA
181 AGGCAGATGGCATTCCCGTCTCGCTCGACAGTTATCAACCCGCGACGCAAGCCTATGCCT
241 TGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGGTTTTCCAGACGCTGCGTTCTATC
301 CGCAATTGGCGATATCATCTGCCAAACTCGTCGTTATGCATTCGGTGCAAGACGGGCAGG
361 CAGATCGGCGCGAGGCACCCGCTGGCGACATCATGGATCACATTGCGGCGTTCTTTGACG
421 CGCGCATCGCGGCGCTGACGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATCCCG
481 GCATGGGGTTTTTTCTGGGGGCTGCTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCG
541 ATGAATTGCGGCTGCGCTTCGATTTGCCGGTGCTTCTGTCTGTTTCGCGCAAATCCTTTC
601 TGCGCGCGCTCACAGGCCGTGGTCCGGGGGATGTCGGGGCCGCGACACTCGCTGCAGAGC
661 TTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACACGAGCCGCGCCCCTTGCGCG
721 ACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCAAGAATTCGTTAACTGCACATTC
781 GGGATATTTCTCTATATTCGCGCTTCATCAGAAAACTGAAGGAACCTCCATTGAATCGAA
841 CTAATATTTTTTTTGGTAAATCTCATTCTGACTGGTTGCCTGTCAGAGGCGGAGAATCTG
901 GTGATTTTGTTTTTCGACGTGGTGACGGGCATGCCTTCGCGAAAATCGCACCTGCTTCCC
961 GCCGCGGAGAGCTCGCTGGAGAGCGTGACCGCCTCATTTGGCTCAAAGGTCGAGGTGTGG
1021 CTTGCCCCGAGGTCATCAACTGGCAGGAGGAACAGGAGGGTGCATGCTTGGTGATAACGG
1081 CAATTCCGGGAGTACCGGCGGCTGATCTGTCTGGAGCGGATTTGCTCAAAGCGTGGCCGT
1141 CAATGGGGCAGCAACTTGGCGCTGTTCACAGCCTATCGGTTGATCAATGTCCGTTTGAGC
1201 GCAGGCTGTCGCGAATGTTCGGACGCGCCGTTGATGTGGTGTCCCGCAATGCCGTCAATC
1261 CCGACTTCTTACCGGACGAGGACAAGAGTACGCCGCAGCTCGATCTTTTGGCTCGTGTCG
1321 AACGAGAGCTACCGGTGCGGCTCGACCAAGAGCGCACCGATATGGTTGTTTGCCATGGTG
1381 ATCCCTGCATGCCGAACTTCATGGTGGACCCTAAAACTCTTCAATGCACGGGTCTGATCG
1441 ACCTTGGGCGGCTCGGAACAGCAGATCGCTATGCCGATTTGGCACTCATGATTGCTAACG
1501 CCGAAGACAACTGG

Key: Base pairs 1- 770 sulphonamide resistance gene *sul2*; 771 – 830 spacer; 831 – 1514 streptomycin resistance gene *strA*.

Appendix 21: Nucleotide sequence for the streptomycin resistance gene cassette *aadA1* and trimethoprim resistance gene cassette *dfrA1*.

1 GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA
61 CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCTCTGAGGAAGAATTGTG
121 AAACTATCACTAATGGTAGCTATATCGAAGAATGGAGTTATCGGGAATGGCCCTGATATT
181 CCATGGAGTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTATTACCTATAACCAATGGCTG
241 TTGGTTGGACGCAAGACTTTTGAATCAATGGGAGCATTACCCAACCGAAAGTATGCGGTC
301 GTAACACGTTCAAGTTTTACATCTGACAATGAGAACGTAGTGATCTTTCCATCAATTAAA
361 GATGCTTTAACCAACCTAAAGAAAATAACGGATCATGTCATTGTTTCAGGTGGTGGGGAG
421 ATATACAAAAGCCTGATCGATCAAGTAGATACACTACATATATCTACAATAGACATCGAG
481 CCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAA
541 GACTTCGCCTCTAACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCA
601 GCAACGGATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATC
661 CGCTGTGCCAGGCGTTAAACATCATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAAC
721 TATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATT
781 TGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTA
841 CGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAA
901 CTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGC
961 ACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGC
1021 AGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTAT
1081 CTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACT
1141 CTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATG
1201 GAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCAT
1261 TTGGTACAGCGCAGTAACCGGCAGAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAAT
1321 GGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGG
1381 ACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTTCACTACGT
1441 GAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAACAATTCGTTCAAGCCGACG
1501 CCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCACTAAGCACATAATTGCTCACAG
1561 CCAAACTATCAGGTCAAGTCTGCTT

Key: Base pairs 1 – 97 part of the 5′CS; 98- 674 *dfrA1* gene cassette; 675 – 1530 *aadA1* gene cassette; 1531 – 1586 is part of the 3′CS.

Appendix 22: Nucleotide sequence for amplicon carrying the trimethoprin resistance gene cassette *dfrA7*.

1 GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA
61 CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTACGGGGGTTGAATT
121 GAAAATTTCATTGATTTCTGCAACGTCAGAAAATGGCGTAATCGGTAATGGCCCTGATAT
181 TCCATGGAGTGCCAAAGGTGAGCAGCTCCTGTTTAAAGCTATCACATATAACCAATGGCT
241 CCTTGTTGGAAGGAAAACATTTGACTCTATGGGTGTTCTTCCAAATCGAAAATATGCAGT
301 AGTGTCGAGGAAAGGAATTTCAAGCTCAAATGAAAATGTATTAGTCTTTCCTTCAATAGA
361 AATCGCTTTGCAAGAACTATCGAAAATTACAGATCATTTATATGTCTCTGGTGGCGGTCA
421 AATCTACAATAGTCTTATTGAAAAAGCAGATATAATTCATTTGTCTACTGTTCACGTTGA
481 GGTTGAAGGTGATATCAATTTTCCTAAAATTCCAGAGAATTTCAATTTGGTTTTTGAGCA
541 GTTTTTTTTGTCTAATATAAATTACACATATCAGATTTGGAAAAAAGGCTAACAAGTCGT
601 TCCAGCACCAGTCGCTGCGCTCCTTGGACAGTTTTTAAGTCGCGGTTTTATGGTTTTGCT
661 GCGCAAAAGTATTCCATAAAACCACAACTTAAAAACTGCCGCTGAACTCGGCGTTAGATG
721 CACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

Key: Base pairs 1 – 97 are part of the 5′CS, 98- 713 is *dfrA7* gene cassette, 714 – 769 part of the 3′CS.

Appendix 23: Nucleotide sequence for the streptomycin resistance gene *strA*, 1214 bp PCR amplicon.

1 TGACTGGTTGCCTGTCAGAGGCAGAGAATCTGGTGATTTTGTTTTTCGACGTGGTGACGG
61 GCATGCCTTCGCGAAAATCGCACCTGCTTCCCGCCGCGGTGAGCTCGCTGGAGAGCGTGA
121 CCGCCTCATTTGGCTCAAAGGTCGAGGTGTGGCTTGCCCCGAGGTCATCAACTGGCAGGA
181 GGAACAGGAGGGTGCATGCTTGGTGATAACGGCAATTCCGGGAGTACCGGCGGCTGATCT
241 GTCTGGAGCGGATTTGCTCAAAGCGTGGCCGTCAATGGGGCAGCAACTTGGCGCTGTTCA
301 CAGCCTATCGGTTGATCAATGTCCGTTTGAGCGCAGGCTGTCGCGAATGTTCGGACGCGC
361 CGTTGATGTGGTGTCCCGCAATGCCGTCAATCCCGACTTCTTACCGGACGAGGACAAGAG
421 TACGCCGCAGCTCGATCTTTTGGCTCGTGTCGAACGAGAGCTACCGGTGCGGCTCGACCA
481 AGAGCGCACCGTTAACCCAGGATGAGAACCTTGAAAGTATCATTGATAGCTGCGAAAGC
541 GAAAAACGGCGTGATTGGTTGCGGTCCAGACATACCCTGGTCCGCGAAAGGGGAGCAGCT
601 ACTTTTTAAAGCATTGACCTACAATCAGTGGCTTCTGGTGGGTCGCAAGACGTTTGAATCT
661 ATGGGCGCACTCCCCAATAGGAAATACGCGGTCGTTACCCGCTCAGGTTGGACATCAAAT
721 GATGACAATGTAGTTGTATTTCAGTCAATCGAAGAGGCCATGGACAGGCTAGCTGAATTC
781 ACCGGTCACGTTATAGTGTCTGGTGGCGGAGAAATTTACCGAGAAACATTACCCATGGCC
841 TCTACGCTCCACTTATCGACGATCGACATCGAGCCAGAGGGGGATGTTTTCTTCCCGAGT
901 ATTCCAAATACCTTCGAAGTTGTTTTTGAGCAACACTTTACTTCAAACATTAACTATTGC
961 TATCAAATTTGGAAAAAGGGTTAACAAAGCTATGCAATCGACGGCAAAAAGCTTCGTTCG
1021 CTTCGCGCACTACGCCTTTTTCCGCGATTGATAGCGACGATATGGTTGTTTGCCATGGTG
1081 ATCCCTGCATGCCGAACTTCATGGTGGACCCTAAAACTCTTCAATGCACGGGTCTGATCG
1141 ACCTTGGGCGGCTCGGAACAGCAGATCGCTATGCCGATTTGGCACTCATGATTGCTAACG
1201 CCGAAGACAACTGG

Key: Base pairs 1- 491 is part of the *strA* gene (Δ*strA)*; 492 – 1059 is *dfrA14* gene cassette; 1060 – 1214 is Δ*strA*.