UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Antimicrobial drug resistance of enteric bacteria from broilers fed antimicrobial growth enhancers and exposed poultry abattoir workers.

By

JAMES WABWIRE OGUTTU

Submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

Date submitted: November 2007

ACKNOWLEGEMENTS

I would like to express my sincere appreciation to the following:

- My supervisor, Prof. CM Veary and Co-supervisor, Dr. Jackie Picard, for their invaluable guidance, and time taken off their weekends to assist with the project
- My wife (Annet Wanyana Oguttu) and children (Jonathan, Abigail and Timothy) for the support during the difficult times whilst working on my project. They are thanked very much for the willingness to spend weekends without me while I carried out the laboratory work
- The supplier of Virginiamycin – Phibro Animal Health
- The University of Pretoria (Department of Paraclinical Sciences: Section Veterinary Public Health) is thanked for sponsoring the entire project
- The Department of Veterinary Tropical Diseases (University of Pretoria) is thanked for making available the Laboratory where the work was carried out
- Prof. Peter Thompson, for his assistance with the statistical analysis of the results
- All the laboratory personnel in the Department of Veterinary Tropical diseases especially Ms. Janita Greyling for the assistance, knowledge, input and support received.
- The company and personnel at all levels of employment for granting permission for the research to be conducted in the abattoir and for the co-operation and assistance given.
- Michelle Kirsten, Departmental Administrator, Department of Paraclinical Sciences, University of Pretoria, is thanked for helping with the editing and formatting of my dissertation.
- All my colleagues at Department of Agriculture and Animal Health, UNISA, are thanked for their support and willingness to stand in for me when I had to be away.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS II

TABLE OF CONTENTS III

LIST OF FIGURES VII

LIST OF TABLES VIII

LIST OF ABBREVIATIONS X

SUMMARY XII

CHAPTER 1 1

INTRODUCTION 1

1.2 AIMS AND OBJECTIVES 3

1.3 THESIS STATEMENT 4

1.4 PROBLEM STATEMENTS 4

1.5 POSSIBLE BENEFITS FROM THIS STUDY 4

CHAPTER 2 6

LITERATURE REVIEW 6

2.1 TERMINOLOGY 6

2.2 CONSEQUENCES OF ANTIMICROBIAL DRUG RESISTANCE IN BACTERIA OF ANIMAL ORIGIN TO HUMAN HEALTH 7

2.3 DEVELOPMENT OF ANTIMICROBIAL DRUG RESISTANCE 12

2.3.1 Determinants of resistance in bacteria 12
2.3.1.1 Intrinsic factors 12
2.3.1.2 Gene transfer within and between bacterial species 13
2.3.1.3 Mutations 14
2.3.2 Drivers of antimicrobial drug resistance in food animals 14
2.3.2.1 Selection pressure 14
2.3.2.2 Method of antimicrobial drug administration 16

2.3.3 Drivers of antimicrobial drug resistance in humans 17
2.3.3.1 Acquisition of resistance by humans from animals 17
2.3.3.2 Antimicrobial selection pressure 19
2.3.3.3 Socio-economic factors 20
2.3.3.4 The role of antimicrobial drug residues in food of animal origin 21

2. 4 DISPERSAL OF ANTIMICROBIAL DRUG RESISTANT ORGANISMS 22

2.4.1 Live animals 22
2.4.2 Food of animal origin 22
2.4.3 Fruit or vegetable from a contaminated environment 23
2.4.4 Contaminated water 23
2.4.5 Human beings 24

2.5 INCLUSION OF ANTIMICROBIALS IN POULTRY FEED 25

2.6 ADDRESSING ANTIMICROBIAL DRUG RESISTANCE AND THE ASSOCIATED PUBLIC HEALTH RISKS 28

2.6.1 Ban or restriction of the use of AMGP 28
2.6.2 Institution of surveillance programmes and research 28
2.6.3 National legal reforms 29
2.6.4 Establishment of guidelines for prudent use of antimicrobials 31
2.6.5 Other approaches 32

2.7 WITHDRAWAL OF AMGP: THE NORDIC AND EU EXPERIENCE 33

2.8 OVERVIEW OF SELECTED ENTERIC BACTERIA 35

2.8.1 *Salmonella* 35
2.8.2 *Escherichia coli* 37
2.8.3 Enterococci 39
2.8.4 *Clostridium perfringens* 41

CHAPTER 3 44

PILOT PROJECT 44

3.1 OBJECTIVES 44

3.2 MATERIALS AND METHODS 44

3.2.1 Specimen collection 44
3.2.2 Reference strains 45
3.2.3 Isolation and identification 45
3.2.3.1 *Salmonella* (serotypes belonging to Group 1) 45
3.2.3.2 *Escherichia coli* 46
3.2.3.3 Enterococci 47
3.2.3.5 *Clostridium perfringens* 48
3.2.4 Storage of isolates 49
3.2.5 Antimicrobial susceptibility testing 49
3.2.6 Results and discussion 49

3.2.7 Conclusion and recommendations ... 52

CHAPTER 4 ... 55

PROJECT: MATERIALS AND METHODS ... 55

4.1 SAMPLING ... 55

4.1.1 Chicken specimens ... 55
4.1.2 Human specimens ... 55

4.2 ISOLATES AND IDENTIFICATION ... 57

4.3 ANTIMICROBIAL USAGE PATTERNS ... 57

4.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING ... 58

4.4.1 Antimicrobial agents ... 58
4.4.1.1 Selection of antimicrobials for testing ... 58
4.4.1.2 Preparation of stock solutions ... 60
4.4.1.3 Preparation of the working solution ... 62
4.4.2 Preparation of bacterial inoculum ... 63
4.4.3 Preparation of the 96 micro well plates ... 63
4.4.4 Incubation ... 64
4.4.5 Determination of MIC's and reading of results ... 64
4.4.6 Controls ... 67
4.4.7 Data analysis ... 68

CHAPTER 5 ... 69

5.1 ISOLATES ... 69

5.2 MINIMUM INHIBITORY CONCENTRATION (MIC) TEST RESULTS ... 72

5.3 ANTIMICROBIAL USAGE PATTERNS ... 100

CHAPTER 6 ... 102

CONCLUSIONS, RECOMMENDATIONS AND QUESTIONS ARISING ... 102

6.1 CONCLUSIONS AND RECOMMENDATIONS ... 102

6.2 QUESTIONS ARISING ... 106

ANNEXURE I: Pilot study disc diffusion results ... 108

ANNEXURE I: Cont. ... 109

ANNEXURE II: Volunteer information leaflet and informed consent....................110
ANNEXURE III: Questionnaire 114
ANNEXURE IV: Panel for determining MIC for research project...........................128

CHAPTER 7 129

REFERENCES 129

LIST OF FIGURES

FIGURE	**4.1**	A viewer that displays the underside of the wells………………….	65
FIGURE	**4.2**	Criteria for interpreting of results………………………………..	66
FIGURE	**5.1**	Percentage resistance of *E. coli* from broilers, abattoir workers and human controls…………………………………………………..	84
FIGURE	**5.2**	Percentage resistance of *E. faecalis* from broilers, abattoir workers and human controls……………………………………………...	90
FIGURE	**5.3**	Percentage resistance of *E. faecium* from broilers, abattoir workers and human controls……………………………………………...	91
FIGURE	**5.4**	Scatter plot for % resistant isolates from packers and broilers for each antimicrobial drug ………………………………………...	99

LIST OF TABLES

TABLE 3.1	Criteria for differentiating *E. faecium* and *E. faecalis*……………..	47
TABLE 3.2	Antimicrobial susceptibility of 10 of 48 *E. coli* isolates…………..	50
TABLE 3.3	Antimicrobial susceptibility of 15 of 35 enterococci isolates……..	51
TABLE 4.1	Antimicrobials included in the MIC panel………………………...	59
TABLE 4.2	Solvents and diluents for preparation of stock solutions of antimicrobial agents………………………………………………..	61
TABLE 4.3	Scheme for preparing dilutions of the various antimicrobial agents……………………………………………………….…...	62
TABLE 5.1	The number of isolates obtained from the different populations…..	69
TABLE 5.2	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. coli* from broilers/farm……...…………………………..	73
TABLE 5.3	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecalis* from broilers/farm………………….…….....…	75
TABLE 5.4	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecium* from broilers/farm………………..…………	76
TABLE 5.5	Minimum inhibitory concentrations of bacitracin against *E. faecalis* isolates………………………………………………….	77
TABLE 5.6	Minimum inhibitory concentrations of bacitracin against *E. faecium* isolates………………………………………………….	77
TABLE 5.7	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. coli* from abattoir workers……..……………………..…	81

TABLE	**5.8**	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. coli* from control group…………………………………	82
TABLE	**5.9**	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecium* from abattoir workers………………………….	86
TABLE	**5.10**	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecalis* from abattoir workers………………………….	87
TABLE	**5.11**	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecalis* from control group……………………………..	88
TABLE	**5.12**	Minimum inhibitory concentrations (MIC's) of antimicrobial agents: *E. faecium* from abattoir workers……………..…………...	89
TABLE	**5.13**	Median MIC of *E. coli* isolates from eviscerators and packers……	94
TABLE	**5.14**	Median MIC of enterococci isolates from eviscerators and packers………………………………………………………………..	95
TABLE	**5.15**	Median MIC of *E. faecalis* isolates from eviscerators and packers………………………………………………………………..	96
TABLE	**5.16**	Median MIC of *E. faecalis* isolates from eviscerators and packers………………………………………………………………..	97
TABLE	**5.17**	Median MIC of *E. coli* isolates from eviscerators and packers….....	98

LIST OF ABBREVIATIONS

AFA:	Antimicrobial feed additive
AMGP:	Antimicrobial growth enhancers
AMR:	Antimicrobial drug resistance
APE:	Antimicrobial performance enhancers
APUA:	Alliance for the Prudent Use of Antimicrobials
ASG:	Antimicrobial Study Group
AST:	Antimicrobial susceptibility test
AUC:	Area under the Curve
CDC:	Centre for Disease Control
CFU:	Colony forming unit
CLSI:	Clinical Laboratories Standards Institute
	[Previously known as National Committee of Clinical Laboratories (NCCL)]
CR:	Colonisation resistance
ESBL:	Extended-spectrum B-Lactamase
EU:	European Union
FDA:	Food and Drug Administration
GIT:	Gastro-intestinal tract
GRE:	Glycopeptide resistant enterococci
HACCP:	Hazard Analysis and Critical Control Point
HIV:	Human Immunodeficiency Virus
HLAR:	High level aminoglycoside resistance
HUS:	Haemorrhagic uraemic syndrome
ISO:	International Organisation for Standardisation
JVARMP:	Japanese Veterinary Antimicrobial Drug Resistance Monitoring Programme
KAA:	Kanamycin aesculin azide agar
KB:	Kirby-Bauer disc diffusion method
LAB:	Lactic acid bacteria
MAC:	Macconkey agar
MBC:	Minimum bactericidal concentration

MIC: Minimum inhibitory concentration

MR: Multi-drug resistant

MRSA: Methicillin resistant *Staphylococcus aureus*

NARMS: National Antimicrobial Resistance Monitoring and Surveillance

NASF: National Antimicrobial Surveillance Forum

NCCL: National Committee for Clinical Laboratory Standards (*vide supra* CLSI)

NE: Necrotic enteritis

OIE: Office Internationale Des Epizooties (World Organisation for Animal Health)

SPS: Sanitary and Phyto - Sanitary Measures

STEC: Shiga-toxin producing *Escherichia coli*

STX: Shiga- toxin

TB: Tuberculosis

UK: United Kingdom

USA: United States of America

UTI: Urinary tract infection

VPH: Veterinary Public Health

VRE: Vancomycin resistant enterococci

WHO: World Health Organisation

XLD: Xylose lysine deoxycholate

SUMMARY

Antimicrobial drug resistance of enteric bacteria from broilers fed antimicrobial growth enhancers and exposed poultry abattoir workers.

By

JAMES WABWIRE OGUTTU

Supervisor:	**Prof CM Veary**
Co-supervisor:	**Dr JA Picard**
Department:	**Paraclinical Sciences**
Degree:	**MSc (Veterinary Science)**

The usage of antimicrobials either as performance enhancers or for prophylactic and therapeutic purposes in food animals, such as chickens, increases the prevalence of antimicrobial drug resistance among enteric bacteria of these animals. This may be transferred to people working with such animals, e.g. abattoir workers, or the products arising from these animals. In this study antimicrobial drug resistance was investigated for selected enteric bacteria from broilers raised on feed supplemented with antimicrobial growth enhancers, and the people who carry out evisceration, washing and packing of intestines in a high throughput poultry abattoir in Gauteng, South Africa.

Poultry farms (n=6) were purposively selected on the basis of allowing for sampling of farms from more than one grow out cycle. Broiler carcases (n=100) were randomly selected per farm five minutes after slaughter and sampled by incising caecae from the rest of the gastro-intestinal tract (GIT). The ends of each caecae were tied off to prevent contamination and to enhance the

culturing of anaerobic bacteria. In the laboratory, caecal contents were selectively cultured for *Clostridium perfringens*, *Escherichia coli*, *Enterococcus faecium*, *E. faecalis*, and vancomycin-resistant enterococci (VRE). *Salmonella enterica* was isolated using pre-enrichment followed by

selective culture. The minimum inhibitory concentration (MIC) micro broth dilution test as prescribed by the Clinical and Laboratory Standards Institute USA (CLSI), previously known as National Committee of Clinical Laboratories (NCCL), was used to determine the susceptibility of the isolates to the following antimicrobials: vancomycin, virginiamycin, doxycycline, trimethoprim, sulphamethoxazole, ampicillin, bacitracin, enrofloxacin, erythromycin, fosfomycin, ceftriaxone and nalidixic acid. The same was done on the faeces of 29 abattoir workers exposed to potentially resistant micro-organisms from broilers and 28 persons used as controls, who had not been equally exposed to potentially resistant micro-organisms from broilers. Both of the human populations had not been treated with antimicrobials within three months prior to sampling. Statistical analysis was done by Fisher's exact test.

No salmonellae and VRE on VRE selective agar (Oxoid UK) were cultured. Two *Clostridium perfringens*, 168 *E. coli*, 20 *E. faecalis* and 96 *E. faecium* isolates from the broiler caecae were cultured. Fifty four (28 and 26) *E. coli*, 24 (21 and 3) *E. faecalis* and 12 (2 and 10) *E. faecium* from humans were cultured. The figures in brackets represent the abattoir workers and human controls respectively. The majority of *E. coli* isolates from broilers had MIC's above the cut off point for the antimicrobials tested. Low resistance was observed among broiler enterococci isolates to vancomycin, virginiamycin, trimethoprim and ampicillin. A comparison of the median MIC's of isolates from abattoir workers (packers) and the control group revealed significant differences in the median MIC's for the following antimicrobials; *E. faecalis*: enrofloxacin (p=0.019). *E. faecium*, trimethoprim (p=0.01), enrofloxacin (p=0.029) and erythromycin (p = 0.03). *E. coli*: trimethoprim (p= 0.012) and ampicillin (p= 0.036). Use of antimicrobials as feed additives causes resistance among enteric bacteria from broilers. Significant differences between median MIC's of abattoir workers (packers) and the control group were observed for therapeutics and not growth enhancers. There was a tendency for isolates from abattoir workers to have a higher median MIC and a higher number of resistant isolates as compared to the control group. In spite of the fact that there was a high level of resistance in the enteric commensal bacteria of broiler caecae, an association could not be shown with that of the human enteric bacteria. It could not be concluded that a significant AMR transfer to poultry abattoir workers existed. This notwithstanding, both the control and

experimental group, carried levels of resistance among their enteric bacteria that could be described as being high.

CHAPTER 1

INTRODUCTION

Low concentrations of antimicrobials (at sub-therapeutic levels) fed to food animals in feed or water have a disease preventing effect and lead to reduced mortality and morbidity, enhanced feed conversion efficiency and improved growth rates (3, 5, 18, 24, 31, 37, 52, 61, 91, 82). However, as has happened in some countries, the voices calling for the usage of antimicrobials as antimicrobial growth promoters (AMGP) to be stopped is growing (3, 6, 54, 84, 85). The reasons cited for this being that:

- there is a possibility of resistant bacterial strains from food producing animals infecting humans (3, 9, 13, 24, 37, 52, 55, 69, 88, 89, 92),
- there is potential for drug resistant bacteria in animals transferring genetic elements, which confer resistance to bacteria that are pathogenic in humans (3, 8, 24, 37, 55, 68, 84, 85, 88),
- when antimicrobials are used in one individual, they affect not only the micro organisms in that individual being treated, but also other people or animals in the environment around or in the neighbourhood of that individual (1, 33), a phenomenon that has led to antimicrobials being designated as “societal drugs” (1). This explains why livestock farm workers and members of their families usually carry a higher incidence of antimicrobial resistant bacteria than the general population (59, 60, 84). For example, reports from the Netherlands suggest that farmers who work with turkeys fed antimicrobials as AMGP are likely to carry a higher level of resistant *Escherichia coli* as compared to their compatriots who worked with pigs that are not fed AMGP (84),
- after animal handlers have picked up resistant bacteria, they could pass them on to the human population at large (60),

- there is potential for antimicrobial usage in animals to induce cross-resistance to antimicrobials used in human medicine. For example, the use of avoparcin as a growth enhancer in poultry has been shown to lead to the development of resistance to vancomycin, which is used to treat enterococcal infections in humans (3, 6, 23, 88),
- with increase in the proportion of antimicrobial resistant microbes like enterococci and *Salmonella* species that are zoonotic, food associated infections are likely to become relatively difficult to treat (37). Though for minor and self-limiting bacterial infections the consequences for this are small, for serious infections, the consequences of antimicrobial failure can be fatal or life threatening, with major long-term disability and significantly increased costs of care (60, 69, 92).
- In Europe despite legislation targeted at controlling the overall usage of antimicrobials in food-producing animals, there have been significant increases in the occurrence of resistance in non-typhoidal *Salmonella* spp. especially to key therapeutic antimicrobials such as fluoroquinolones and extended-spectrum β–lactamases (6, 79).

However, South Africa is unique among countries that have large animal populations under intensive systems by still allowing the use of AMGP. At the same time, there is little information available on the subject of antimicrobial drug resistance in animals in South Africa. Work that has been done in the past has been from carcasses, based on antimicrobial susceptibility of bacterial pathogen isolates, and not faecal samples. In these studies (37, 53), it was found that 98 - 100% isolated *Salmonella* were resistant to tetracyclines (used frequently as a growth promoter and for treatment of *Salmonella* infections). Of the staphylococci isolates tested resistance to both tetracycline and oxacillin was 39-70%, while resistance among the enterobacteriaceae isolates to tetracycline and streptomycin was 34-60%. It is noteworthy that a large proportion of the bacterial flora on fresh poultry in these studies exhibited multiple antimicrobial drug resistance. Although the veterinary profession in South Africa is aware of the emergence of antimicrobial resistance and the need to have it investigated, surveillance programmes for antimicrobial resistance are just in their infancy (60).

In South Africa "mala" (intestines) from chickens fed AMGP and possibly carrying micro-organisms that are resistant to antimicrobials, are processed (cleaned and packed) by abattoir workers prior to being sold to consumers. This implies that abattoir workers are exposed to potential resistant micro-organisms during their work, and could hence be at risk of developing resistance among their enteric flora (66). Therefore, given that no work to date has been done to investigate the situation among poultry abattoir workers in South Africa, this project addresses a problem about which little is known in this country and yet valuable from a veterinary public health point of view.

1.2 AIMS AND OBJECTIVES

The primary objective of this study was to investigate whether abattoir workers who eviscerate, wash and pack intestines (with potentially resistant bacteria) from chickens fed feed medicated with antimicrobials, carry a high prevalence of resistant enteric bacteria as compared to people who do not work in poultry abattoirs. This would be achieved by conducting a comparative study of the level of antimicrobial drug resistance of isolates from the abattoir workers whose work includes "mala" washing and packing and from people not associated with the abattoir.

This study also sought to elucidate the following aspects of antimicrobial drug resistance among caecal microflora of chickens:

1. Occurrence of antimicrobial resistance in selected zoonotic (*Salmonella*), animal pathogen (*Clostridium perfringens*) and indicator bacteria (*Escherichia coli* and *Enterococcus faecium* and *E. faecalis*) isolated from broilers on a group of farms in the Gauteng area where antimicrobials are included in the feed given to the poultry;
2. Level of vancomycin resistance among enterococci isolated from poultry, given that avoparcin was in the past extensively used in the poultry industry in South Africa;
3. Level of vancomycin resistance in enterococci isolated from exposed poultry abattoir workers; and
4. Antimicrobial usage patterns on the broiler farms where the broilers referred to in paragraph 1 above are reared.

1.3 THESIS STATEMENT

Use of antimicrobial feed additives in food animals e.g. broilers, results in a high prevalence of resistance among their enteric bacteria, and this resistance is reflected among abattoir workers as a result of resistance transfer.

1.4 PROBLEM STATEMENTS

1. Feeding poultry on feed containing antimicrobial feed additives leads to high levels of resistance among enteric organisms from broilers, which is mirrored among isolates from exposed abattoir workers due to transfer of resistance from broilers to abattoir workers.
2. Though the use of avoparcin as an antimicrobial feed additive in South Africa ceased six/seven years ago after the European manufacturers stopped its production, resistance to vancomycin against which avoparcin causes cross resistance, can still be detected among broiler isolates.
3. When avoparcin was used in poultry flocks in South Africa, abattoir workers and people not associated with poultry picked up resistance, which can still be detected to date.

1.5 POSSIBLE BENEFITS FROM THIS STUDY

a) Assessment of the prevalence of resistance among isolates from broilers, abattoir workers and humans not associated with the poultry industry in South Africa.

b) Assessment of the level of resistance to the glycopeptide vancomycin against which avoparcin induces cross resistance.

c) Assessment of the risk of acquiring antimicrobial drug resistance as a result of handling intestines from broilers fed AMGP. This by studying the patterns of resistance in the two populations (control group and abattoir workers), which could in turn form a basis for possible intervention.

d) Previous work done in South Africa (20) suggests that both *S.* Typhimurium and *S.* Enteritidis are frequently isolated from chickens. This study will assess as to whether or not this is still the situation with respect to intestinal carriage of non-typhoidal *Salmonella* in the poultry flocks sampled.

The chapter that follows (literature review) consists of a section that describes some of the terms used in the literature review. This is followed by a discussion of the consequences of antimicrobial drug resistance in bacteria of animal origin on human health, determinants of resistance, how resistance is transferred, why antimicrobials are included in poultry feed, how antimicrobial drug resistance is being contained internationally and an overview of the importance and trends of antimicrobial drug resistance among selected enteric bacterial species.

CHAPTER 2

LITERATURE REVIEW

2.1 TERMINOLOGY

Veterinary Public Health is defined by the World Health Organisation (WHO) as the sum of all contributions to the physical, mental and social well-being of humans through an understanding and application of veterinary science, or as a component of public health activities devoted to the application of professional veterinary skills, knowledge and resources to the protection and improvement of human health.

A bacterial isolate is classified as **resistant** to a specific antimicrobial when it is not inhibited by the minimum inhibitory concentration (MIC) of that antimicrobial drug that normally inhibits the growth of the susceptible members of that species (60). A resistant bacterium is also described as one that does not respond to one or more of the drugs commonly used to treat infections caused by the group (92).

Break point, (based on clinical studies) is the concentration of the antimicrobial, below which an isolate is classified as susceptible and above which as resistant (3).

Antimicrobial resistance could also manifest as **tolerance**, which is considered present when the minimum bactericidal concentration (MBC) is significantly greater (generally 32 – fold) than the MIC. The MBC is defined as the concentration of an antimicrobial that kills 99% of the bacteria tested (60). This type of resistance is attributed to lack of autolytic enzymes particularly in streptococci and also seen when β-lactams bind to transpeptidase that result in growth inhibition, but not bacterial death (60).

Cross-resistance is a phenomenon whereby bacteria that develop resistance to an antimicrobial are also resistant to other antimicrobials to which they may never have been exposed. This is attributed to the fact that a common mechanism for achieving resistance exists (59) within the class, but can extend beyond the class.

Guidelines for **prudent use of antibacterials in animals** in general, are recommendations which must be always be followed by veterinarians when administering antimicrobials to animals in order to reduce the use of antimicrobials to the lowest indispensable level. They constitute the rules of veterinary science which are to be complied with during any use of antimicrobials in animals and which must be observed each and every time an animal is treated properly in accordance with the drug legislation (82).

The parameter describing the relationship between the antimicrobial concentration and the length of time that the concentration remains in serum is called the **Area under the curve (AUC)**. This parameter is important to the life and death of bacteria in vivo (31).

AUC: MIC ratio in full stands for Area under the Curve to Minimum Inhibitory Concentration ratio. This is a pharmacodynamic parameter that represents the degree to which the serum concentration and time exposure of the antimicrobial exceed the minimum needed to interfere with the bacterial life cycle. The higher the AUC: MIC ratio, the greater the probability of maximum eradication of the organism, and the less likelihood of development of resistance in the targeted bacteria (31).

2.2 CONSEQUENCES OF ANTIMICROBIAL DRUG RESISTANCE IN BACTERIA OF ANIMAL ORIGIN TO HUMAN HEALTH

The increase in resistance among isolates from food animals that has been observed in a number of countries (6, 22, 73, 84, 89) adds a new significance to food associated disease, making antimicrobial drug resistance a public health dilemma (5, 17, 28, 30, 35, 72, 89). Due to the increase in resistance rates, it is recommended that physicians are aware that patients taking antimicrobial agents for any reason are at risk of acquiring antimicrobial-resistant food borne infections (6). While for minor and self limiting bacterial infections, the consequences

for the host of antimicrobial failure are small, for serious infections, the consequences can be fatal or life threatening, with major long-term disability and significantly increased costs of care (6, 20, 60, 68, 73, 92). An increase in the prevalence of resistance in some significant pathogenic bacteria like S*almonella* spp. may lead to a large increase in hospitalisation rate, mortality and morbidity, since drug resistant micro organisms tend to exhibit predilection to cause serious disease (22, 42, 57, 73, 86). It is actually known that in *Salmonella* spp. the genetic determinants for *Salmonella* virulence and antimicrobial resistance can occur on the same plasmid (37). In the USA, studies show that bacteraemia caused by VRE is associated with markedly higher death rates than bacteraemia due to antimicrobial-sensitive strains of enterococci (24). Fifteen percent (15%) of human isolations of multi-drug (MR) *S.* Typhimurium DT 104 have been reported to be associated with cases of septicaemia (79). While in developing countries infections with organisms like S*almonella* spp. is associated with invasive illness, and often results in septicaemia associated with high mortality, it is not the case in developed countries. In the latter, outbreaks of food-borne infections are usually self-limiting and antimicrobial therapy is not normally indicated (79). Which means that burden of antimicrobial drug resistance among food associated diseases is likely to be higher in the developing countries as compared to the developed world.

When food borne pathogens develop resistance, more so multiple resistances, it leads to physicians having to alter their treatment as the infection will not respond to any commonly used antimicrobial substances (6, 20, 22, 54, 60, 79). Failure to notice resistance in time on part of the physician could mean loss of a life (60, 68). For example, in 1998, a 62 year old Danish woman died when the food poisoning she contracted from eating S*almonella*-infected pork failed to respond to the antimicrobial ciprofloxacin (92). Resistance among zoonotic organisms like *Salmonella* spp. and VRE limits the therapeutic options available to veterinarians and physicians in the treatment of diseases caused by such organisms (17, 28). This is particularly the case when humans acquire infections due to fluoroquinolone resistant and extended-spectrum β-lactamase (ESBL) producing multidrug-resistant *E. coli* (78, 86). The drugs that have to be replaced are in most cases the cheap and effective first-choice or "first-line" drugs (64, 93, 94). Physicians have to switch to "third-line" drugs which are frequently more expensive and in many countries prohibitive with the result that some diseases cannot be treated where resistance to first-line drugs is widespread (20, 93).

In sub-Saharan Africa and South-East Asia, antimicrobial-drug resistance is being increasingly recognised in pathogens that commonly cause infections in health-care settings, rendering available antimicrobial agents ineffective and further diminishing the list of already scarce effective agents (12, 20). For bacterial infections particularly in critically ill patients due to nosocomial infections, given the remarkable abilities of bacteria to adapt and overcome hostile mechanisms used by antimicrobials, physicians are faced with the prospect of a post antimicrobial era (17).

When microbes develop resistance, and fail to respond to treatment, the consequence is an increased number of infected people moving in the community. Subsequently the general population is exposed to an increased risk of contracting resistant strains of infection (93, 68). This is especially true if there is a co-infection with Human Immunodeficiency Virus (HIV). In such instances, the result is not only a rapid progression in the infected individual, but also a potential multiplier effect on the dissemination of the resistant pathogen to the rest of the population (28).

Following an increase in the frequency of antimicrobial resistant zoonotic pathogens such as *Campylobacter* spp., *Salmonella* spp. and VRE, the result is development of a reservoir of resistant organisms that can act as a source of infection in humans (17, 29, 43, 88). For example, outbreaks of *Acinetobacter* infections, *Pseudomonas aeruginosa*, and ESBL-producing *Klebsiella pneumoniae* have been reported following development of resistance among these organisms (17, 59). Von Baum and Marre (2005) *inter alia*, state that ESBL-positive enterobacteriaceae apart from being resistant to a wide variety of β-lactam antimicrobials including third generation cephalosporins and monolactams, also pose a major challenge to clinical microbiology laboratories in that they are difficult to detect by standard diagnostic procedures. Meaning that ESBLs and other resistant organisms may go undetected in routine susceptibility tests, depending on the test panel used (29, 67, 86). When microbes develop resistance, and fail to respond to treatment, the consequence is an increased number of infected people moving in the community. Subsequently the general population is exposed to an increased risk of contracting resistant strains of infection (68, 93). This is especially true if there is a co-infection with Human Immunodeficiency Virus (HIV). In such instances, the result is not only a rapid progression in the infected individual, but also a potential

multiplier effect on the dissemination of the resistant pathogen to the rest of the population (28).

At the time drugs like avoparcin, virginiamycin, and avilamycin (which belong to the same classes as the human drugs vancomycin, quinupristin-dalfopristin (Synercid), and evernimicin respectively) were approved as growth promoters, they were considered of little or no significance in human medicine. However, because of the emergence of multiple resistant bacteria causing infection in humans, and more so increasing resistance in Gram-positive pathogenic bacteria, antimicrobial drugs used as growth promoters have attracted renewed attention as potentially useful for human therapy (88). Actually some of these classes of antimicrobials have become important last resort drugs in the treatment of such infections (3). Therefore while antimicrobial drug categories currently used in human therapeutics are clearly known, it may not be clear what new antimicrobial drugs, or derivatives, may in the future be used in human therapeutics even if not used therapeutically today. For example, as pharmaceutical companies continue with their discovery efforts, active analogues of animal-use drugs have been developed as important classes of valued human therapeutics. However, because of years of chronic use as AMGP, resistant bacteria are already in the environment which thwart the efficacy of these new antimicrobials and transfer resistance traits, in some cases even before the new human therapeutics have been introduced (10, 88). The implication of this is that drugs like Synercid (a combination of two streptogramins) and ziracin (belonging to everninomicins class) approved to treat VRE may have been compromised by the use of related antimicrobials in animal feed (88, 90). The problem is further compounded by the fact that new classes of antimicrobial drugs are not available, which leaves development of new drugs by modifying old drugs that have been used in agriculture as AMGP for decades as the only hope (88).

In some instances, it is no longer possible to talk about empiric antimicrobial therapy. For example, in Germany where the empirical treatment for uncomplicated community acquired UTI (urinary tract infection) in non-pregnant women used to be trimethoprim-sulphamethoxazole (TMP-SMX) because it was considered superior to β-lactams, studies done in 1996 showed resistances of up to 18% to this combination. This prompted a recommendation that TMP-SMX be used as empiric treatment only in areas where the

resistance rates in uropathogenic *E. coli* is less than 10 or 20%. With development of resistance to fluoroquinolones, these drugs are no longer recommended for initial oral or intravenous monotherapy, but in their place, cefepime, ceftazidime, piperacillin/tazobactum or carbapenem have been suggested as suitable agents for empiric monotherapy in cases of unexplained fever in neutropenic patients with cancer (86). Von Gottberg (87) is of the view that due to increasing resistance being observed in South Africa, as has already happened in the USA, it won't be long before it becomes necessary to change what is currently considered empirical therapy for meningitis from consisting of ceftriaxone or cefotaxime (meant to cover penicillin-resistant isolates) to include vancomycin instead.

Antimicrobials have come to be termed as 'societal drugs', for the simple reason that when given to one person, they affect not only the micro-organisms in the person being treated, but also those in the people and the environment around that person (59, 63). In light of this, it is therefore possible that the use of antimicrobials in animals affects not only the micro-organisms in the animals being treated, but also humans sharing the environment with the animals on antimicrobial drug treatment.

With development of resistance all the gains made in terms of reduced threat posed by infectious diseases, the dramatic drop in deaths from diseases that were widespread, untreatable and frequently fatal, ease of suffering of millions of people over the years and major gains in life expectancy experienced in the later part of the last century following development of antimicrobials (20, 42, 93, 94), are seriously jeopardised. For example, in Estonia, Latvia, and parts of Russia and China over 10% of tuberculosis (TB) patients have strains resistant to the two most powerful TB medicines (94).

The development and dissemination of antimicrobial drug resistance can no longer be ignored. It is a problem that demands immediate attention (60). In the next section, factors that promote the development of resistance in bacteria, food animals and humans, the extent of antimicrobial usage in poultry and reasons for inclusion of antimicrobials in poultry feeds are discussed. Mention is also made of the various classes of antimicrobials commonly used in poultry, and how they relate with those used in humans.

2.3 DEVELOPMENT OF ANTIMICROBIAL DRUG RESISTANCE

Rises in resistance where antimicrobials are used, indicate the great capacity of these bacteria to overcome the antimicrobial pressures that we apply. Therefore given time and drug use, antimicrobial resistance will emerge. In view of this, there are no antimicrobials to which resistance has not or will not eventually appear (31, 59), and that wherever antimicrobials are used, resistant bacteria are present (31, 57, 68). Factors that influence development of antimicrobial drug resistance can be placed into the following categories: factors that determine resistance in bacteria, drivers of resistance in food producing animals and factors that influence antimicrobial drug resistance in humans.

2.3.1 Determinants of resistance in bacteria

2.3.1.1 Intrinsic factors

Resistance to antimicrobial agents in some instances is a characteristic of microbes which makes them resistant to certain antimicrobial agents (20, 57, 94). This is responsible for the intrinsic or natural resistance that is seen in certain bacteria, and occurs because the normal antimicrobial target in the bacterial cell is not present, not susceptible, cannot be reached by the antimicrobial (e.g. because the bacterial cell is impermeable to the antimicrobial) or due to the presence of natural degrading enzymes. This type of resistance however, is not of concern to clinicians. The type of antimicrobial drug resistance that concerns clinicians, which also is an integral part of a bacterium's own defence system, is that seen in micro-organisms to antimicrobials to which they are normally susceptible (68).

Within any population of micro-organisms, a few of the microbes may have some resistance genes. This explains resistance detected in both Gram-negative and Gram-positive organisms even before there was wide spread use of penicillin (17, 60, 92, 94). It is hypothesized that adaptation to antimicrobials by bacteria, is an essential survival strategy particularly for microbes having their main environment within the host (20, 31, 54, 93). For food-borne pathogens like salmonellae, enterococci, *Campylobacter* and *Escherichia coli*, the host environment is most important and so withstanding the different challenges in the host, e.g. antimicrobial resistance is of prior importance for survival of these genera. With organisms that have their major living environment outside the host, adaptation to non-host environment

is of higher priority than surviving within the host. This could therefore explain why acquired resistance is not common among organisms like *Listeria monocytogenes* and *Yersinia enterocolitica* (54).

Inherent differences in resistance to antimicrobials have been observed within a genus. For example, in one study while all of the *Campylobacter jejuni* isolated in the programme were susceptible to macrolide antimicrobials, only 48.4% of the *Campylobacter coli* isolated were resistant to the same drugs (43). Susceptibility to macrolids, tetracycline and quinolones has also been observed in Japan as being higher in *C. coli* than in *C. jejuni* (43). Differences in the pattern of resistance have also been observed in enterococci, with most clinical isolates of VRE (vancomycin resistant enterococci) being *E. faecium*, while it is less common in *E. faecalis* (16). Results from studies in Spain and other countries also suggest that resistance is more common in *E. faecium* than *E. faecalis* (3, 20, 40, 50, 78). Commensal bacteria from animals such as members of the enterobacteriaceae, staphylococci and *Pasteurella* spp. readily develop resistance to commonly used antimicrobials. On the other hand β-haemolytic streptococci and clostridia tend to remain fully susceptible to penicillin G (84).

2.3.1.2 Gene transfer within and between bacterial species

After bacteria have developed resistance, genes encoding resistance can be passed onto other strains of commensal organisms or even far more virulent organisms such as *Staphylococcus aureus* (3, 24, 50, 59, 60, 68, 79, 80, 81, 84, 93,). How this takes place has been described elsewhere in detail (17, 28, 60, 69, 80, 86, 92). An aspect of the gene transfer that is particularly worrisome is that genes resistant to a number of antimicrobials can move *en mass* from one microbe to another, thereby enabling a single horizontal transfer to confer multi-drug resistance (56, 59). It is in fact thought that the impact of the resistance of enterococci in the human intestinal tract could be mainly based on transfer of resistance elements rather than the transfer of resistant strains (50). Actually a view is held that direct transfer of genetic information is responsible for sudden increases in resistance, as compared to development of resistance through stepwise incremental remodelling of microbe, which often appears as gradual increasing minimum inhibitory concentrations (59).

Given the multicentric nature of the emergence of VRE in Europe, it has been hypothesized that the likely source of *van*A and *van*B genes is horizontal transfer of genes from

glycopeptide producing micro-organisms (that must protect themselves against these products), to enterococci via one or more bacterial intermediaries (17). Anaerobic bacteria in human faeces have also been implicated as possible sources of *vanB* genes for enterococci (17).

2.3.1.3 Mutations

Mutations are implicated in the emergence of resistance (59, 86). When the TEM-1 β-lactamases were first reported in *E. coli* in the 1960s, soon after the introduction of ampicillin therapy, these enzymes could not hydrolyze cephalosporins. However, by the 1980s, under strong pressure of treatment with these β-lactam drugs, many bacteria with TEM-mediated β-lactamase resistance became resistant to the extended spectrum cephalosporins through a series of amino acid substitutions in the TEM enzyme. Mutation is blamed for the ability of β-lactamases to counter inhibitors e.g. sulbactam and clavulanic acid, that clinicians had thought would be used to protect some β-lactam antimicrobials from degradation by bacteria, and for the more than 50 different TEM β-lactamase mutants that have been described (59). A single point mutation in *gyrA* encoding the bacterial DNA gyrase can confer high-level resistance, as evidenced by some studies where fluoroquinolone-resistant strains rapidly replaced susceptible *Campylobacter* in treated chickens following a genetic change in the organisms (42).

2.3.2 Drivers of antimicrobial drug resistance in food animals

2.3.2.1 Selection pressure

Selective pressure from the use of antimicrobial drugs has been implicated in the amplification of antimicrobial drug resistance in animals (3, 4, 10, 13, 17, 59, 60, 68, 79, 80, 84, 86, 88, 92,). This is because exposure of a bacterial population with resistant members to an antimicrobial gives the resistant members a competitive edge over non-resistant members (10, 17, 31, 59, 92, 68,). This is particularly true when exposed to anti-anaerobic antimicrobials, glycopeptides or any broad-spectrum antimicrobial (10, 14, 17, 24, 59, 84,). According to Bager *et al* (13), this phenomenon accounts for the fact that though the specific pressure exerted say by the use of avoparcin disappeared, glycopeptide resistant enterococci (GRE) would still have a competitive advantage if subjected to drugs that they were co-

resistant to. Actually amplification of resistant microbes by antimicrobial usage is implicated in the circulation of resistant organisms like drug resistant enterococci in the environment (45, 68).

Results of a number of studies involving different methods show that after the introduction of an antimicrobial in veterinary practice, resistance in pathogenic bacteria and/or faecal flora increases (4, 17, 28, 42, 54, 57, 59, 84, 88, 92). For example, when use of virginiamycin in Denmark increased from 1995 to 1997, it was followed by a corresponding increase in the occurrence of virginiamycin resistance among *E. faecium* isolates in broilers from 27,4% in 1995 to 66,2% in 1997. A similar pattern was also observed following the introduction of avilamycin as a feed additive (3). A study done in the USA showed that chickens naturally colonised with fluoroquinolone-susceptible strains began excreting resistant strains after two days of doses of enrofloxacin, a drug commonly used for prophylaxis in the poultry industry (42). Countries where fluoroquinolones are approved for use in its animal population, drug resistance prevalence of up to 29% to fluoroquinolones among *Campylobacter* isolates have been observed (4). With the initiation of the use of the fluoroquinolones in food animals in many countries, an increase in the proportion of campylobacter and salmonella isolates resistant to this group of drugs has been observed (9, 28, 32, 68). Therefore the increasing use of antimicrobials in animals, fish and in agriculture has been identified as one of the causes of the development of antimicrobial drug resistance being observed worldwide (3, 5, 13, 14, 17, 28, 42, 57, 59, 60, 65, 70, 71, 80, 81, 86, 89, 92, ,).

Since antimicrobial usage exerts selection pressure, antimicrobial resistance profiles of pathogenic food isolates reflect the animal treatment with antimicrobial substances (43). For example, in Austria where tetracycline ranks among the most often used drugs in animal husbandry, next to quinolone resistance, resistance to tetracycline is seen most often in all genera of bacteria tested (54). It has also been shown in Austria that quinolone resistance was higher (as high as 40%) in poultry isolates as compared to pork and beef isolates because the fluoroquinolone ciprofloxacin is often used to prevent *Salmonella* infections in poultry (54). This clearly contrasts with Australia that has adopted a policy of restricting fluoroquinolone use in poultry and hence has very low levels of resistance among *Campylobacter* isolates to ciprofloxacin (4). Results of studies by the Japanese Veterinary Antimicrobial dug resistance

monitoring Programme (JVARMP) (43) indicate a significant difference in the resistance of *C. jejuni* isolates from cattle, broilers and layers to aminoglycosides, tetracyclines and quinolones ($P < 0.01$, individually). This trend has also been observed in the Netherlands where there was an increase in resistance against carbadox following its introduction as AMGP and for prevention of swine dysentery in pigs, while in poultry where carbadox was not used, no resistance was observed (84). A study (78) done in Spain demonstrated that faecal enterococci from broilers had a higher resistance rate as compared to those from layers. The same reasoning could explain with the exception of a few cases, why resistance against the different categories of antimicrobials is more prevalent in enterococci strains from farm animals than those from pets (20).

2.3.2.2 Method of antimicrobial drug administration

Oral treatment is the predominantly used route in administering drugs to large flocks (61, 82). Disadvantages associated with this method of drug administration in poultry include inadvertent under-dosing due to reduced bioavailability, which is likely to arise due to in-homogenous mixtures, chemical degradation of a drug, and reduced feed intake by the diseased animals including indiscriminate antimicrobial use (82). Given that whenever the AUC: MIC ratio is not maximised, the likely result is development of resistance (31), there is a likelihood that administration of antimicrobials orally for prophylactic purposes results in development of resistance. The likelihood of this happening for that matter is high since the antimicrobials in these instances are often given at sub-therapeutic levels (42).

By minimising the time that sub-optimal drug levels are present in the infected tissue compartment, the emergence of resistant pathogenic populations can be prevented (21, 31, 59, 93). By implication therefore, poorly planned or haphazard use of these medicines is an important risk factor in the development of resistance currently being observed (94). In developed countries particularly, injudicious use of antimicrobials in food producing animals is blamed for the antimicrobial drug resistance in zoonotic salmonellas (79).

2.3.3 Drivers of antimicrobial drug resistance in humans

2.3.3.1 Acquisition of resistance by humans from animals

Circumstantial and epidemiological evidence of the existence of transfer of resistance genes coding for resistance from animals to humans as a cause of resistance among the human isolates has been cited by a number of authors (6, 14, 17, 42, 88, 92). The levels of VRE (*vanA* resistance) found in faecal samples of healthy humans outside hospitals in Sweden was at some stage very low compared to other EU countries that still used avoparcin extensively as an AMGP (84). However, when avoparcin was banned in the EU, there was a concomitant fall in the prevalence of VRE in the region (17). In Germany, farms or areas where avoparcin had previously been used proved to have a high prevalence of VRE, even among people that were not associated with the hospitals (3, 13, 17, 88, 92). This was not the case in the USA where avoparcin was never approved for growth promotion purposes due to concerns of avoparcin being a carcinogenic agent (13, 17, 24, 88). In the USA there were no reports of ciprofloxacin-resistant human *Campylobacter* spp. isolated prior to 1992. From 1997 to 1999, however, there was an increase in the number of resistant isolates from 13% to 18%, which coincided with the licensing of fluoroquinolones for use in the treatment of colibacillosis in poultry (18, 84, 89). A similar association was observed in the Netherlands, where the emergence of fluoroquinolone-resistant human *Campylobacter jejuni* infections followed the advent of its use in poultry in 1987. In Spain rates as high as 80% of campylobacter displaying resistance to fluoroquinolones, have been recorded (84, 89). The increasing resistance to quinolones observed in humans in the Netherlands, Britain and Spain is thought to have been as a result of the use of the same class of drugs in animals (59). From 1975 to the mid-1980s there was a substantial increase in the incidence of Multiple Resistant (MR) *S.* Typhimurium from production animals, and a concomitant increase in multi-resistant isolates from humans. This increase was due to a sequential acquisition of plasmids and transposons coding for drug resistance to a wide range of antimicrobials: ampicillin (A), chloramphenicol (C), gentamicin (G), kanamycin (K), sulphonamides (S), tetracyclines (T), and trimethoprim (TM) (giving rise to R-type ACGKSSuTTm) (79). Resistance genes against antimicrobials that are or have only been used in animals, for example the aminoglycoside apramycin, have been observed in human isolates and more so in organisms that are strictly human pathogens, like shigellae (40, 79).

Van den Bogaard *et al* (84) and other authors (24, 40, 88) report transfer of resistant bacteria as usually being from animals to humans. It is postulated that the higher the prevalence of resistance in the animal population the greater the extent of transfer of resistance from animals to humans (60, 84). In view of this, even in the absence of specific pressure amongst humans, development of resistance among human isolates is still possible due to transfer of resistance via members of say, enterobacteriaceae (60). This could possibly explain why persons exposed to farm animals and abattoir workers have a considerably higher percentage of antimicrobial resistant *E. coli* in their intestinal flora (43, 60, 84). The ability of organisms to move from animals to humans has lead to suggestions by some authors that both human and animal populations of bacteria constitute an overlapping reservoir of resistance (40, 60, 86). This thinking is supported by studies in which identical Tn1546 variants among VRE from both farm animals and human beings were recovered, indicating a common human and animal reservoir for *vanA* elements (17). Therefore the argument that the use of AMGP in animals plays a role in the emergence of resistance among isolates from humans is not without merit. In the light of this, it is not surprising that a lot of attention has been focused on food-producing animals as one of the potential sources of antimicrobial-resistant bacteria for humans (61, 69).

However, though the use of antimicrobials in veterinary medicine is implicated in the development of resistance in human beings (3, 10, 13, 14, 17, 24, 28, 36, 40, 42, 46, 50, 57, 59, 68, 84, 88, 92), there is no complete consensus on the significance of antimicrobial use in animals, or resistance in bacterial isolates from animals, on the development and dissemination of antimicrobial resistance among human bacterial pathogens (20, 31, 56, 68, 69, 82, 88). For example, the link between the emergence of multiresistant salmonella in humans and on-farm antimicrobial use is unknown or contested (28, 72). Whereas it is known that VRE colonisation is quite common in healthy people and farm animals following the use of avoparcin as a growth promoter, its role in nosocomial infections is said to be insignificant (14, 17, 88). A recent study in Sweden suggests that the animal route of drug resistant enterococci transmission from food animals to humans is negligible. The study presupposes that the route of circulation of drug–resistant enterococci from patients in hospitals is through hospital and urban sewage, and then via treatment plants to surface water and possibly back to humans (45). Therefore the role of antimicrobial use in veterinary

medicine in the development of resistance in humans is a subject that remains to be fully understood and on which a substantial amount of research still has to be done (3, 13, 31, 60).

2.3.3.2 Antimicrobial selection pressure

Compared to the role played by the spread of resistant bacteria from farm animals to humans, antimicrobial use in human medicine is considered a major factor in the development of resistance among human isolates (17, 24, 49, 60, 65, 79, 82, 84, 89). Selective pressure, both in and outside of the hospital environment, is considered the most important determinants in the development and spread of antimicrobial resistance (59). Events such as the evolution of multi-resistant tuberculosis and methicillin-resistant *Staphylococcus aureus* have been linked to medical, and not veterinary, use of antimicrobials (36). Some studies done in Brazil, England and Wales on resistance patterns in *Salmonella enteritidis* isolates obtained from humans and poultry showed no relationship between the resistance patterns of isolates from the two sources, suggesting that food producing animals bred in these countries may not be the primary sources of drug resistant observed in human isolates (28).

In the light of this, some authors suggest that much of the resistance observed in human medicine could be attributed to inappropriate use in humans, while antimicrobial use in animals selects for resistant food-borne pathogens (49, 60, 64, 89), and that resistance observed in humans and animals could be two unrelated events (48). This is also supported by the differences that have been observed in the antimicrobial drug resistance among VRE isolated from food and that from clinical material, with the former in some cases tending not to show the same resistances as those from clinical material (50). This also explains the existence of two strains of *E. faecium*: one (*vanA*) said to have developed as a result of antimicrobial use in food animals while the other (*vanB*), not found in animals and is due to vancomycin use in human health care settings (68). It also accounts for VRE isolates from animals, though similar to those from healthy individuals as has been shown in Europe, differ from those recovered from patients in hospitals (17). This dual cause of antimicrobial drug resistance explains why there are differences between human and animal isolates in terms of resistance to the therapeutically most important antimicrobials (78). Antimicrobial selection pressure in human medicine explains why Spain with a high rate of self medication without prescription (83) and an out patient consumption of 275 tons per annum of antimicrobials, has one of the highest resistance rates for community-acquired pathogens in humans (86). In

countries like Finland, levels of resistance among human isolates remains favourable for most pathogenic bacteria. The reason for this difference being that consumption of systemic antibacterial drugs among the Finns and hence selection pressure has remained unchanged or even declined over the years (57).

There is no linear relationship between antimicrobial usage in humans and the development of resistance. For example in Japan, where vancomycin injections have been used for the treatment of methicillin resistant *Staphylococcus aureus* infections, a low prevalence of *van*A vancomycin-resistant enterococci from humans has been observed (41). Actually fewer than 50 cases of *vanA* or *vanB*-type VRE isolates had been reported by 2000. On the contrary, in the USA wide-spread use of vancomycin in hospitals has been characterised by an alarming level of VRE infections in the hospitals (17). Antimicrobials are not currently recommended for the treatment of *E. coli* infections in humans (57, 89). Therefore resistance observed in shiga-toxin producing *E.* coli O157:H7, (STEC O 157:H7) of which cattle are thought to be the main reservoir suggests that medical use of antimicrobial plays a limited role in the wide spread occurrence of antimicrobial drug resistance in this group of human pathogens. It can therefore be concluded that the increasing level of resistance seen in STEC *E. coli* O157:H7, is due to agricultural use of antimicrobials and not their use in the hospital setting (89).

2.3.3.3 Socio-economic factors

Socio-economic factors as drivers of resistance among human isolates are important in both developed and developing countries (20). In the latter, antimicrobials are available over the counter and are hence easily accessible, leading to overuse (20, 62, 64, 94). This is believed to account for resistance rates of 90% among human isolates to tetracycline in West Africa where misuse of this group of antimicrobials has been practiced for many years (64). Besides that, in developing countries under use has also been identified as an important cause of the development of resistance (20, 59, 62). This is because in poorer countries, patients are either unable to afford the full course of the medicines to be cured of their illness, can only purchase counterfeit drugs on the black market, or receive sub-optimal doses. In the view of this, resistance would therefore most likely be a problem in countries like Bangladesh where 8 out of 10 brands of ampicillin on the market are said to be substandard, and in Africa where antibacterial misuse is unregulated and antimicrobials sold within the continent are often of a substandard quality (20, 62, 64, 67, 94). The use of substandard drugs selects for resistant

pathogens during treatment even if the diagnosis was correct and in this way, favour the selection of resistant pathogens (64, 94). Besides that, data from developing countries suggests that prevalence of resistance is not only in the high range, but is also increasing (63).

In developed countries, overuse has been identified as the main concern as far as development of resistance is concerned. This includes subtler ways like prescribing broad spectrum antimicrobials when microbiologic evidence indicates that a narrower spectrum drug would be sufficient, and prescribing antimicrobials because of patient pressure, when the odds are that the infection is viral, rather than bacterial (10, 30, 59, 65, 93, 94).

Inappropriate use of antimicrobials as a contributor to the rise of antimicrobial drug resistance is also a function of the behaviour of general practitioners, and a result of promotional efforts of the drug industry. The later are responsible for the high expectation by the general public that antimicrobials cure almost any illness. Lack of time also pushes the general practitioner to prescribe despite lack of a clear indication for antimicrobials (20, 83).

2.3.3.4 The role of antimicrobial drug residues in food of animal origin

Humans could acquire resistance among their enteric organisms by ingesting antimicrobials that remain as residues in animal products, as this allows for selection of antimicrobial resistant bacteria in the consumers of such products (60).

Antimicrobial resistance is a complex problem involving myriad interactions between humans, animals, drugs and the environment (20, 92). However, out of this complexity a simple truth emerges: antimicrobials breed resistance, no matter where they are taken. Therefore it does not make sense to cut the problem into pieces, which has seen veterinarians and medical practitioners pointing fingers at each other. What we are seeing could be a cumulative effect of both medical and veterinary use of antimicrobials over the years, and what we need at this time is learn all we can about the various factors that promote resistance, and use the knowledge gained to make decisions about how and where antimicrobials should be used. The next section addresses ways through which resistant organism or genes can be spread from one place to another.

2. 4 DISPERSAL OF ANTIMICROBIAL DRUG RESISTANT ORGANISMS

2.4.1 Live animals

Cases of outbreaks of infections with antimicrobial drug resistant bacteria in the animals have been reported in a number of countries following importation of live animals (17, 80, 86). For example, sea gulls and exotic birds imported from Indonesia and Hong Kong are said to have introduced multi-drug *S.* Typhimurium DT104 into Great Britain (89). Van den Bogaard *et al* (84) are of the view that farmers are at a greater risk of picking up resistance from food animals than abattoir workers and the general urban population, emphasising the role live animals play in transmission of resistant bacteria.

2.4.2 Food of animal origin

When food producing animals are preferentially colonised by antimicrobial drug resistant bacteria, the consequence is a greater contamination of food with potential pathogens to the consumer during slaughter and or food preparation, (5, 80, 84, 86). This is enhanced by the fact that use of antimicrobials has the potential to disturb the colonisation resistance (CR) known as the "gut barrier" of the intestinal flora of animals exposed to certain antimicrobial drugs. With reduced CR, the minimal infection or colonization dose of pathogenic or resistant bacteria is significantly lowered. When this occurs, these animals excrete these bacteria over a longer period of time as well as in higher numbers compared to animals with an intact intestinal flora. This enhances dissemination of resistant bacteria within a group of animals, and increases chances of contaminating carcases with these bacteria during slaughter (59, 84). This has been demonstrated for most broad-spectrum antimicrobials and for certain AMGP e.g. avoparcin, and to a lesser extent virginiamycin and tylosin. Avilamycin and bacitracin on the other hand seem not to disturb the CR in the dosages used for growth promotion, while flavomycin has been shown to provide a certain protection against *Salmonella* spp. (84).

Poultry products particularly, are considered a likely source of resistant organisms including *Campylobacter* spp., VRE and multidrug resistant *Salmonella* spp. for humans through the food chain (9, 18, 28, 42, 78, 84). Imported slaughtered chickens were implicated in the

spread of VRE to Japan and Denmark from countries using avoparcin as a growth promoter (41, 88). The risk here is appreciated when consideration is given to the fact that at one time a country like Japan imported 1, 2 million tons of slaughtered chicken a year from countries where avoparcin was being used in poultry flocks.

It is recognised that zoonotic bacteria after acquiring resistance in the food-animal host can be transmitted to humans through the food chain (42, 68, 79, 80, 92). For example, in Denmark cases involving MR *S.* Typhimurium that was not responsive to fluoroquinolone antimicrobials in patients are said to have been due to MR *S.* Typhimurium that was associated with pork of Danish origin and was resistant to nalidixic acid (79). One way through which humans can acquire resistance among their enteric organisms is by directly ingesting resistant organisms from food of animal origin (17, 68, 80, 81, 84, 86,). This is supported by the fact that intestinal carriage of enterococci strains following ingestion of antimicrobial-resistant *E. faecium* and glycopeptide resistant enterococci (GRE) from chicken and pork is possible (13, 40, 74, 88). Besides that people who use only sterilised food or strict vegetarians, tend to carry a significantly low level of resistance (17, 84, 88). One investigation carried out in a Muslim country revealed that only VRE poultry variants occurred in that country, and that the pig variant types were absent. The explanation for this is that Muslims do not eat pork, and are hence not exposed to VRE variants from pigs (40).

2.4.3 Fruit or vegetable from a contaminated environment

If chicken like any other food of animal origin contaminates kitchen surfaces and later vegetables or fruits to be eaten are placed on the same surfaces, such foods become a vehicle for carriage of resistant microbes. Shredded lettuce in particular has been implicated in outbreaks of MR *S.* Typhimurium DT 104 in the UK and other European countries (Germany, the Netherlands and Iceland) (18, 80).

2.4.4 Contaminated water

In 1998 there was a water-associated out-break of *E. coli* O157:H7 that was resistant to streptomycin, sulfisoxazole, and tetracycline in Missouri (89). Water associated outbreaks of antimicrobial drug resistant bacteria have also been reported involving other bacteria species

e.g. *Campylobacter* spp. and *S. typhi* in places like the Indian sub continent, Southeast Asia and Tajikistan. For example in 1997 an outbreak of multi-resistant *S. typhi* occurred in Tajikistan in which 6000 cases were recorded. Of interest in this outbreak, was that the epidemic strain exhibited a decreased susceptibility to ciprofloxacin. Contaminated ground water has been mentioned as a possible source of antimicrobial resistant bacteria occurring in both animal and human food chain (32, 92).

2.4.5 Human beings

Close family members of farm workers tend to carry a higher level of resistance in their enteric organism than the general public (60). Thus humans and especially those working with animals e.g. farm workers and/or abattoir workers can act as vehicles of transferring resistance and or resistant organisms to the general public.

Improved means of transport and globalisation in trade give greater significance to transfer of antimicrobial resistance (20, 67, 68, 94). A notable example is that of two cases of ciprofloxacin resistant *C. jejuni* infections in patients reported in Australia but suspected to have picked up these resistant organisms from chicken they had ingested in Europe (2). Nel (59) also cites authors who reported a multi-drug resistant bacterium that was traced from Spain through Portugal, to France, Poland, the United Kingdom, South Africa, the United States and Mexico. In the case of the resistant *C. jejuni*, though it is not known as to whether patients concerned transferred the ciprofloxacin resistant *C. jejuni*, this has the potential of acting as a source of an outbreak and moreover once genes have been introduced they are difficult to get rid off (59). Outbreaks of certain strains of *Salmonella* spp. have broken out in developed countries, particularly in patients with a recent history of return from areas where the resistant strains are endemic. Residents and visitors to developing countries tend to acquire antimicrobial resistant *E. coli* as part of their normal flora (63).

Factors that enhance person to person transmission of antimicrobial drug resistance include: crowded dwellings like student hostels and health care settings, non-compliance with hygienic standards like hand disinfection or barrier precautions and understaffing especially in health care settings (17, 59, 63), presence of patients with a high-density intestinal colonisation with

resistant microbes such as VRE (59), and colonisation pressure which is the number of colonised patients present each day (17).

2.5 INCLUSION OF ANTIMICROBIALS IN POULTRY FEED

It was in 1950 after it had been reported that the addition of streptomycin to chicken feed increased the growth rate of chickens, that the practice of adding antimicrobials to commercial feed for cattle, pigs, and chickens gained impetus (80). For example, approximately 24.6 million pounds of antimicrobials are given to animals each year in the USA (as growth promoters) at sub-therapeutic amounts in their feed, compared to 3 million pounds consumed by humans (90, 92). While in 1978 it was estimated that 48% of the total antimicrobials in the USA went into animal feeds, recent studies estimate that 70% of this nation's antimicrobials find their way into animal production facilities for non-therapeutic uses (10, 36). In South Africa, the antimicrobial market constitutes the largest sector in veterinary drugs (60), while in Denmark 105 tonnes of antimicrobials were consumed for growth promotion alone in 1996 (86). In the EU, before the use of antimicrobials as AMGP was banned, approximately 50% of all antimicrobial agents used annually were given to animals (84). Internationally antimicrobial drugs represent the largest portion of pharmaceutical sales; both in volume and dollar value of any drugs used in animal production (60). It is estimated that the annual world wide consumption of antimicrobials is 100,000 to 200,000 tonnes (86), and of this the largest quantities are used as regular supplements for prophylaxis or growth promotion purposes (28, 88).

In food animals, antimicrobials are used for non-therapeutic purposes at sub-therapeutic levels for growth promotion, increasing feed efficiency and decreasing waste production (4, 10, 24, 28, 31, 37, 53, 57, 61, 69, 70, 72, 79, 82, 84, 89, 92). Through the use of antimicrobials in food animals, it has been possible to enhance production efficiencies that have contributed to the availability of a reasonably priced and plentiful food supply (31, 60, 93). The National Academy of Sciences estimates that the ban of AMGP in the USA would raise a person's annual meat bill by $5 to $10 (10). Though the mechanisms by which AMGP achieve growth enhancement is not clearly understood, it is thought that AMGPs reduce normal intestinal flora, which otherwise would compete with the host for nutrients (88). In intensive poultry

husbandry where birds are raised in overcrowded areas at optimal temperature and low light intensity to enhance growth rates and mass increases, and shorten the production cycles, sub-therapeutic doses of antimicrobials are administered routinely via feeds and water to raise feeding efficiency and rate of weight gain (31, 37, 53). In some instances antimicrobials are used at low doses in animal feed as a means of lowering the percentage of fat while increasing the protein content in the meat (4). When antimicrobials are used for purposes such as growth promotion, increasing feed efficiency and decreasing waste production, they are referred to as feed savers, antimicrobial growth promoters or antimicrobial performance enhancers (APE) (84, 88).

Besides production enhancement, antimicrobial drugs are also health management tools licensed to be used for supporting good husbandry practices aimed at not only prevention, but also for therapeutic purposes i.e. treatment of diseases (6, 18, 28, 31, 37, 39, 57, 60, 61, 69, 72, 82, 92,). This is possible because the antimicrobials and especially AMGP are believed to reduce harmful gut bacteria, which would otherwise reduce performance by causing sub clinical disease (88). In this way they are used for the prevention and treatment of bacterial associated infectious diseases. Particularly in events where animals/birds are fed feed heavily contaminated with infectious bacteria e.g. carcass meal, edible plastic, sewage, petrochemical residues and excrement, antimicrobials are used to suppress the outbreak of epidemics (37). Antimicrobials are also administered to food-producing animals for welfare reasons, measured in terms of animal being free of diseases (68, 80, 82). In broiler production particularly, AMGP have a protective effect against necrotic enteritis caused by *Clostridium perfringens* toxins (91). Antimicrobials as growth promoters also help control zoonotic pathogens such as S*almonella* spp., C*ampylobacter* spp., *E. coli* and enterococci (4, 82), and in this way help in producing food that is safe for human consumption in terms of food-borne diseases (82). Given the benefits that accrue from inclusion of antimicrobials in feed for broilers, this category of chickens spend 40 days of their 42 days life on antimicrobials (90).

Most antimicrobials used as AMGP are highly effective against Gram-positive bacteria (70, 84, 88), with the exception of carbadox and olaquindox, which are mainly active against Gram-negatives (84). The concentration used in feed varies with each antimicrobial agent. However the concentration often used is referred to as sub-therapeutic (not to be confused

with sub-MIC levels). Meaning that the resultant concentration in the gastrointestinal tract of the animal is likely to be sufficient to inhibit the susceptible bacteria and markedly affect the composition of bacterial gut flora (88). In the EU before legislation was passed prohibiting the use of AMGP, drugs that were extensively used as growth promoters included the macrolides (tylosin and spiramycin), avoparcin, bacitracin, virginiamycin and oligosaccharide (avilamycin) (3, 24, 41, 50, 57, 84, 85). However, between 1997 to 1998 avoparcin, ardamycin, bacitracin, virginiamycin tylosin and spiramycin were banned as AMGP in the EU. Which meant that only a few substances (monensin, salinomycin, avilamycin and flavophospholipol), could legally be used as growth promoting agents in the EU (85). However, as from 01/01/2006 inclusions of antimicrobials in animal feed as AMGP, with the exception of coccidiostats have since been banned in the EU (4). In the USA, 17 classes of antimicrobials are approved for growth promotion and feed efficiency, including tetracyclines, penicillin, macrolides, lincomycin (analogue of clindamycin), and virginiamycin (analogue of quinupristin/dalfopristin) (6). In South Africa, the following drugs are registered and hence available for use as AMGP and for improving feed efficiency in poultry (7): tetracyclines, penicillins, tylosin, flavomycin, zinc bacitracin, olaquindox, kitasamycin, avilamycin and ionophores.

In general, the antimicrobial classes used for therapeutic or prophylactic purposes in animals are similar to those used in human medicine, although some unique non-human use classes are available too. The class of drugs similar to those used in human medicines includes drugs like tetracyclines, sulphonamides, macrolides, beta-lactams, cephalosporins and fluoroquinolones, while the non-human use class includes pleuromutilins and polyether ionophores (39, 82).

Notwithstanding that there are benefits that accrue from inclusion of antimicrobials in poultry feed (4, 10, 24, 28, 31, 37, 53, 57, 61, 70, 72, 79, 82, 84, 85 89, 92), there is a need to regulate the practice of including antimicrobials in animal feed. A discussion of the advantages of this and efforts made so far to regulate the practice follows.

2.6 ADDRESSING ANTIMICROBIAL DRUG RESISTANCE AND THE ASSOCIATED PUBLIC HEALTH RISKS

2.6.1 Ban or restriction of the use of AMGP

The Swann report of 1969 was the first to recommend the exclusion of antimicrobials that are used in both humans and animals for therapeutic purposes from feed (33, 77). When it was later discovered that the use of AMGP analogues caused cross resistance with therapeutically important antimicrobials and that this resistance can cross to humans, it was recommended in 1977 that the use of antimicrobial drugs as production enhancers or for non-therapeutic purposes be terminated, particularly if the antimicrobial drug is used for human medical purposes, or if it is known to be selective for cross-resistance to antimicrobial drugs in human medicine (10, 59, 60, 88). Subsequently in 1999 the EU decided to ban the use of bacitracin, avoparcin, spiramycin, tylosin and virginiamycin as AMGP (6, 86, 91, 92), the reason being their structural relatedness to antimicrobials agents used in human medicine and veterinary medicine (6). Consequently WHO, Food and Drug Administration (FDA), APUA, the USA congress and some major food service companies, now advocate for the withdrawal of antimicrobial in food animals (3, 6, 10, 42). It is argued that in the place of antimicrobial drugs as AMGP, alternative strategies like mass vaccination, new feeding systems, increased infection control measures and improved management practices be adopted (59, 86, 28).

2.6.2 Institution of surveillance programmes and research

In 1999 as a response to the concerns expressed by the WHO and Office Internationale Des Epizooties (OIE), a number of organisations were established in a number of countries based on the "global principles for containment of antimicrobial resistance in animals intended for food" (3, 60, 94). The purpose was to have groups that monitor changes in antimicrobial susceptibilities of zoonotic bacteria (*Campylobacter* spp. and S*almonella* spp.), indicator bacteria (*E. coli* and *E. faecalis* and *E. faecium*) and animal pathogens from food producing animals on farms (43, 60), with the possibility of using data from such programmes as a basis for the implementation of an antimicrobial drug resistance control (63).

Surveillance can be carried out at various levels (36, 57, 71), and these are:

i. Diseased animals with bacteria isolated from pathological samples,
ii. Healthy animals with sentinel/indicator bacteria isolated from the intestinal flora from animals at slaughter houses, and
iii. Food contaminants isolated from food.

The Denmark approach to surveillance puts emphasis on a few categories, such as pigs, broilers, layers, and dairy cows in their monitoring programme. This is because within each of these production categories, the tendency is to have a limited variation in their breeds and production methods, hence providing a homogenous population suitable for studying changes in bacterial populations living in these reservoirs (3).

The importance of surveillance and research in mitigating antimicrobial drug resistance is well captured by the words of the National Academy of Science quoted in William's paper (92) which says that: "until more accurate data on animal antimicrobial use, and patterns plus rates of resistance transfer to humans are available, actions aimed at regulating antimicrobials cannot be implemented through science-driven, well-validated, justified process".

2.6.3 National legal reforms

If the benefits of surveillance in addressing antimicrobial drug resistance are to be realised, appropriate laws have to be enacted where they are lacking. Law is needed to make reporting of information a legal duty and also to deal with the tensions sometimes arising between individual privacy rights and the community's interest in being protected from infectious diseases (30).

For example, it has been suggested that quarantine measures be applied as is done for other exotic diseases to prevent inadvertent importation of resistant/multiresistant bacteria into countries where they are not already present (68). Although the WHO mandates its member states to report outbreak of diseases like plague, cholera, and yellow fever (30), the reporting of antimicrobial drug resistance is not catered for. Likewise in South Africa, while the

Animal Diseases Act 35 of 1984 (8) mandates reporting of diseases categorised as controlled diseases, reporting of antimicrobial resistance is not catered for.

National legal reforms taken in one or a few countries are bound to suffer if other countries do not take similar action (30). Likewise creation of new international legal duties would be undermined if similar duties were not translated into national law. Therefore a national and international legal strategy is the way to go if antimicrobial drug resistance is to be contained. Things that could be considered are implementation of mandatory guidelines in the drug legislation, adaptation of drug registration and label instructions for antimicrobials to the rules of prudent guidelines and legally based limitations of the amounts of antimicrobials to be prescribed and dispensed for use in farm animals intended for production of human food (30, 82).

Bager *et al* among others (13) are of the view that the process of licensing a drug take into consideration the fact that once resistance develops it is difficult to cure. The FDA's strategy to control antimicrobial drug resistance includes among other measures revision of the pre-approval safety assessment for new animal drug applications (18, 30, 80,), i.e. adopting rigorous guidelines for approving and evaluating animal antimicrobial drugs used in food-producing animals (5, 10, 18, 68). For example, FDA's centre for veterinary medicine proposes a stronger regulatory approach when approving new antimicrobial drugs for use in food animals. According to the new proposals, drugs of highest importance to human health – those used to treat serious or life threatening disease in humans and for which there is no alternative treatment would be subjected to the strictest criteria for approval for animal use. Drug sponsors are required to carry out tests to show their product's potential to induce antimicrobial resistance as part of pre-registration application (18).

Lack of secure patent in some countries, due to inadequate legislation on intellectual property rights, acts as a deterrent to pharmaceutical companies from carrying on with research and development activities on new drugs (30). Equally complex and costly regulatory approval procedures that pharmaceutical companies face in some countries like the USA can be detrimental to the development of new antimicrobials. Hence for development of new drugs to catch up with the rate of resistance development, there is a need to streamline drug

approval regimes and adopt "expedited approval of new antimicrobials" as an incentive for development of new drugs (30).

2.6.4 Establishment of guidelines for prudent use of antimicrobials

It has been shown that failure to adopt prudent use guidelines for third generation cephalosporins and other substances leads to development of resistance (3, 86). It is therefore important to develop acceptable antimicrobial utilization strategies not only in human medicine, but also in animals and agriculture (35). This would lead to a minimisation of development or even reduction of resistance among pathogens (10, 30, 35, 82, 86, 93).

Such measures include antimicrobials being applied against certain microbes only after antimicrobial susceptibility testing (AST) has been done to choose the correct antimicrobial (54, 82, 86). Where the aetiological agent belongs to a bacterial species in which resistance to commonly used antimicrobial agents has been documented or could arise, AST should be carried out as a matter of necessity (39). Ungemach *et al* (82) suggest that performance of AST to selected specific antimicrobial be mandatory when switching therapy to another antimicrobial, especially if therapy does not involve fixed antimicrobial combinations; when the antimicrobial is not used in compliance with the label instructions (other dosage or animal species than designated); and regularly in cases of repeated or long-term use in larger animal herds. It is recommended as part of improved rational use of antimicrobials that use is made of pathogen-specific, rather than broad-spectrum antimicrobials when possible. Furthermore, prophylactic use of antimicrobials should be restricted only to proven or exceptional indications (e.g. immunosuppression, peri-operative), and drug dosages and lengths of therapy should always be optimized (39, 59, 82). Other measures that could lead to improved rational use of antimicrobials include physicians receiving appropriate and continuing education from both drug companies and well balanced sources (5, 30, 59, 83). The FDA in line with this has already developed educational programmes and media bulletins about judicious use of antimicrobials targeted at farmers and veterinarians (5).

When guidelines for prudent use of antimicrobials are enforced, veterinarians are forced to make a more precise clinical and microbiological diagnosis, to acquire a detailed knowledge

of the features of anti-bacterials, to keep a sufficient assortment of various antimicrobials, issue fewer prescriptions and markedly reduce treatment days, with a trend towards more therapeutic indications instead of prophylaxis. All of these have a potential to reduce the amount of antimicrobials consumed and the consecutive development of resistance (82).

2.6.5 Other approaches

Other measures discussed here include adoption of risk analysis principles in making decisions that relate to antimicrobial drug resistance, and dissemination of information on antimicrobial drug resistance.

Risk analysis can assist regulators in the decision making process, by determining the actual risk to human health from antimicrobial use in animals (risk assessment) and the requirements for risk minimisation (risk management and risk communication) (68). Risk assessment quantified for the first time the magnitude of the dangers to humans of eating chicken contaminated with fluoroquinolone resistant *Campylobacter* spp. It showed that the number of people infected with fluoroquinolone-resistant *Campylobacter* spp. from eating chicken rose from an estimated 8, 782 in 1998 to 11, 477 in 1999 (18).

Though there is no agreed upon approach to risk analysis that has been developed and aimed at minimising the impact of resistance on humans without putting the food-production industry at a disadvantage, some authors have suggested a novel risk analysis that involves risk assessment for three interrelated hazards: the antimicrobial (chemical agent), the antimicrobial-resistant bacterium (microbiological agent) and the antimicrobial-resistant gene (genetic agent). In this risk analysis, they also suggest a risk minimisation which includes control of antimicrobial use and/or reduction of the spread of bacterial infection and/or prevention of transfer of resistance determinants between bacterial populations (68).

Highlighting the magnitude of the problem of antimicrobial resistance has been used by organisations like WHO, OIE and others. This is evidenced from the several meetings that have convened over the years (11, 30, 59, 60,). In 1997, WHO convened a meeting in Berlin Germany under the title" The Medical Impact of the Use of Antimicrobial in Food Animals".

In June 1998 another meeting was convened, this time in Geneva Switzerland. The title of the meeting was "The Use of Quinolones in Food Animals and the Potential Impact on Human Health". There was yet another meeting in held in September 1998 in Denmark, this time the title of the meeting was, "The Microbial Threat". In March 1999 the OIE also held a meeting entitled "The Use of Antimicrobials in Animals - Ensuring Protection of Public Health". Information disseminated through these meetings led to a number of decisions and/or recommendations aimed at curbing antimicrobial drug resistance development world wide. In South Africa, both the National Antimicrobial Surveillance Forum (NASF) and the Antimicrobial Study group (ASG) have been tasked with the collection of data from as many laboratories as possible from the medical field (60, 87). On the veterinary side, the antimicrobial working group is tasked with the establishment of the national veterinary antimicrobial resistance surveillance and monitoring programme (60). The expectation is that data collected in some of these programmes will be presented regularly, with co-ordination, collation and dissemination of relevant facts to clinicians in the private and public sector (87).

A lot has been done both nationally and internationally to contain the practice of including antimicrobial usage and the associated problem of resistance due to the practice of adding antimicrobials at sub-therapeutic levels. Unfortunately little has been done in developing countries because of factors relating to poverty and inadequate resources (20). However, are there any benefits to withdrawing/banning antimicrobial usage as growth promoters or enhancers? A look at how the Nordic and EU countries benefited from implementing these measures follows.

2.7 WITHDRAWAL OF AMGP: THE NORDIC AND EU EXPERIENCE

One obvious benefit of withdrawing AMGP is a drop in the amount of antimicrobials used in the animal industry. For example, in Norway the result of withdrawing antimicrobials as AMGP was a drop in the amount of antimicrobials used in production by 25% over the period 1995 – 2000 (91). In Germany in the period 1997 to 1999, the non-therapeutic usage of antimicrobials as AMGP in farm animals declined by 51% from 3494 to 786 tonnes due to the ban of various antibacterial feed additives (82).

Restricting the use of AMPG in 1969 and implementation of appropriate legislation in the UK, is credited with disappearance of resistant organisms like MR *S.* Typhimurium DT 29 disappearing from both animals and humans at some stage (79). In Denmark, the ban on the use of avoparcin as a growth promoter in 1995, led to a decline in the occurrence of glycopeptide-resistant *E. faecium* (GRE) in broilers from 72, 7% in 1995 to 5, 8% five years later. Following the withdrawal of the macrolide tylosin as a growth promoter in poultry, resistance declined from 46,7% to 28,1% for tylosin and from 76,3% to 12,7% for erythromycin (1, 3, 42, 88). When virginiamycin was eventually banned in Denmark, the occurrence of virginiamycin resistance decreased to 33, 9% in 2000 (3). A similar pattern was also observed following the withdrawal of avilamycin (3). There is evidence to suggest that restricting fluoroquinolone use to therapeutic indications only in food animals decreases rates of fluoroquinolone-resistant *Campylobacter* spp. (42).

Withdrawal of antimicrobial use as feed additives has potential of lowering the level of resistance observed among healthy individuals. For example, the GRE carrier rate among healthy humans in Germany decreased from 13% in 1994 to 4% in 1997 following the German ban of avoparcin in 1996 (1, 3, 6, 84, 88). A similar decrease was also observed not only among food animals, but also among humans in the Netherlands and other European countries following the ban of AMGP (3, 84).

Following withdrawal of AMGP in Denmark, the production results of food animals remained constant or even increased (3, 6, 42, 94). In Sweden, farmers continued raising pigs almost as cheaply as before following withdrawal of AMPG, (3, 91, 92). In fact, the growing rate remained as good as in countries using AMGP in slaughter pigs (91). In South Africa too, production records from farms where the withdrawal of antimicrobial growth enhancers has been implemented, have shown no remarkable effect on the growth performance of broilers on these farms (John Alga, Company farm manager, personal communication, 2005). However, there have been cases where withdrawal of AMGP resulted in an increased use of therapeutic antimicrobials (85, 86). It is also reported that with the advent of the banning of growth promoting antimicrobials, *Cl. perfringens* induced necrotic enteritis and subclinical disease have become important threats to poultry health (34). In Norway, there was a temporary increase in necrotic enteritis after avoparcin was banned, but this was before

narasin (an ionophore feed additive) became available, which made the increase negligible (38). The Swedish experience (91, 92) actually shows that with appropriate disease control measures in place, the expected outbreaks of necrotic enteritis following withdrawal can be prevented. Consequently, the assumption that the banning of AMGP would be followed by an increased consumption of antimicrobial drugs for therapeutic use in slaughter poultry, and hence increased selective pressure for development of antimicrobial resistance for therapeutic antimicrobials (as was feared in Norway) (38) is not strong enough to justify continued use of AMGP in food animals.

2.8 OVERVIEW OF SELECTED ENTERIC BACTERIA

2.8.1 *Salmonella*

The genus *Salmonella* currently includes 2400 different serotypes that are ubiquitous in the environment and can colonise and cause disease in a variety of food producing and non-food producing animals. In food producing animals this colonisation is favoured by intensive animal production (9, 16, 84). Zoonotic salmonellae exhibit a clonal nature, are random in their infection dynamics, and are easily recovered in the environment (72). On the contrary, non-zoonotic salmonellae such as *S. typhi*, *S. gallinarum* and *S. pullorum* are highly host specific.

Salmonellae are the predominant cause of food-borne infections in many countries, with poultry considered the most important source of these pathogens as compared to other foods of animal origin (9, 16, 28, 73, 90). The two most important serovars in humans are *S.* Enteritidis and *S.* Typhimurium. While the former is associated with pandemics of human infections due to eating raw or lightly cooked shell eggs and egg containing products, the later is more prevalent in the porcine, ovine and bovine meat industries (28, 60). In the U K, Europe and the USA, *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow and *S.* Hadar are the most important serotypes spread through food. Of these species *S.* Enteritidis, *S.* Virchow and *S.* Hadar are normally associated with poultry and poultry products, while S. Typhimurium has a more ubiquitous host range (9, 79). However, since the 1980s *S.* Enteritidis has emerged as the most frequently isolated from cases of human salmonellosis in Europe, and continues to be the most frequently isolated serotype from human cases (9). In contrast, *S.* Wien, *S.*

Typhimurium, *S.* Johannesburg *and S.* Oranienburg have exemplified out breaks of salmonellosis in the developing world like the Indian subcontinent, South East Asia, South and Central America and Africa.

Fluoroquinolones are the drugs of choice for treating human salmonellae infections, while other antimicrobials are not clinically effective and contribute to a prolonged carrier status (6, 57, 73). However, there are increasing reports describing decreasing susceptibilities to antimicrobial agents such as fluoroquinolones and expanded-spectrum cephalosporins, drugs of choice in cases of life threatening salmonellosis due to multidrug-resistant strains (6, 73, 79, 89,). A recently concluded seven-year study in Spain revealed that ampicillin resistance in *Salmonella* species had increased from 8% to 44%, tetracycline resistance from 1% to 42%, chloramphenicol resistance from 1,7% to 26%, and nalidixic acid resistance from 0.1% to 11% (89). A similar observation was made in the UK, where resistance in *S.* Typhimurium more than doubled between 1981 and 1989, and isolates resistant to third generation cephalosporin ceftriaxone (the drug of choice in invasive infections caused by strains resistant to ciprofloxacin) have more than doubled since 1998 (89). In the USA, resistance to tetracycline in *Salmonella* species increased from 9% in 1980 to 24% and ampicillin resistance increased from10% to 14% (89). A recent survey in Portugal revealed that only 25% of the *Salmonella* isolates obtained were susceptible to all antimicrobials, 39% were resistant to one antimicrobial and 36% were resistant to two or more agents of different groups (9). In the Indian subcontinent and South East Asia, it is a norm for *S. typhi* (non zoonotic) strains to exhibit multidrug resistance (79).

The incidence of human infections with MR *S.* Typhimurium DT104 has increased dramatically in the last decade. A distinct feature associated with most DT104 isolates is a multiple antimicrobial resistance phenotype to ampicillin, chloramphenicol/florfenicol, streptomycin, sulphonamides, and tetracycline (ACSSuT) (79, 89). Additionally, DT104 isolates in Great Britain have also acquired resistance to trimethoprim and aminoglycosides and like in Denmark, demonstrated decreased susceptibility to fluoroquinolones (84, 89). Further still, the majority of MR DT104 isolates possess a unique chromosomal gene cluster that encodes for the complete spectrum of the ACSSuT resistance phenotype (89). On the contrary, *S.* Enteritidis isolates susceptible to most antimicrobials have been reported in the

UK. The reason for this, being that *S.* Enteritidis although wide-spread in the poultry flocks does not cause clinical symptoms in affected flocks and so animals are not usually treated with antimicrobials in the advent of infection. In view of this, the two serotypes are exposed to different selection pressures, thus the difference in resistance levels and patterns (84). This is confirmed by Antunes *et al* (9) who cite a number of authors that also report resistance to be less prevalent in *S.* Enteritidis as compared to other strains.

Although the presence of *Salmonella* in production animals poses a significant food hygiene risk, treatment of infected production animals with antimicrobials is not recommended. In South Africa *Salmonella* infections are controlled diseases, and so treatment is not prescribed but rather eradication (8). This approach in Finland is credited with the low levels of resistance figures among *Salmonella* isolates (57).

2.8.2 *Escherichia coli*

Escherichia coli a common inhabitant of the human and animal intestinal tract is a Gram-negative, facultative aerobic organism, and a member of the Enterobacteriaceae family (62, 86). Pathogenic *E. coli* fall into two groups: the first one is the urogenic group, which is the predominant causative organism of urinary tract infections (UTI), is also frequently isolated in neonate meningitis and Gram-negative nosocomial and community-acquired infections. The other is the enteric group that often causes childhood enteritis and bacteria-related traveller's diarrhoea (86). Among the enteric *E. coli,* Shiga-toxin (Stx) producing *E coli* (STEC) O157:H7 and non-O157:H7 have been identified as aetiological agents for haemorrhagic colitis and haemorrhagic uraemic syndrome (HUS) in humans (86). However, of the two, O157:H7 serotype is considered as being the most significant and has been associated with large food-borne outbreaks in North America, Europe, and Japan (57, 89). Non-O157 STEC food-borne outbreaks have also been reported and the common isolate serotypes in these cases are O26 and O111 (89). The Centre for Disease Control (CDC) estimates that *E coli* O157:H7 causes approximately 73,000 illnesses and 61 deaths each year in the USA while non-O157 STEC account for an additional 37,740 cases with 30 deaths (89).

There is no consensus as to whether antimicrobials should be recommended for treatment of *E. coli* O157:H7 infection in humans (57, 86, 89). The major concern is that antimicrobial treatment of *E. coli* and especially STEC infections may worsen the disease by inducing the release of Shiga-toxin(s) (the cause of HUS) and also enhances the transfer of virulence factors in vivo (86, 89). However, in Japan, it has been shown that antimicrobial (fosfomycin) therapy significantly reduces the number of infected children that develop HUS, and that some antimicrobials do not stimulate Shiga toxin release *in vivo* (89). The implication of this is that, antimicrobials may in the future be routinely administered or are already considered necessary to help treat STEC related illnesses (54, 89). This notwithstanding, there is already a narrow choice for medication available for the treatment of enteric *E. coli* (54), due to the high prevalence of resistant STEC strains, isolated from humans, and animals as well as the presence of intergrons conferring multi-resistance (86). Further still, multiple-drug resistant O157:H7 from food, animals and humans are increasingly being encountered (89, 86). The most frequently reported resistance phenotype of *E. coli* O157:H7 and O157: NM isolates being to streptomycin-sulfisoxazole-tetracycline, which accounts for over 70% of the resistant strains. Increasing resistance to fosfomycin, the drug of choice for paediatric gastrointestinal infections due to STEC infections in Japan, has also been documented (89).

Non-O157 STEC isolated from humans and animals have also developed antimicrobial resistance phenotypes, and many are resistant to multiple antimicrobials commonly used in human and veterinary medicines (89). As a rule, resistance levels in *E. coli* are usually high for broad-spectrum penicillins and trimethoprim, and low for third-generation cephalosporins and nitrofurantoin (86).

When studying resistance levels of bacteria from persons involved in animal handling, such as abattoir workers, *E. coli* is said to be the organism of choice as a model (60). This is because *E. coli* strains efficiently exchange genetic material with pathogens such as *Salmonella*, *Yesinia* and *Vibrio* species, as well as pathogenic *E. coli* (63). Further more, studies with *E. coli* are of particular relevance because this species is a commensal in both humans and animals. This makes commensal *E. coli* a useful indicator of the antimicrobial resistance in bacteria in the community (63).

2.8.3 Enterococci

Enterococci spp. are part of the lactic acid bacteria (LAB) and their characteristics include being ubiquitous in occurrence, their habitat consisting of the intestinal tract of humans and animals plus a variety of foods and feeds. Therefore enterococci are not only considered faecal contaminants (indicators of poor hygiene), but also as normal parts of food microflora (50).

When they cause disease, the clinical features of enterococcal infections are variable, and may include any anatomical site, and may be life threatening during bacteraemia and endocarditis. In fact enterococci are now viewed as emerging major nosocomial pathogens, and are considered the second most common cause of nosocomial infections in the USA (14, 17, 40, 68). Almost all nosocomial enterococcal infections caused by either *E. faecalis* or *E. faecium* arise in the urinary tract or intra-abdominally (17). This genus has the ability to cause serious infections when immunity of the host is low, and have been associated with critically ill people for a long time (14, 17, 69, 88).

Presently there are about twenty validly published species of enterococci (50), but of these, the four predominant species in poultry intestinal flora are *E. faecalis*, *E. faecium*, *E. hirae* and *E. durans* (78). However, of these four, only *E. faecalis* and *E. faecium* have public health significance in that they are the most frequently isolated species in humans, and are associated with antimicrobial drug resistance (17, 40, 50). In the USA alone, these two species account for approximately 85% and 10% of clinical isolates respectively (40, 50). While it is reported that *E. faecium* is the most important nosocomial pathogen especially among immuno-compromised individuals (51, 14), and that it is commonly associated with greater morbidity and mortality, *E. faecalis* is reported as the most common cause of enterococcal infections (40). In some parts of the world, like Britain, an increase in the proportion of enterococci among blood culture isolates of between 3% in 1971 and 12% in 1985 has been observed (17).

Glycopeptide resistance and high level aminoglycoside resistance (HLAR) are often associated with *E. faecalis* and *E. faecium* from both animals and humans (50, 78), which makes the use of enterococci strains in the food industry a potential public health hazard

(enterococci are applied in food fermentation processes or for the improvement of the sensorial quality of foods and as probiotics in food and feed). This is because the *van*A gene cluster encoding for vancomycin resistance in animals and human VRE (14, 88) that is located on a transposon designated Tn1546, can spread from one enterococcal species to another as well as to other pathogenic bacteria, for example, *S. aureus* (88). The European Commission has established a testing scheme regarding antimicrobial resistance for bacteria used in animal nutrition. The objective is to ensure that before an *Enterococcus* strain is used as a starter or probiotic culture in feed, presence of transferable resistances is excluded (50).

Enterococci tend to be resistant to many antimicrobials (88), but where pressure to select for resistance does not exist, *E. faecalis* and *E. faecium* are generally susceptible to avilamycin, erythromycin, vancomycin and virginiamycin. The exception to this rule is *E. faecalis*, which is intrinsically resistant to the streptogramin virginiamycin (3). In Spain, the traditional treatment for enterococcal infections is penicillin usually in combination with an aminoglycoside. However, in patients with hypersensitivity to penicillin or cases of infections due to β-lactam resistant enterococci, glycopeptides especially vancomycin, are the drugs of choice (50, 78). *Enterococci* constitute one of the best examples of the bacterial quest for survival. For years these organisms were viewed as harmless inhabitants of the gut flora, but have now acquired resistance to multiple antimicrobials, making vancomycin one of the last available compounds that still exhibit efficacy to these organisms (17, 88). Worldwide emergence of glycopeptide-resistant *Enterococci* plus HLAR, coupled with the increase in their occurrence, poses a serious threat to the continued possibility of curing infections in humans (3, 51, 78), more so in immuno-compromised patients (17).

On the other hand, *E. faecium* and *E. faecalis* are recommended as indicator bacteria for resistance to antimicrobial agents that are active against Gram-positive bacteria, while *E. coli* serves as an indicator for Gram-negative organisms (60).

VRE first claimed clinical attention in a renal unit in the UK in 1986. This was followed by reports in France in the same year and then other parts of Europe. Three years later (1989) it was found in the USA, where they have become endemic as nosocomial infections (1,14,17). While in 1989 all enterococcal blood isolates in USA were susceptible to vancomycin,

between 1995 and 2000 the proportion of resistant strains increased from 12.8% to 25.9% respectively (17). A prevalence rate of up to 15.4% VRE human strains has been reported in the USA (78, 88), whilst in a study done in Spain a 1.8% prevalence rate was found (78). In the USA the emergence of nosocomial *E. faecium* infections was first characterised by increased resistance to ampicillin in 1980s, followed by a rapid increase of VRE (17, 51). The risk factors that influence acquisition of VRE by humans are exposure from insufficiently heated food or cross-contaminated ready to eat foods. Heavy uses of vancomycin and probably also third-generation cephalosporins, including travellers returning from abroad, tourists, asymptomatic faecal carriage of VRE by the community and imported food are prerequisites for frequent VRE infections in hospitals (14, 88). The occurrence of VRE in food of animal origin is well documented (50, 88). Worth noting is the fact that *vanA* containing enterococci is the most common in Europe and America, and is said to be responsible for high level vancomycin and teicoplanin resistance (17). Two antimicrobials with activity against VRE have been introduced, both of which have only bacteriostatic activity. One of them (quinupristin-dalfopristin) is only active against *E. faecium* and worse still, transferable resistance to the combination has been described. Furthermore, prolonged treatment of VRE with the other, linezolid, has already been associated with the development of resistance and treatment failure in VRE infections. Consequently the limited antimicrobial possibilities continue to make prevention of the spread of VRE a major health-care issue in developed countries (17).

2.8.4 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, anaerobic, spore forming, and non-motile bacterium, able to produce various toxins and enzymes responsible for the associated lesions and symptoms. *Clostridium perfringens* strains are categorised into five toxinotypes: A, B, C, D, and E, based on the production of four major toxins (α, β, ε, ι) (25, 47, 85).

The incidence of *Cl. perfringens* in the intestinal tract and in processed meat of poultry is high; with 75% to 95 % testing positive when intestinal contents of chickens are analysed (85). *Clostridium perfringens* is also widespread in the environment, such as water and soil.

It has also been shown that the intestinal droppings of wild birds contain high numbers of *Cl. perfringens* and that free living birds can suffer from necrotic enteritis (85).

Colonisation of poultry with *Cl. perfringens* is suggested to take place very early in the life of animals, and can be transmitted within the integrated broiler chicken operation, starting from the hatchery. It is has actually been shown that *Cl. perfringens* contamination found on processed broiler carcasses can originate in the breeder operation and can be transmitted through the hatchery and grow-out operations (25, 85). Other sources of infection include environmental sources such as contaminated feed, water or any part of broiler production or plant (85).

Clostridium perfringens infections in poultry may present as acute clinical disease or subclinical disease. The acute form of the disease leads to increased mortality in the broiler flocks, which can account for 1% loss per day for several consecutive days during the last weeks of the rearing period. In the subclinical form, damages to the intestinal mucosa caused by *Cl. perfringens* leads to decreased digestion and absorption, reduced weight gain and increased feed conversion ratio. It has also been shown that the sub-clinical form of *Cl. perfringens* causes cholangiohepatitis and leads to an increased number of condemnations at poultry processing plants due to liver lesions (25, 85). Both necrotic enteritis and the subclinical forms of *Cl. perfringens* infections are caused by *Cl. perfringens* type A, and to a lesser extent type C (85).

Clostridium perfringens in poultry constitutes a risk factor for transmission to humans through the food chain, and is one of the most frequently isolated bacterial pathogens in food borne diseases in humans after others like *Campylobacter* and *Salmonella*. Worthy of note, is that poultry amongst other foods has been associated with outbreaks of *Cl. perfringens* (47, 85). In humans *Cl. perfringens* causes two types of food borne diseases; type A diarrhoea and type C necrotic enteritis, caused by enterotoxin-positive *Cl. perfringens* type A strains and *Cl. perfringens* type C strains, respectively (85).

Besides vaccination (still in the experimental stages), control of coccidiosis, use of competitive exclusion products and probiotics, and nature of feed, inclusion of growth

promoting antimicrobials have been used to prevent colonisation of *Cl. perfringens* in poultry (27, 85). Almost all growth promoting agents are known to have effect on colonisation of *Cl. perfringens* in poultry and the prevention of necrotic enteritis (85).

Clostridium perfringens is known to be susceptible to clindamycin, rifampicin, tetracyclines, chloramphenicol, metronidazole and penicillin. Of these, penicillin has been the drug of choice for prophylaxis and treatment of clostridial infections since the Second World War. In allergic patients chloramphenicol is the recommended treatment (76). However, studies show that clindamycin, rifampicin and metronidazole exhibit superior toxin suppression and rapid bacterial killing compared to penicillin, and hence better outcomes during therapy. Besides that a decreased susceptibility of *Cl. perfringens* to penicillins has been described (76).

CHAPTER 3

PILOT PROJECT

3.1 OBJECTIVES

The objective of the pilot study was to:

- Determine the practicality of sampling and processing one hundred samples (suggested in the research protocol as the sample target) on the same day the caecal harvesting is done;
- Assess the isolation rate and hence prevalence of each of the bacteria under study;
- Assess the level of antimicrobial drug resistance among isolates from the broiler flocks under study using the Kirby-Bauer disc diffusion method; and
- Run a trial test for the “Vancomycin Resistant Enterococci” (VRE) plates (Oxoid, UK) and assess the prevalence of VRE.

3.2 MATERIALS AND METHODS

3.2.1 Specimen collection

One hundred caecae were randomly collected from slaughtered broilers approximately five minutes after slaughter at one high throughput poultry abattoir in South Africa. Sample collection was done at a point on the slaughter line after the first inspection point, where carcasses with defects are identified and removed either to be condemned or to be cut up as portions. The aim of sampling from this point on the slaughter line was to ensure that the chickens sampled had been healthy before slaughter and therefore fit for human consumption.

The specimens were harvested by incising the caecae off the rest of the gastrointestinal tract. Harvesting of caecae was done using sterilised scissors and forceps. The caecae were then tied off at the open end so as to maintain an anaerobic internal environment. This was done to augment the survival of *Cl. perfringens* specifically as well as to prevent cross-contamination.

The caecae were placed in separate sterile plastic bags and conveyed in an insulated polystyrene container with at least 3 frozen ice packs to the laboratory. The specimens were processed within three hours of harvesting. In the laboratory each caecum was cut open, and one fraction (approximately 0.5gm) of caecal content from each sample was inoculated onto the relevant media to culture and isolate the different bacteria. For *Salmonella*, inoculum was first added to pre-enrichment medium before isolation on relevant media.

After the inoculum for isolation of different bacteria had been obtained, the rest of the caecal content was stored at minus 86 °C for the duration of the study.

3.2.2 Reference strains

The reference stains used in this study, were obtained from the bacteriology laboratory of the Department of Veterinary Tropical Diseases University of Pretoria. The following strains were used:

i. *Escherichia coli* ATCC 25922 and
ii. *Staphylococcus aureus* ATCC 29213.

3.2.3 Isolation and identification

3.2.3.1 *Salmonella* (serotypes belonging to Group 1)

Salmonella was isolated according to a standard method describe by Antunes *et al* (8) with some minor modifications. Initially, 25gm of sample was aseptically added to 200mls of the pre-enrichment medium, buffer peptone water (Oxoid, UK) and incubated at 42 °C for 48 hours. Thereafter, 1ml of incubated pre-enrichment mixture was added to 10 ml of Rappaport-Vasilidis broth (Difco, MI, USA) and incubated at 42 °C for 24 hours. A loopful of the broth was then streaked onto split Petri dishes (Plastopro Scientific, SA) with XLD (Difco, MI, USA) and Brilliant Green agar (Difco, MI, USA). These were in turn incubated at 37 °C. The plates were examined for the presence of typical colonies of *Salmonella* after 24 hours. Red colonies with a black centre on XLD agar (Difco, MI, USA) and pink colonies on brilliant green agar (Difco, MI, USA) were selected for sub-culturing onto blood agar for purification. The inoculated blood agar plates were then incubated at 37 °C for 18-24 hours.

Salmonella enteric group I was identified as follows:

- Colony characteristics e.g. no swarming on blood agar and uniformity of colonies and non-lactose fermenting
- Gram negative rods
- Catalase positive,
- Oxidase negative,
- Spot indole negative,
- Citrate positive
- Malonate negative,
- Dulcitol positive,
- Lysine positive,
- H_2S positive.

3.2.3.2 *Escherichia coli*

MacConkey agar (Oxoid UK) was initially aseptically inoculated with a swab of caecal content, and then spread onto the agar using an inoculation loop. The plates were then incubated at 37º C for 18 to 24 hours. After which plates were examined for uniformity of colonies, and one presumptive *E. coli* colony (large pink colonies due to lactose fermentation) from each plate was identified for purification by sub-culturing onto blood agar. The inoculated blood agar plates were then incubated at 37°C for 18-24 hours.

Identification of *E. coli* was carried using the following criteria:

- Uniformity of colonies,
- Gram negative rods,
- Catalase positive,
- Citrate negative,
- Oxidase negative,
- Spot indole positive,
- Lactose positive.

3.2.3.3 Enterococci

Kanamycin Aesculin Azide agar (KAA) plates (Oxoid, UK) were inoculated as described above to isolate enterococci. The plates were incubated at 37°C for 24 hours, and examined for typical colonies (small white colonies with a black halo due to aesculin fermentation). Presumptive enterococci colonies were sub-cultured onto blood agar and incubated at 37°C for 18-24 hours.

Enterococci were identified using the criteria below:

- Uniformity of colonies and haemolysis on blood agar,
- Gram positive cocci
- Tolerance to bile aesculin,
- Growth in 6.5% NaCl,

To differentiate between *E. faecium* and *E. faecalis*, the criteria depicted in Table 3.1 were used.

Table 3.1: Criteria for differentiating *E. faecalis* and *E. faecium*

TESTS	*E. faecium*	*E. faecalis*
Pyruvate	-	+
Arabinose	+	-
Lactose	+	+
sorbitol	-	+
Growth in 6.5% NaCl	+	+
Gram's stain	+	+
Haemolysis on blood agar	alpha	beta

3.2.3.4 Vancomycin resistant enterococci (VRE)

VRE selective agar (Oxoid, UK) was initially aseptically inoculated with a swab of caecal content, which was then spread onto the agar using an inoculation loop. The inoculated plates were incubated at 37 °C. After 24 hours the plates were inspected for growth. Though the majority of the VRE plates (Oxoid, UK) exhibited fermentation of aesculin, suggesting growth of vancomycin resistant organisms, only 18 plates grew discrete colonies. The presumptive VRE isolates (small white colonies with a black halo due aesculine fermentation) were subsequently sub-cultured onto blood agar and incubated at 37 °C for 18-24 hours.

3.2.3.5 *Clostridium perfringens*

Clostridium selective agar plates were inoculated as described above to isolate *Cl. perfringens*. Plates were incubated at 37 °C in the anaerobic chamber for 18 to 24 hours. Presumptive colonies (black colonies) were picked and inoculated onto blood agar. Inoculated blood agar was in turn incubated under the same conditions and examined after 48 hours of incubation for the presence of beta-haemolytic colonies.

Though no *Cl. perfringens* (as only two isolates had been cultured) were identified and stored at this stage, criteria that were to be used for *Cl. perfringens* identification are outlined below:

- A double zone of beta haemolysis on blood agar
- Gram positive squat rods
- No or minimal aerobic growth
- Catalase negative
- Non-motile
- On lactose-egg-yolk-milk agar it is lecithinase and lactose positive, non-proteolytic and lipase negative.

Only pure colonies (obtained on blood agar) of the different bacteria were subjected to the identification criteria to identify the isolate to species level.

3.2.4 Storage of isolates

Pure strains of overnight growth of each of *E. coli* and *Enterococcus* species positively identified according to the above criteria were inoculated into brain heart infusion broth (CA Milsch) placed in sterile 2ml cryotubes (Labretoria, SA) and stored at minus 86°C. Since no *Salmonella* was confirmed as belonging to *Salmonella enterica* Group I, none was stored. Of the 18 presumptive colonies of enterococci isolated on VRE plates, none was positively identified as being either *E. faecalis* or *E. faecium*, hence no VRE was stored.

3.2.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility (resistance) of the isolates was determined by the disc diffusion method, as described by the Clinical and Laboratory Standards Institute (CLSI) formerly called National Committee for Clinical Laboratory Standards (58). Antimicrobial susceptibility testing was only done for *E. coli* and enterococci isolates and not for *Cl. perfringens* and *Salmonella* due to low numbers and failure to isolate these species respectively.

Fifteen enterococci isolates and 10 *E. coli* isolates were subjected to antimicrobial susceptibility testing (AST). The following antimicrobial impregnated discs (Oxoid, UK) were used; ampicillin-AMP (10 µg), Baytril-ENR (5 µg), fosfomycin-FOS (30µg), neomycin-N (30 µg), sulphamethoxazole/trimethoprim-SXT (30 µg), doxycycline-DOX (30 µg), lincospectin-LS (109 µg), Lincomycin-MY (2 µg), gentamicin-CN (10 µg), vancomycin-VN (30 µg). Susceptibility or resistance of the *E. coli* isolates to eight antimicrobials (AMP, ENR, FOS, N, DOX, LS, MY, and CN) was determined, while that of the enterococci isolates was against ten antimicrobials (SXT, ENR, N, AMP, MY, FOS, DOX, LS, CN, and VN).

3.2.6 Results and discussion

From the 100 caecae sampled, *Salmonella* (n = 0), *Cl. perfringens* (n = 2), VRE (n = 0), enterococci (n = 35) and *E. coli* (n = 48) were isolated. Two species of enterococci targeted in this study i.e. *E. faecalis* (n = 5) and *E. faecium* (n = 30) were obtained. These findings with respect to the prevalence of the two species (*E. faecalis* and *E. faecium*) in poultry are in

contrast with those of studies done in the Canary Islands, Spain (78) that report *E. faecalis* as the predominant species in broilers.

Resistance or susceptibility was determined for antimicrobials used commonly for therapy and prophylaxis in chickens. No AMGP were included at this stage. This is because the bacteriology laboratory of the Department of Tropical Diseases does not routinely test for resistance or susceptibility against antimicrobials used as growth promoters and hence did not have discs for these antimicrobials.

The isolates for which susceptibility was determined in the pilot study and the AST results are indicated in Annexure I.

Table 3.2: Antimicrobial susceptibility of 10 of 48 *E. coli* isolates

Antimicrobial agent	**Number (%) resistant**
Ampicillin (AML)	6 (60)
Baytril (ENR)	10 (100)
Fosfomycin (FOS)	8(80)
Neomycin (N)	9 (90)
Sulpha-trimethoprim (SXT)	4 (40)
Doxycycline (DOX)	10 (100)
Lincospectin (LS)	9 (90)
Lincomycin (MY)	10 (100)
Gentamicin (CN)	5 (50)

Table 3.3: Antimicrobial susceptibility of 15 of 35 *Enterococci* isolates

Antimicrobial agent	Total number (%) of resistant isolates
Ampicillin (AML)	0 (0)
Baytril (ENR)	7 (47)
Fosfomycin (FOS)	6 (40)
Neomycin (N)	14 (93)
Doxycycline (DOX)	15 (100)
Lincospectin (LS)	15 (100)
Lincomycin (MY)	15 (100)
Gentamicin (CN)	1 (7)
Sulpha-trimethoprim SXT)	0 (0)
Vancomycin (VN)	2 (13.3)

Of the 48 *E. coli* isolates, only 10 were subjected to AST. The prevalence of antimicrobial resistance among *E. coli* isolates (n=10) to different antimicrobials is indicated in Table 3.2. Out of the 35 *Enterococci* isolates obtained, only 15 were subjected to AST. The prevalence of antimicrobial resistance among *Enterococci* isolates (n = 15) is indicated in Table 3.3.

All the *E. coli* isolates (100%) were resistant to the three or more antimicrobials; DOX, LS and MY, making it the predominant phenotype, followed by ENR, FOS, N (80%) and ENR, FOS, N, DOX, LS, MY (60%). The resistance rate observed for the antimicrobials named in these phenotypes was expected given that these antimicrobials are used on a regular basis in poultry. A resistance (50%) rate to gentamicin among the *E. coli* was higher than expected. This could be attributed to the low sample size that was tested. However, a rate of resistance to gentamicin (6.5%) that is still considered high has been reported in the recently published report by the South African National Veterinary Surveillance and Monitoring Programme for Resistance to antimicrobial Drugs (68). This is of concern given that gentamicin is only indicated for use in feline, canine and equine species in this country (7).

Of the fifteen enterococci isolates subjected to the AST, only two (13.3%) showed resistance to vancomycin, with six intermediate (needing higher than recommended dose). This relatively low level of resistance (compared to other isolates) was expected, given that

avoparcin ceased to be available on the South African market six years ago, and is hence not currently used in the flocks under study. However, the presence of resistance could be explained by the fact that while avoparcin was used in this country resistance to vancomycin developed, but has since not disappeared completely even after cessation of avoparcin use in South Africa. This is in agreement with what other authors have reported on the occurrence of VRE in countries where avoparcin had been withdrawn after many years of use.

The enterococci isolates in general showed a pattern similar to that of *E. coli* in that most isolates (100 %) were resistant to at least two or three antimicrobials. The predominant phenotype like *E. coli* was DOX, LS, MY (100%). However, unlike *E. coli*, this was followed by N, DOX, LS, MY (90%). Further still, unlike *E. coli*, a low level of resistance (1 %) to gentamicin was observed among the enterococci isolates. However, 100% *E. coli* and enterococci isolates were susceptible to potentiated sulphonamides (sulpha/trimethoprim) compared to 0% of enterococci.

3.2.7 Conclusion and recommendations

Given that the two are commensal organisms, the results obtained show that the flocks under study had a high intestinal carriage of both *E. coli* and enterococci as was anticipated. However, this could not be said of VRE, *Salmonella enterica* Group I and *Cl. perfringens* due to the low numbers (n = 2) in the case of the latter and failure to isolate the former on VRE plates (Oxoid, UK). Therefore the chance of getting a sufficient number of *Salmonella* and *Cl. perfringens* and VRE was likewise low.

Salmonella Enteritidis is a controlled disease in South Africa (8). It is therefore expected of farmers to implement measures aimed at preventing *Salmonella enterica* Group I infections in their flocks. The control measures employed include (among others) vaccination of parent stock with *S.* Typhimurium vaccines that offers cross protection against *S.* Enteritidis. With reference to the farms under study, there is an on-going in-house monitoring of *Salmonella* at the abattoir. It is also the policy of the company to condemn (not to be released for sale for public consumption) any batch of broilers that test positive for *Salmonella* at slaughter in the abattoir.

However, for *Cl. perfringens*, given that the organisms have a high incidence in the intestinal tract of chickens (85) this low isolation rate could have been due to the methodology used in isolation of *Cl. perfringens*, or more feasible, that the flocks sampled had been on antimicrobials and coccidiostats, which have been blamed for low isolation rates in some studies (47). It was decided that adjustment would have to be made in terms of adhering to conditions conducive for isolation of anaerobic bacteria. For example plating out the inoculums would have to be carried out in the anaerobic chamber and not on the bench.

Based on the practicality of sampling and being able to culture the selected organisms within 3 hours of sampling, it was decided that 100 caecae be randomly collected as in the pilot study. However, based on the isolation rate of the selected organisms in the pilot study, out of the 100 samples brought to the laboratory, 25 to 30 would be randomly selected for isolation of *E. coli*, enterococci, *Cl. perfringens* and VRE. Since the results obtained here suggest prevalence of zoonotic salmonellae as being low, it was decided that all the 100 samples collected from each farm be inoculated onto the relevant media to isolate *Salmonella*. The reason for this was to “cast the net” as wide as possible, to boost the chances of isolating zoonotic *Salmonella*.

The results of the pilot study agree with cited studies (9, 77) that report high prevalence of resistance among isolates from animals fed antimicrobial medicated feed. In view of this it was agreed to proceed with the project and include an assessment of the level of resistance among abattoir workers stationed in evisceration and “mala” (intestine) packing sections of the abattoir. The objective was to establish whether the level of resistance observed in the broiler isolates would be reflected in the human isolates. This would then be used to suggest the existence of transfer of resistance between the two populations.

The findings of this pilot study suggested that a sufficient number of *E. coli* and enterococci would be obtained, thus providing Gram-positive and Gram-negative bacteria for use to determine MIC’s. Because avoparcin in this country ceased to be used in poultry at least six years ago, a low number of VRE was anticipated. It was therefore hoped that by modifying the isolation technique e.g. ensuring conditions conducive for the isolation of anaerobic organism (in the case of *Cl. perfringens*) and the incubation of VRE plates at 42ºC instead of

37ºC (as was the case in the pilot study) the isolation rate of these two genera would be improved. It was therefore envisaged that this coupled with the plating out of a large number of samples (500 in case of salmonellae) would yield a sufficient number of isolates in the course of the project.

In conclusion the project looked promising in that:

i. A sufficient number of each of a Gram-positive (*Enterococcus* spp.) and Gram-negative (*E. coli*) isolates would be obtained for use in establishing the prevalence of resistance among these categories of organisms.
ii. The project would provide information on the prevalence of zoonotic *Salmonella* (on the farms under study), said in previous work (16) to be prevalent in SA poultry flocks.
iii. The project would also provide an idea of the distribution of the two *Enterococcus* species (*E. faecalis* and *E. faecium*) in the poultry flocks under study.
iv. The presence of a few vancomycin-resistant enterococci (as per the AST) reinforced the need to determine the prevalence of these bacteria in the flocks under study.

CHAPTER 4

PROJECT: MATERIALS AND METHODS

4.1 SAMPLING

4.1.1 Chicken specimens

The sampling procedure and handling of the specimens from the chicken carcasses was done as described in the pilot study. Sampling was carried out during the period July 2005 to December 2005.

Chickens are brought for slaughter at the abattoir used in the study in cycles that last seven weeks, during which all the chickens raised in a single growing cycle are slaughtered out. Sampling was therefore carried out once every four weeks so as to sample chickens from more than one grow out cycle. Five farms (n = 5) were sampled, which together with the one farm sampled in the pilot study brought the total number of farms sampled over the entire sampling time to 6 (n = 6). Selection of poultry farms was purposive in that the sampling date was set as the last Thursday of every month. Therefore the farm slaughtered out on that date was consequently selected for sampling. However, chickens selected for sampling were randomly selected off the slaughter line approximately five minutes after slaughter, and the caecae harvested as described in the pilot project. A total of 500 broilers were sampled from five farms over the six months period of the study, bringing the total number of broilers (including the hundred sampled in the pilot study) to 600 (n = 600).

4.1.2 Human specimens

Selection of humans for sampling was purposive so as to ensure that the subjects sampled had not been on antimicrobial treatment for at least three months prior to sampling. However, participation in the project by the abattoir workers and the control group was on a volunteer basis. Volunteers had to complete informed consent forms (Annexure II) approved by the

Medical Ethics Committee of the Faculty of Medicine, University of Pretoria as proof that their participation was on a voluntary basis.

Sampling of humans started in January 2006 and went on till June 2006. Full co-operation from the humans proved to be a problem, hence the protracted sampling period. This was to allow for repeated visits to the abattoir for a series of information sessions with the identified control group to solicit their participation in the project, and hence to allow for the sampling of as many people as possible. It was only after a full commitment on the part of these people had been achieved, that sampling began.

Only abattoir workers located in the evisceration and intestine (mala) packing areas of the abattoir were included in the study group. Furthermore, only people in the designated areas who had not been on any form of antimicrobial therapy for at least three months prior to sampling were requested to provide a faecal sample. This was ascertained by asking the volunteers whether they had been on antimicrobials (various forms of oral medication were described) within three months prior to the date of sampling, and by consulting with the community health nurse at the abattoir. Out of a possible 44 people, 29 volunteered and qualified to participate in the study. All volunteers were kept anonymous by assigning them numbers in the place of their names. This made it impossible to identify the volunteer and the sample he or she had provided. However, later on in the study, this turned out to be a disadvantage as a second round of sampling could not be organised to increase on the sample size. The reason for this being that it was difficult at this stage to rule out the possibility of sampling some people more than once.

The control group consisted of students and workers at the Faculty of Veterinary Science, University of Pretoria. Like the experimental group (abattoir workers), selection was purposive, and only people who had not been on antimicrobials for at least three months prior to sampling were requested to provide a sample. However, unlike the experimental group, there was no means of verifying whether or not they had been on antimicrobials other than asking them. In addition, people identified and selected to act as the control group were required not to have been in contact with poultry on AMGP or handled feed mixed with antimicrobials during the period of sampling or for at least three months prior to sampling.

Twenty eight people accepted, completed informed consent forms and qualified to serve as the control group. Like the experimental group, people who served as the control group were kept anonymous.

The abattoir workers and the people that formed the control group were each given a bottle with a spoon to collect the morning stool. The spoon was used to scoop off either the first or last faeces from the anal area. Both groups were implored not to pick the faecal sample for submission from the ground or toilet. The sample bottles with stool were dropped off at the company clinic (in a cooler box with ice packs) located on the premises of the abattoir as the volunteers reported for work in the morning. The samples were then transported to the bacteriology laboratory of the Department of Tropical Diseases Faculty of Veterinary Science. In the laboratory isolation of VRE, *Salmonella*, enterococci and *E. coli* was carried out.

4.2 ISOLATES AND IDENTIFICATION

As explained above, samples (n=100) were collected from broilers during each sampling session. Of these, 25-30 caecae were randomly selected for isolation of *E. coli*, *E. faecalis*, *E. faecium, Cl. perfringens* and VRE. To enhance isolating salmonellae, all the hundred samples were plated out for specific isolation. Isolation and identification of the different isolates was as described in Chapter Three paragraph 3.2.3. After identification to species level, pure overnight growth isolates were inoculated into brain heart infusion broth (Oxoid, UK) in sterile 2ml cryotubes (Labretoria, SA) and stored at minus 86˚C.

4.3 ANTIMICROBIAL USAGE PATTERNS

With the help of a structured questionnaire (Annexure III), a survey of the antimicrobial usage patterns over the period 2004 to 2005 was carried out to determine the types of antimicrobials used on the farms under study. This was followed by other short structured questionnaires conducted telephonically and by E-mail to obtain additional information during the course of the study, but not originally envisaged as necessary for the interpretation of results.

4.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING

4.4.1 Antimicrobial agents

4.4.1.1 Selection of antimicrobials for testing

Antimicrobials to be subjected to the minimum inhibitory concentration (MIC) test are listed in Table 4.1 below. Of these, ceftriaxone, erythromycin, nalidixic acid and vancomycin are not registered for use in poultry in South Africa. Ceftriaxone was included although cephalosporins have never been used in the nation's poultry flocks and it is only registered for use in humans. It would be used to assess the prevalence of resistance to cephalosporins, drugs used extensively in human medicine. Resistance to erythromycin is an indication of early resistance against the macrolide class of antimicrobials and nalidixic acid is an early indicator of resistance development in the fluoroquinolones. All the members of the tetracyclines and sulphonamides respectively have the same mode of action and can therefore be represented by one antimicrobial i.e. doxycycline in the case of the tetracyclines and sulphamethoxazole in the case of the sulphonamides. The ampicillin and the slightly more lipid soluble amoxycillin are analogues and thus the more stable ampicillin was used in AST as a representative of the beta-lactam antimicrobials. Virginiamycin, though not used in this particular flock for the sampling period, was included because of the potential for it to cause cross resistance in *Enterococcus* spp. to synercid, macrolides and lincosamides (antimicrobials with potential for use in humans), and which led it to being withdrawn as AMGP in the EU (23). The rest of the drugs had been used in the flocks under study for the duration of the sampling period, and more so bacitracin was included as the only AMGP that was used in these flocks.

As recommended (44), pure antimicrobial powders were obtained directly from the representatives of the manufacturers (Sigma-Aldritch, USA), with the exception of virginiamycin which was obtained from a commercial source (Philbro Animal Health, South Africa). All the antimicrobial agents were supplied with a lot number, potency (µg or international units (IU) per mg powder, or as a percentage active ingredient), including their expiry dates. Storage of these agents before and during antimicrobial susceptibility testing was according to the manufacturers recommendations.

Table 4.1: Antimicrobials included in the MIC panel

Antimicrobial	Group	Activity#	Human medicine#	Poultry
1. Bacitracin*	Peptide antibiotic	G +v	+	+
2. Virginiamycin*	Streptogramin/ Peptide antibiotic	G +v	-	+
3. Trimethoprim	Antibacterial Diaminopyrimidine	B	+	+
4. Fosfomycin	Peptide antibiotic	B	-	+
5. Doxycycline	Tetracycline	B	+	+
6. Ampicillin	Penicillin/β-lactam	B	+	+
7. Sulphamethoxazole	Sulphonamide	B	+	+
8. Enrofloxacin	Fluoroquinolone (3rd generation)	B	-	+
9. Vancomycin	Glycopeptide/Peptide antibiotic	G +v	+	–
10. Erythromycin	Macrolide	B	+	–
11. Nalidixic acid	Quinolone (1st generation)	B (G –v)	+	–
12. Ceftriaxone	Cephalosporin (3rd generation) /β-lactam	B	+	–

\# G +v = means active against Gram-positive organisms
G -v = means active against Gram-negative organisms
B = means broad spectrum
B (G-v) = broad spectrum but activity mainly against Gram negatives;

\# + = used in poultry or humans in SA; – = not used in poultry or humans in South Africa.

* Antimicrobial performance enhancers

4.4.1.2 Preparation of stock solutions

Preparation of stock solutions for the selected antimicrobials was done following a protocol described by the CLSI and ISO (44, 58). To determine the weight of the agents, the following formula that gives allowance for the potency was used (44, 58):

Weight of powder (mg) =

$$\frac{\text{Volume of stock solution [to be constituted (mL)] x desired concentration (mg/L)}}{\text{Potency of powder (mg/g)}}$$

The potency was provided by the suppliers, while the volume of the stock solutions to be prepared was set in house as 100 ml. The concentration was determined by doubling the starting concentration (highest concentration on test panel - see Annexure IV), giving the working concentration. This was in turn multiplied by 10 to get the desired (stock) concentration, which was then used to compute the desired weight of the antimicrobial. For example, the starting concentration for vancomycin was 256 µg/l (Annexure IV). To get the concentration used in the formula above, 256 µg/l was multiplied by two to get a working concentration of 512 µg/l. This was in turn multiplied by 10 to get the concentration (5120 µg/l) used in the formula for calculating the required weight of the powder (mg). A calibrated analytical balance was used to weigh antimicrobial agents, which were dissolved in 100 ml of solvent to make the stock solution. Where drugs must be dissolved in a solvent that is different from the diluent, only enough solvent to solubilize that antimicrobial agent powder was used, and the final volume made up with the appropriate diluent.

Table 4.2: Solvents and diluents for preparation and diluting of stock solutions of antimicrobial agents

Antimicrobial	Solvent	Diluents
Vancomycin	Water	Water
Virginiamycin	Minimum volume of ethanol fraction 95% to dissolve , then add water to make up volume	Water
Doxycycline	Water	Water
Trimethoprim	Half volume of water, a minimum volume 0,1 mol/L lactic acid to dissolve, then make up to total volume with water	Water
Sulphamethoxazole	Half volume water, a minimum volume 1mol/NaOH to dissolve, then make up total volume of with water	Water
Ampicillin	Phosphate buffer 0,1 mol/l, pH 8.0	Phosphate buffer 0,1 mol/l, pH 6,0
Bacitracin	Water	Water
Enrofloxacin	Half volume of water, then add NaOH 1 mol/L drop wise to dissolve	Water
Erythromycin	Ethanol volume fraction 95%	Water
Fosfomycin	Water	Water
Ceftriaxone	Water	Water
Nalidixic acid	Half volume of water, a minimum volume 1 mol/L NaOH to dissolve, then make up to total volume with water	Water

The solvents and diluents used in the mixing and diluting the stock solutions are listed in Table 4.2. With the exception of virginiamycin, bacitracin and fosfomycin, solvent and diluents used for all the antimicrobials were adopted from the CLSI and ISO documents (44, 58). Since bacitracin and fosfomycin are water soluble, water was chosen for the two as a solvent and diluent. Steven *et al* (75) suggest ethanol 95% (solvent for erythromycin) and water as solvents and diluents respectively for virginiamycin. The different phosphate buffer solutions used as diluents and or solvents were prepared in house using the prescribed recipes

of the Bacteriology Laboratory, Department of Tropical Diseases, University of Pretoria. Stock solutions were sterilized by filtering the solutions through a 0,22 µl sterile filter (Millipore, South Africa).

4.4.1.3 Preparation of the working solution

Table 4.3: Scheme for preparing dilutions of the various antimicrobial agents

Antimicrobial agent	Stock solution (µg/ml)	Dilution ratio	Working solution (µg/ml)	Starting concentration (µg/ml)
Virginiamycin	1280	1:10	128	64
Doxycycline	2560	1:10	256	128
Trimethoprim	640	1:10	64	32
Sulphamethoxazole	40960	1:10	4096	2048
Ampicillin	640	1:10	64	32
Bacitracin*	200	n/a	200	100
Enrofloxacin	904	1.56:5	16	8
Erythromycin	2560	1:05	512	256
Fosfomycin	2560	1:10	256	128
Ceftriaxone	1280	1:5	256	128
Nalidixic acid	2560	1:10	256	128

* Concentrations measured in units/ml

The working solution was prepared by diluting the stock solution using the recommended diluent (Table 4.2) in the ratio given above (see Table 4.3). This gave a concentration that was double that of the starting solution. The concentration ranges on the test panel (Annexure IV) to be tested were calculated so that the break points for the antimicrobials tested against

the organisms was at least two concentrations above the lowest concentration or two concentrations below the starting concentration. A total of eight concentrations for each antimicrobial agent were tested (Annexure IV). Note that resistance of *E. coli* to vancomycin, and *E. faecalis* to virginiamycin and nalidixic acid and *E. faecium* to nalidixic acid was not tested.

4.4.2 Preparation of bacterial inoculum

Isolates stored in brain heart infusion broth (Difco laboratories, USA) were reactivated by thawing the organisms in the 2 mL cryotubes (Labretoria, South Africa) and thereafter culturing onto Columbia blood agar (Oxoid, UK) to which 5% horse blood was added, and incubated at 37ºC overnight. Four or five overnight pure colonies (to avoid selecting atypical variant colonies) from blood agar plates were emulsified in saline water, while adjusting the turbidity of the inoculated saline water to visibly compare to that of the 0.5 McFarland turbidity standards. The rational for this as explained in the CLSI document (58), was to ensure that after inoculation, each well contained approximately 5 x 10^5 colony forming units per ml (CFU/mL). After this, 10µl of the test organism emulsified in 0.9% saline water was placed in 20ml of cation adjusted Mueller-Hinton broth (in test tubes) (Oxoid, UK) that meet the requirements for testing non-fastidious organisms as stipulated in both the ISO/FDIS 20776-1: 2006(E) and CLSI document (44, 58). The cation adjusted Mueller-Hinton broth with the inoculum was then dispensed into sterile plastic Petri dishes (PlastoPro, South Africa) to facilitate picking up the inoculum to inoculate the sterile, round bottomed 96 micro well plates (Sterilab, South Africa).

4.4.3 Preparation of the 96 micro well plates

Using a micropipette, 100µl of the diluent was dispensed in all the wells with the exception of the first row. Thereafter 200 µl of the working solution of each antimicrobial was dispensed into its appropriate uninoculated first well as indicated in Annexure IV. With the help of a sterile multi-channel pipette 100µL of the antimicrobial solution was transferred to the second well, mixed three times and transferred serially to all the wells in the column, with the last 100 µl being discarded into disinfectant solution. This resulted in a two-fold dilution series of each antimicrobial. Two control wells were present in each plate, to which no antimicrobials

were added. One served as the positive growth control with 100 µl of the bacterial suspension added and the other as a negative control and contained cation-adjusted Mueller-Hinton broth.

Fresh starting solutions and dilutions of 96 well plates were prepared each time the tests were performed to avoid repeated freeze-thaw cycles which accelerate the degradation of some antimicrobial agents particularly β-lactams (44).

4.4.4 Incubation

To prevent drying, each tray was sealed with a sterile plastic sheet (Amersham, South Africa). Inoculated plates were incubated at 35º C for 18-20 hours in an aerobic incubator in stacks of strictly four trays, to allow for even incubation temperature distribution between the trays (60).

4.4.5 Determination of MIC's and reading of results

Reading of test results was done with the help of a viewer mirror that displays the underside of the wells (Figure 4.1).

The criteria used in the interpretation of the results (Figure 4.2) as seen in the viewer, is adapted from that used by Nel in her MSc dissertation (60). This criterion caters for instances where the appearance of certain wells does not conform to the criteria for testing procedures. For example, some wells appeared as fading end-points. This occurred for only sulphonamides and was considered normal. Other appearances that did not conform to the criteria included "skips", where growth occurred at lower concentrations, skipped one or more concentrations and grew again. In this case if only one well was skipped, the higher concentration was accepted as the MIC. Some discrepancies also occurred where there was contamination or mixed growth. Where the contamination involved one well, the results were also accepted. However, where it was considered that the discrepancies affected the test results, plates were discarded and tests run again. The MIC's of isolates were determined as the lowest concentration that inhibited bacterial growth in the wells (Figure 4.2) (60). Isolates were then classified as either resistant or susceptible using resistance break points published by the CLSI, 2004 Veterinary monitoring of antimicrobial resistance in Spain report and the

2005 report on Swedish antimicrobial utilisation and resistance in veterinary medicine as reference points (available at: www.sva.se). Isolates with MIC values above these reference break points were record as being resistant while those that had MIC's below the reference break points were considered as susceptible.

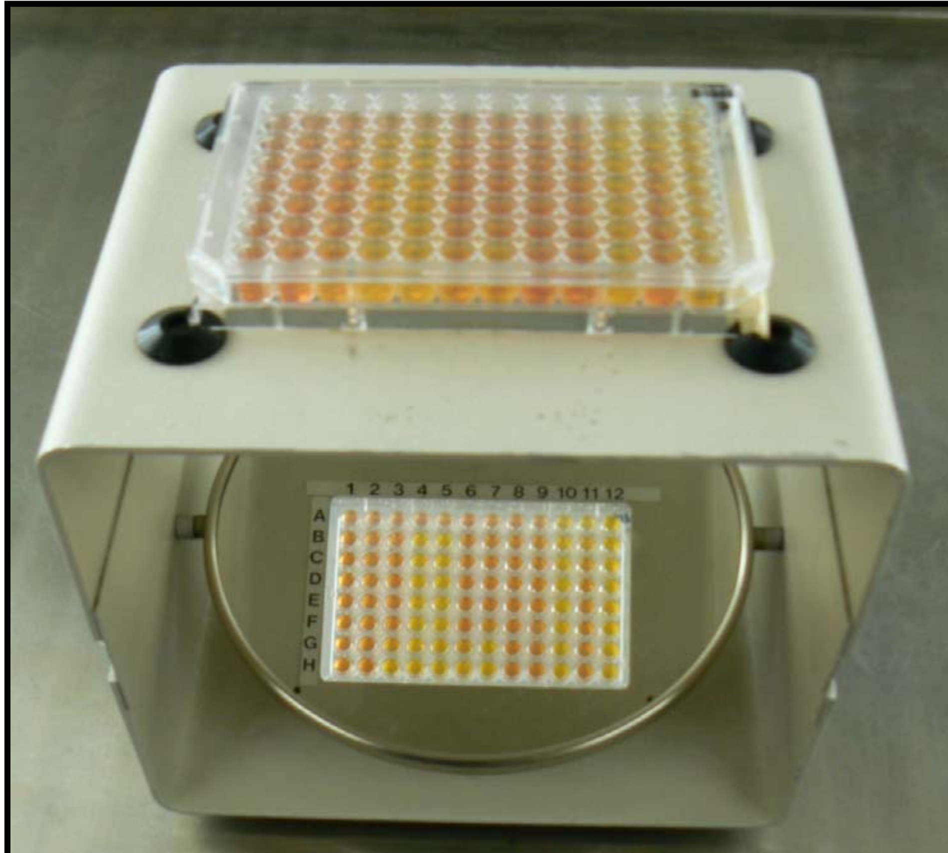

Figure 4.1: A viewer that displays the underside of the wells

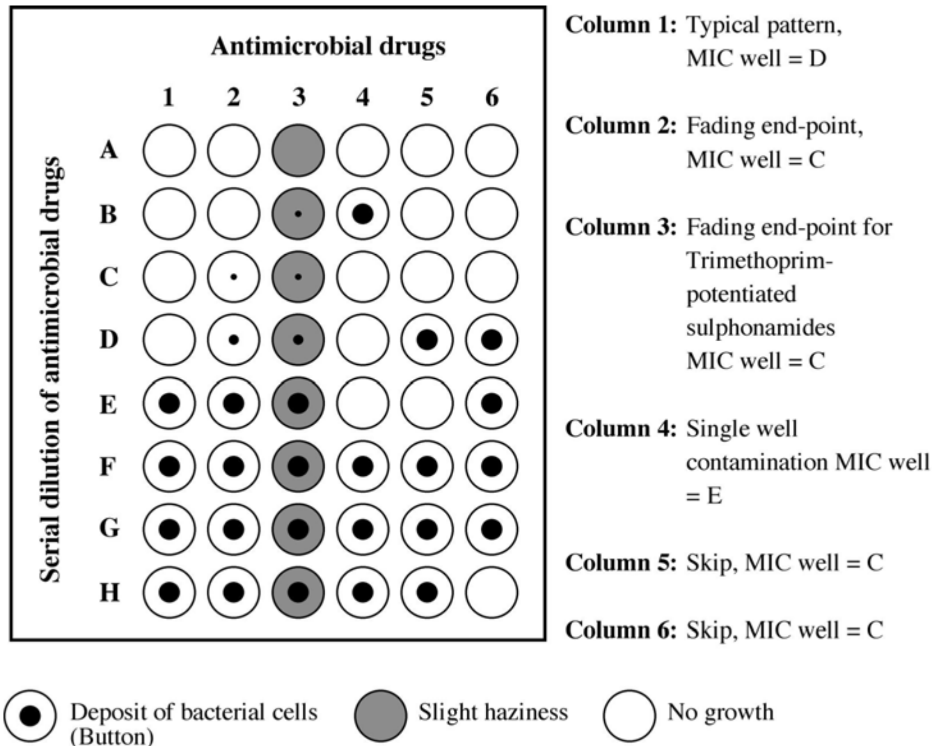

Figure 4.2: Criteria for interpreting of results (60)

In Figure 4.2, A-H indicates the different dilution concentrations in descending order of the antimicrobial drugs that were tested. Rows 1-6 were used for the simultaneous testing of different antimicrobial drugs. Where buttons of bacteria are visible, the bacteria are still viable, but where the buttons are no longer visible or growth was less than 50%, the antimicrobial drug concentration at the point inhibited the growth of the bacteria (60). According to the criteria, the first well for each antimicrobial in which there was no growth or growth was less than 50% and hence determined as the MIC was marked off with a cross on the test panel (Annexure IV).

All isolates were identified and labelled as follows:

- Specimen number
- Date of isolation
- Bacteria genera
- Group from which it was isolated.

Susceptibility for all *E. coli* isolates was established for only antimicrobials for which inherent resistance is not reported e.g. doxycycline, trimethoprim, sulphamethoxazole, ampicillin, enrofloxacin, fosfomycin, ceftriaxone and nalidixic acid. Hence drugs like bacitracin, vancomycin, virginiamycin and erythromycin to which inherent resistance is known to occur where not included in the test panel. The same principle was applied when selecting antimicrobials to include in the test panel for enterococci isolates, and so AST was only performed for the following antimicrobials: vancomycin, virginiamycin, doxycycline, trimethoprim, sulphamethoxazole, ampicillin, bacitracin, enrofloxacin, fosfomycin, and erythromycin. Others for which inherent resistance is a problem e.g. nalidixic acid were not included.

4.4.6 Controls

The reference strains used are described under the pilot project (paragraph 3.2.2). Reference strains were tested each time new batches of microdilution plates were inoculated. Results of these tests were compared with expected values given by the CLSI (Table 4, Document M31-P, VOL. 14 NO. 20) for the following antimicrobials: erythromycin, ampicillin, tetracycline, vancomycin, and trimethoprim/sulphamethoxazole. For others antimicrobials not listed in the CLSI document but were tested, the control stains should have been susceptible to published MIC's. When the MIC's of the reference strains did not fall between the required ranges, the results were discarded and test run again. Growth was not expected in the negative control wells as they were not inoculated with organisms. But when growth occurred in these wells, it indicated contaminated Mueller-Hinton agar, and so results were discarded and tests run again.

Ten microlitres (of the test organisms and the control strains used to inoculate the 96 micro well plates) were inoculated on to blood agar each time tests were run to test for purity and bacterial concentration of the inoculum. These plates were incubated at 35ºC for 18-20 hours in an aerobic incubator. If the colonies on the blood agar were not uniform, it indicated contamination and so results were discarded and tests repeated. Likewise inoculums had to yield 30 – 50 colonies per 10 micro litres inoculated onto blood agar plates for the results to be accepted.

4.4.7 Data analysis

Recorded data were analysed by Professor Peter Thompson from the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria. The statistical package used was Stata 8.2 (StataCorp, College station, TX, USA). Medians of the MIC values were compared between groups using the Wilcoxon rank-sum test. The following comparisons were done:

- The median MIC's of eviscerators and the packers,
- The median MIC's of the packers and the control group,
- Percentage comparison of resistant isolates from broilers and abattoir workers,
- Percentage resistant isolates from broilers and the control group,
- Percentage resistant isolates from abattoir workers and the control group.

Spearman's rank correlation test was also done to determine the correlation coefficient. The following correlation studies were done:

- Correlation of resistance between *E. coli* isolates from broilers and abattoir workers.
- Correlation of resistance between enterococci isolates from broilers and abattoir workers.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 ISOLATES

Table 5.1 The number of isolates obtained from the different populations sampled

Source	Bacteria species	Number of isolates stored
1. Broilers	*Escherichia coli*	168
	Enterococcus faecalis	20
	E. faecium	96
	Salmonella	0
	Clostridium perfringens	2
	Enterococci on VRE plates	0
2. Abattoir workers	*Escherichia coli*	28
	Enterococcus faecalis	21
	E. faecium	2
	Salmonella	0
	Clostridium perfringens	0
	Enterococci on VRE plates	0
3. Control group	*Escherichia coli*	26
	Enterococcus faecalis	3
	E. faecium	10
	Salmonella	0
	Clostridium perfringens	0
	Enterococci on VRE plates	0

The number of isolates cultured is summarised in Table 5.1. The proportions of *E. faecium* and *E. faecalis* [isolated on KAA-(Oxoid, UK)] in broilers, as in the pilot study, differed from what was obtained in a study done in the Canary islands, Spain (78). In the latter *E. faecalis* tended to be more prevalent (making up 63% of the isolates), while 8.1% of the isolates were *E. faecium*, and rest (*E. mundtii*, *E. casseliflavus*, *E. durans* and *E. hirae*) constituting the remaining 27.3%. However, the findings of this study agree with what was observed in Denmark (48), where more *E. faecium* (52%) than *E. faecalis* (15%) were isolated from broilers. One reason that could explain this inconsistency, is that suggested by Kuhn *et al* (48), who are of the view that the distribution of the two species among isolates from the abattoir is not only dependent on the animal species but also the geographical region.

Previous studies done in South Africa (16) suggest that *S*. Enteritidis and *S*. Typhimurium are commonly isolated from poultry flocks in South Africa. However, findings of this project suggest otherwise, as no isolate of these species was obtained. As was the case in the pilot study, no vancomycin resistant *E. faecalis* and *faecium* (VREF) were isolated on VRE plates. This could be due to the very low levels of VRE in the population studied or the selective method used, given that enrichment of the inoculum in broth before plating out onto selective agar was not done.

After three farms had been sampled, only two *Cl. perfringens* isolates from broilers had been cultured, which meant that number of *Cl. perfringens* isolates that would be obtained would be very low and would not give statistically meaningful results. According to Kalender and Ertas (47), flocks fed on feed containing antimicrobials and coccidiostatic drugs tend to yield a low number of *Cl. perfringens* (5%). This could explain failure to isolate *Cl. perfringens* as the broilers sampled were on antimicrobials (the registered sulfonamide plus trimethoprim) and salinomycin, clinacox and monensin as coccidiostatic drugs.

The numbers of both *E. faecalis* and *E. faecium* isolated from abattoir workers differed greatly, with the percentage of *E. faecalis* being much higher (92%) compared to *E. faecium* (8%). The prevalence of *E. faecalis* and *E. faecium* observed among abattoir workers also differed from what Klein (50) reports for humans, with more *E. faecalis* than *E. faecium* being cultured from the group in this study. The distribution of the two species among abattoir

workers differed from that observed in poultry isolates in that, while *E. faecalis* was more predominant in abattoir workers, *E. faecium* was the predominant species in broilers. However, these results agree with those from studies done in Sweden where more *E. faecalis* were obtained in clinical isolates, hospitalised patients, and hospital sewage (80%, 57%, and 54%, respectively) (48). The fact that the distribution of the two species of enterococci differed between broilers and abattoir workers, with the later having *E. faecalis* as the predominant species as opposed to broilers, suggests that there is no or minimal movement of enterococci from the broiler carcasses to the abattoir workers sampled. This could be attributed to workers wearing gloves when handling intestines, as was observed during the visits to the abattoir during sampling.

Workers at the abattoir under study are regularly screened for *Salmonella* as part of the control programme to prevent contamination of poultry meat by workers with these organisms. In view of this, it was expected that no *Salmonella* would be cultured from this group of people.

The distribution of the *Enterococcus* species in the control group was in agreement with Klein's (50) findings among humans i.e. fewer *E faecalis* (n= 3) than *E. faecium* (n= 10) isolates. While the distribution of the two species among the control group differed to that observed among isolates from abattoir workers, it was similar to that observed for poultry isolates i.e. *E. faecium* carriage was higher than that of *E. faecalis*. As with the experimental group, no VRE and *Salmonella* isolates were obtained. Attempted isolation of *Cl. perfringens* was terminated before sampling of control group commenced. The reason for this was that *Cl. perfringens* isolates had not been cultured from the experimental group. Hence there would be no results for comparative purposes.

5.2 MINIMUM INHIBITORY CONCENTRATION (MIC) TEST RESULTS

The percentage MIC distribution of each bacterium and group from which the isolates were obtained, is summarised in Tables 5.2 to 5.12. The concentrations of bacitracin were measured in Units/ml and not µg/ml as was the case for other antimicrobials. Hence the MIC distribution for bacitracin (Tables 5.5 and 5.6) were not included in the same tables with other antimicrobials.

The percentages of isolates with MIC's higher than the cut off points were indicated as percentage resistant. These tables give a comparison of the MIC's from the different populations (broilers, abattoir workers and the control group) as well as the distribution of the MIC's in each dilution range for each antimicrobial drug. The areas that are not shaded depict the dilution range tested for each antimicrobial and the occurrence of the isolates for each dilution. The shaded areas represent the dilution ranges that fell outside the tested ranges. Isolates that had MIC's higher than the tested ranges were indicated in the shaded areas. The isolates that had MIC values lower than the tested ranges were either grouped with the ones that fell in the lowest concentration tested or indicated as belonging to the lowest concentration. The individual MIC values for *E. faecalis*, *E. faecium* and *E. coli* tested are not reflected in this document due to the large size of the file (90 pages of spread sheet) in which they were recorded.

Table 5.2: Minimum inhibitory concentrations of antimicrobial agents: *E. coli* from broilers/farm (n = 168)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Doxycycline	98.2								0.6	1.2	8.3	36.9	28.6	23.2	1.2				
Trimethoprim	33.9					51.8	5.4	4.2		4.8	30.4	2.4	1.2						
Sulphamethoxazole	78.7											8.1	6	4.2	3	2.4	4.2	5.4	66.7
Nalidixic acid	90.5							4.2	3	2.3	3	6	11.9	10.7	58.9				
Enrofloxacin	75.6		8.3	4.8	11.3	15.5	8.3	3	0.6	1.2	47.0								
Ceftriaxone	39.3						60.7	2.4	1.8	23.8	10.1	0.6		0.6					
Ampicillin	75						25	5.4	2.4	2.2	4.8	4.8	55.4						
Fosfomycin	98.2								0.9		0.9	4.2	7.1	7.1	79.8				

[a] The white fields denote range of dilutions tested for each substance.

MIC's above the range are given as the closest to the range in shaded areas, while isolates with MIC less than the range tested were grouped together with those with the lowest MIC.

The vertical bars represent the reference cut-off point**.**

The majority of the *E. coli* isolates from broilers (Table 5.2) had MIC values that were considered resistant to doxycycline (98.2%), sulphamethoxazole (78.7%), ampicillin (75%), enrofloxacin (75.6%) fosfomycin (98.2%) and nalidixic acid (90.5%) all of which, with the exception of fosfomycin are used in humans in South Africa. These findings are consistent with previous and recent studies that reported a high level of resistance among isolates from broilers in South Africa (37, 53, 69).

A high level of resistance (90.5%) observed against nalidixic acid is an early indication of resistance development to the quinolone group of antimicrobials as a result of cross resistance. In view of this, the high resistance to nalidixic acid is probably as a result of using enrofloxacin, a drug widely used in the poultry industry in South Africa and to which a high levels of resistance (75.6%) was observed. This is confirmed by SANVAD in the recently published report in which the resistance rate to enrofloxacin recorded was 65.2% (69).

The prevalence of resistance to ceftriaxone (39.3%) among broiler isolates although low compared to what was observed for other antimicrobials was not expected. It is reported that exposure of *E. coli* to low levels of tetracycline induces an expression of genetic loci that regulates susceptibility to cephalosporins, penicillin, chloramphenicol, tetracycline, nalidixic acid and fluoroquinolones (59). Since the flocks sampled had been on tetracycline at the time of sampling, this could account for the level of resistance observed to ceftriaxone (a cephalosporin) even though the isolates tested had not been exposed to these antimicrobials at the time. It is known that the primary cause of resistance in a large number of Gram-negative bacilli like *E. coli* is the ability to generate ESBL, enzymes which can inactivate the penicillin and cephalosporin class antibiotics. In addition, this type of resistance is known to manifest rapidly (59). It is therefore also possible that these *E. coli* isolates exhibit ESBL. Alternatively, this resistance to ceftriaxone could be attributed to cross resistance with amoxicillin, a β-lactam to which the isolates were exposed.

Table 5.3: Minimum inhibitory concentrations of antimicrobial agents: *E. faecalis* from broilers/farm (n = 20)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	5							95					5						
Doxycycline	95									5		50	45						
Trimethoprim	20				80								20						
Sulphamethoxazole	70												10		20	15	10	10	35
Ampicillin	0				60	20	20												
Enrofloxacin	55			20	25	40		5	10										
Erythromycin	100											5	5	5	5	80			
Fosfomycin	95												5	15	80				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the reference cut-off point.

Note: Bacitracin results were not include here because its MIC's were measured in Units/ml (Table 5.5)

Table 5. 4: Minimum inhibitory concentrations of antimicrobial agents: *E. faecium* from broilers/farm (n = 96)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	2.1								97.9				1	1					
Virginiamycin	0					9.4	8.3	26	34.4	16.7	5.2								
Doxycycline	96.9									3.1	4.2	54.2	38.5						
Trimethoprim	0				99			1											
Sulphamethoxazole	92.7											2.1	2.1	1	2.1	4.2	10.4	7.3	70
Ampicillin	12.4				13.5	22.9	22.9	12.5	10.4	5.2	10.4	1	1						
Enrofloxacin	92.7		1.04	3.1	3.1	5.2	12.5	25	44.8	2.1	3.1								
Erythromycin	100											1	9.4	3.1	5.21	81.3			
Fosfomycin	99												1			99			

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the reference cut-off point**.**

Note: Bacitracin results were not include here because its MIC's were measured in Units/ml (Table 5.6)

Table 5.5: Minimum inhibitory concentrations (MIC's) of bacitracin against *E. faecalis* isolates

Source	% resistant	No of *E. faecalis* isolates (%) with the following MIC's (Units/ml) for bacitracin									
		≤ 0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	> 100
Farm (n = 20)	60		5	5	10	5	5	10	25	10	25
Workers (n=21)	9.5		47.7	9.5		4.8	14.3	14.3	9.5		
control group (n =3)	0		33.3		66.7						

[a] The white fields denote range of dilutions tested for each substance.
MIC's below or above the range are given as the closest to the range in shaded areas.
The vertical bars represent the reference cut-off point.

Table 5.6: Minimum inhibitory concentrations (MIC's) of bacitracin against *E. faecium* isolates

Source	% resistant	No of *E. faecium* isolates (%) with the following MIC's (Units/ml) for bacitracin									
		≤ 0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	> 100
Farm (n = 96)	44.7		4.2	3.1	1	1	21.9	24	33.3	6.3	5.1
workers (n = 2)	0			50	50						
control group (n = 10)	0		20	20	20	10	20	10			

[a] The white fields denote range of dilutions tested for each substance.
MIC's below or above the range are given as the closest to the range in shaded areas.
The vertical bars represent the reference cut-off point.

The majority of enterococci isolates from broilers had MIC values for vancomycin (Tables 5.3 and 5.4) considered as susceptible and hence low levels of resistance (5% for *E. faecalis* and 2.1% for *E. faecium*) were observed. The difference in the percentages observed between the two species (*E. faecalis* and *E. faecium*) to vancomycin in this study could not be explained. As has been reported (14, 69), it was anticipated that vancomycin resistance would be higher in *E. faecium* as compared to *E. faecalis*. However, the low rates of resistance reported here were anticipated since avoparcin an analogue of vancomycin was not used in the flocks studied, and has not been available for use in South African poultry flocks since its production was stopped after it was banned in the EU. The resistance observed here is due to the fact that once resistance to specific antimicrobials develops, it has a tendency to persist at low levels even after the drug has been withdrawn (3, 13, 17, 43, 57, 59), meaning that once the problem has been created it takes a while before it can be remedied, if at all. This is because when the antimicrobial pressure is removed, the genetic material containing the resistance gene is retained. Hence withdrawal of the relevant antimicrobials only results in a reduction of the prevalence of resistant strains, but does not completely eliminate them (59). For example, 3 to 6 years after the ban of avoparcin, resistant *E. faecium* could still be found among broilers and pigs in Denmark (3, 13, 17). In Finland it was observed that resistance levels among enterococci isolates remained at 11% for avoparcin, 19% for bacitracin and 17% for virginiamycin in studies conducted after the use of these antimicrobials as feed additives had been discontinued (57). Where the all-in all-out system is practiced, the prevalence of resistant microbes seems to gradually decline, and may only be reduced over time given that successive generations do not have direct contact with the intestinal flora of adults. On the contrary, this is not the case in animals like pigs grown on a continuous production system, and their young become exposed to the intestinal microflora of older ones early in life (13). It is therefore possible that if the isolates studied here were from pigs, a higher level of resistance could have been observed. Worthy of note is that the work did not substantiate the expectation that poultry VRE isolates tend to carry *van*A genes that have been associated with very high MIC values (≥ 128 µg/ml).

The majority of *E. faecalis* isolates from broilers (see Tables 5.3 and 5.5) had MIC values described as resistant to doxycycline (95%), sulphamethoxazole (70%), enrofloxacin (55%), erythromycin (100%), fosfomycin (95%) and bacitracin (59%). Among *E. faecium* isolates from broilers, high levels of resistance were observed for the following antimicrobials: doxycycline (96.9%), sulphamethoxazole (92.7%), enrofloxacin (92.7%), erythromycin (100%) and fosfomycin (99%).

The 100% resistance observed for erythromycin for both *Enterococcus* spp. was not expected since erythromycin is not registered for use in poultry in South Africa and no macrolide that could have caused cross resistance with erythromycin had been used in these flocks during sampling. However, it is possible that Fosbac plus T (drug that contains tylosin –a macrolide) or tylan (a macrolide), drugs that are widely used in the poultry industry in South Africa to treat mycoplasmosis, could have been used on the farms sampled. The phenomenon where usage of one drug leads to persistence and dissemination of resistance to a related antimicrobial has been described. For example, in the UK, following the milk-borne outbreak of MR *S.* Typhimurium DT 104 with decreased susceptibility to ciprofloxacin, there was an enhanced persistence and dissemination of resistance due to the use of the related antimicrobial marbofloxacin during the outbreak (79). It is also believed that drug application may not only select for resistance against the applied drug, but also for multiple resistance phenotypes having a selection advantage (54, 59, 92). It is also known that organisms that are resistant to one drug are likely to become resistant to others. This multi-drug resistance is attributed to at least two phenomena: cross-resistance within a class of antibiotics and genetic loci which can regulate resistance to multiple classes of antibiotics (59). Especially when resistance is genetically mediated, it is postulated that genes resistant to a number of antimicrobials can move *en mass* from one microbe to another, thereby enabling a single horizontal transfer to confer multi-drug resistance (56, 59). In the light of this, in South Africa where antimicrobials are extensively used in the poultry industry for growth enhancement there could be resistance to antimicrobials that have not been used in the poultry flocks.

There were differences in the level of resistance to trimethoprim (20% and 0%), ampicillin (0% and 12.4%), enrofloxacin (55% and 92.7%) and bacitracin (60% and 44%) (Table 5.3, 5. 4 and 5.5), observed for *E. faecalis* and *E. faecium* isolates

respectively from broilers. This suggests a difference in the development of resistance between the two species to these agents when subjected to the same selection pressure.

Since broilers are short life species (reared for 35-42 days), and that the farms under study (according to the questionnaire completed) practice an all-in all-out system of rearing broilers, with poultry houses thoroughly cleaned, washed and disinfected before new batches of broilers are brought into the poultry houses, the high level of resistance observed here, demonstrates the ability of bacteria to develop resistance quickly or the ability of a few resistant bacteria that survive to quickly re-populate the flock when exposed to antimicrobial selection pressure. A study done in the USA with *Campylobacter* showed that chickens naturally colonised with fluoroquinolone-susceptible strains began excreting resistant strains after 2 days of doses of enrofloxacin, a drug commonly used for prophylaxis in the poultry industry (42).

Table 5.7: Minimum inhibitory concentrations of antimicrobial agents: *E. coli* isolates from abattoir workers (n =28)

Antimicrobial agent	No. of isolates (%) with the following MIC’s[a] (µg/ml)																	
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Doxycycline	46.5						10.7	28.6	7.1	7.1	3.6	17.9	10.7	14.3					
Trimethoprim	32.1				46.4	14.3	3.6	3.6				3.6	28.6						
Sulphamethoxazole	67.9											3.6	7.1	10.7	10.7	3.6		3.6	60.7
Ampicillin	42.9				3.6	14.3	32.1	7.1			3.6	3.6	35.7						
Enrofloxacin	17.9		67.8		14.3		10.7	3.6			3.6								
Fosfomycin	46.4								7.1	25	14.3	7.1		7.1	39.3				
Ceftriaxone	10.7						89.3	7.1	3.6										
Nalidixic acid	21.4						50	14.3	7.1	7.1			10.7	3.6	7.1				

[a] The white fields denote range of dilutions tested for each substance.

MIC’s below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the MIC cut off point**.**

Table 5.8: Minimum inhibitory concentrations of antimicrobial agents: *E. coli* isolates from the control group (n = 26)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																	
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Doxycycline	34.6					23.1	0	19.2	15.4	7.7	19.2	7.7	7.7						
Trimethoprim	26.9				57.8	3.9	7.7	3.9					26.9						
Sulphamethoxazole	46.2										3.9	19.9	19.2	11.5					46.15
Ampicillin	30.9				7.6	23.1	34.6	7.7			3.9	3.9	23.1						
Enrofloxacin	19.2		69.2	3.9	7.7	3.9	3.9			7.7	3.9								
Fosfomycin	34.6								7.7	23.1	30.8	3.9		3.9	30.8				
Ceftriaxone	3.9						96.2	3.9											
Nalidixic acid	11.6						46.2	23.1	11.5	3.9	3.9	3.9			7.7				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the MIC cut-off point.

The numbers of *E. coli* isolates from the abattoir workers with median MIC values above the cut-off point (% resistant) were lower than was observed for poultry isolates for the following antimicrobials: doxycycline (p < 0.001), enrofloxacin (p < 0.001), fosfomycin (p < 0.001), ceftriaxone (p =0.003) and nalidixic acid (p < 0.001). For trimethoprim (p = 1.00), sulphamethoxazole (p = 0.228) and ampicillin (p = 0.350), no significant differences were observed when the median MIC values of the two groups were compared. The three antimicrobials for which no significant difference in the prevalence of resistance was observed are drugs that are widely used in both humans and broilers, while the former groups includes antimicrobials extensively used in poultry.

Escherichia. coli isolates from people not associated with the abattoir (control group), likewise had lower levels of resistance compared to the broilers. Significant differences were observed for doxycycline (p < 0.001), sulphamethoxazole (p < 0.001), ampicillin (p = 0.002), enrofloxacin (p < 0.001), fosfomycin(p < 0.001), ceftriaxone (p < 0.001), and nalidixic acid (p < 0.001), the exception was trimethoprim (p = 0.654) for which a close level of percentage resistance to that observed among *E. coli* isolates from poultry was recorded. These findings are similar to was observed when the level of resistance among *E. coli* isolates from broilers and abattoir workers were compared.

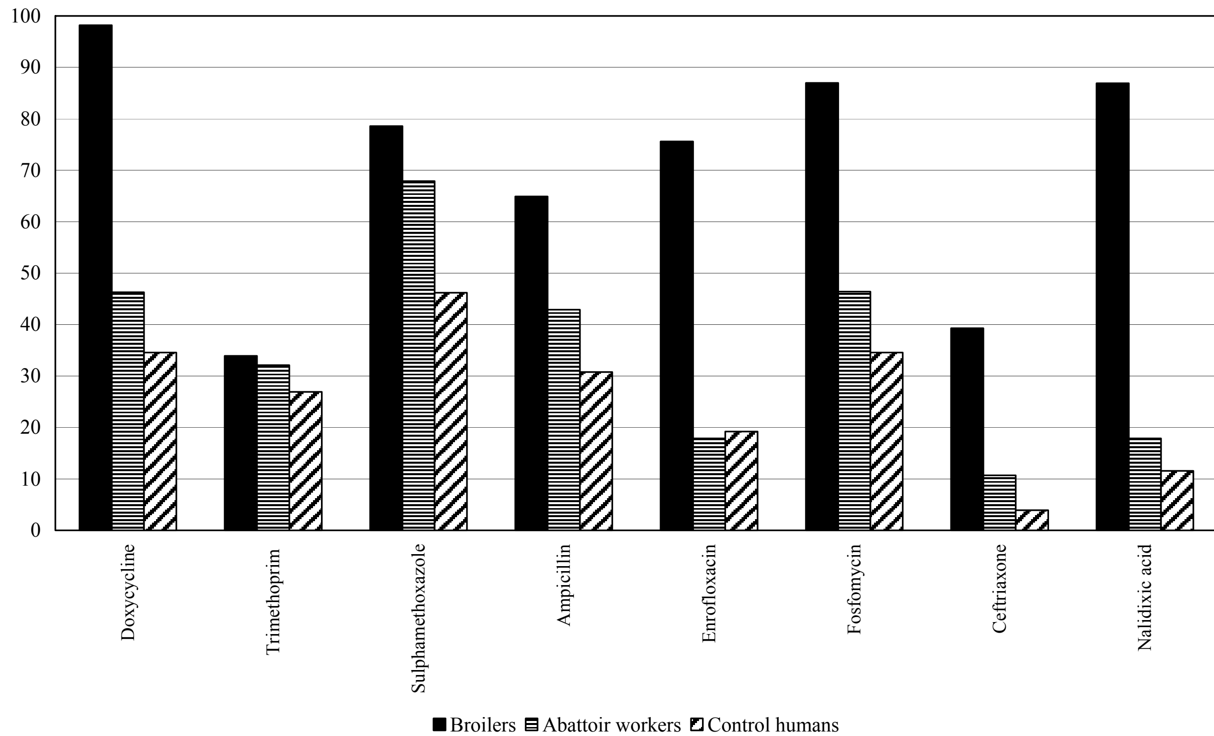

Figure 5.1: Percentage resistance of *E. coli* from broilers (n=168), abattoir workers (n=28) and human controls (n=26) to antimicrobials tested in this study

Figure 5.1 illustrates that the prevalence of resistance observed in the three populations (broilers, workers and control group) differed. With the exception of trimethoprim, it is clear that the prevalence in the broilers is much higher than in the two human populations sampled. This could be attributed to the fact that the conditions of antimicrobial usage in farm animals favour the development of resistance as compared to humans (31).

In addition, figure 5.1 also shows that the level of resistance tended to be higher among abattoir workers (with the exception of enrofloxacin) compared to the control group. This is consistent with the reports that people working with animals fed feed with AMGP tend to carry a higher level of resistance to such antimicrobials as compared to those who do not (60, 84). However, a statistical analysis showed no significant differences for all the antimicrobials ($p > 0.1$). Thus an association between resistance among isolates from offals and carcass on one hand and abattoir workers on the other, could not be proven. The only exception was sulphamethoxazole where abattoir workers had resistance level similar to that of the broiler isolates. Not withstanding these findings, with the exception of ceftriaxone to which, 3.9% *E. coli* isolates from the control group (Table 5.8) had MIC's considered resistant, resistance among *E. coli* isolates from the two human populations tested, could still be described as being high or similar to what it was in Europe

before the usage of feed growth enhancers was abolished (14, 17, 88). However, these findings are much lower than in countries where antimicrobials are easily accessible and are available over the counter. For example in Nigeria, where antimicrobials are easily accessible over the counter, a prevalence of up to 90% resistance to tetracycline among human isolates has been recorded (62). This could be attributed to the less stringent regulatory mechanisms in such countries as compared to South Africa, where antimicrobials are not easily accessibility over the counter in human medicine.

The difference in terms of the number of *E. coli* isolates with MIC's above the cut-off point for fosfomycin (Table 5.7 and 5.8) between the workers (46.6%) and the control group (34.6%) was not significant ($p = 0.418$) as also illustrated by Figure 5.1. This implies that the humans in South Africa not associated with the poultry industry (like poultry abattoir workers), carry resistance to fosfomycin. This is explained by the fact that fosbac is widely used as a feed additive in the country's poultry flocks. Wherever antimicrobials are used extensively in a country's animal population, there is a tendency for the general human population and not only people working with animals to carry high levels of resistance. No significant difference was observed when rates of resistance to other antimicrobials used extensively in poultry e.g. enrofloxacin ($p = 1.0$) in the two groups (control and abattoir workers) were compared. However, the higher percentages of resistant isolates from abattoir workers as compared to the control group also indicates ($p = 1.0$) that people working with animals fed feed containing antimicrobials carry a higher level of resistance compared to those not associated with such animals.

Another finding of interest among the human *E. coli* isolates was the 32.1% from abattoir workers and 26.9% of the isolates from the control group that had MIC's considered resistant to trimethoprim (Tables 5.7 and 5.8). This level of resistance is almost similar to what was observed with *E. coli* isolates from the broilers (resistance rate of 33.9%). Given that potentiated sulphonamides are registered for use in humans and chickens, these results imply that the selection pressure for resistance against trimethoprim is great in the three populations. Since abattoir workers and control group had close levels of resistance among their isolates, this also implies that the resistance observed in abattoir workers is not necessarily linked to resistance observed in the isolates from the broiler intestines, the abattoir workers wash and pack in the course of their working.

Table 5.9: Minimum inhibitory concentrations of antimicrobial agents: *E. faecium* from abattoir workers (n = 2)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	0							100											
Virginiamycin	0							100											
Doxycycline	0						50			50									
Trimethoprim	100											100							
Sulphamethoxazole	100																		100
Ampicillin	0				50	50													
Enrofloxacin	100										100								
Erythromycin	50								50	50									
Fosfomycin	100														100				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the MIC cut-off point.

Note: Bacitracin results were not include here because MIC's were measured in units/ml (Table 5.5)

Table 5.10: Minimum inhibitory concentrations of antimicrobial agents: *E. faecalis* from abattoir workers (n = 21)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	9.5							90.5				4.8	4.8						
Doxycycline	66.7						23.8			9.5	14.3	33.3	19.1						
Trimethoprim	23.8				61.9	4.8	4.8			4.8	14.3	4.8	4.8						
Sulphamethoxazole	71.4									4.8			9.5	4.8	9.5	9.5	4.8	9.5	47.6
Ampicillin	0				76.2	19.1	4.8												
Bacitracin	9.5																		
Enrofloxacin	52.4		9.5	19.1	19.1	19.1	4.8				28.6								
Erythromycin	81							14.3	4.8		19.1	19.1	9.5	4.8		28.6			
Fosfomycin	90.5												9.5	19.1	71.4				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas.

The vertical bars represent the MIC cut-off point.

Note: Bacitracin results were not include here because MIC's were measured in units/ml (Table 5.4)

Table 5.11: Minimum inhibitory concentrations of antimicrobial agents: *E. faecalis* from control group (n = 3)

Antimicrobial agent	No. of isolates (%) with the following MIC's[a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	0							100											
Doxycycline	66.7						33.4				33.2	33.4							
Trimethoprim	33.3				66.7						33.3								
Sulphamethoxazole	66.7														33.2			33.4	33.4
Ampicillin	0				33.4	33.4	33.2												
Enrofloxacin	33.3			33.4	33.2		33.4												
Erythromycin	66.7								33.4	33.4						33.2			
Fosfomycin	100														100				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas

The vertical bars represent the MIC cut-off point.

Note: Bacitracin results were not include here because MIC's were measured in units/ml (Table 5.4)

Table 5.12: Minimum inhibitory concentrations of antimicrobial agents: *E. faecium* from control group (n = 10)

Antimicrobial agent	No. of isolates (%) with the following MIC's [a] (µg/ml)																		
	% resistant	≤ 0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048
Vancomycin	0							100											
Virginiamycin	0					10	50	30	10										
Doxycycline	40						60					10	20	10					
Trimethoprim	0				80				10	10									
Sulphamethoxazole	80										10			10		10		20	50
Ampicillin	10				60	30						10							
Enrofloxacin	50			30	20	30	20												
Erythromycin	100									20	10				70				
Fosfomycin	100													10	90				

[a] The white fields denote range of dilutions tested for each substance.

MIC's below or above the range are given as the closest to the range in shaded areas

The vertical bars represent the MIC cut-off point.

Note: Bacitracin results were not include here because MIC's were measured in units/ml (Table 5.5)

Only two *E. faecium* isolates were obtained from abattoir workers vide supra in subsection 5.1, and both of these isolates were resistant to trimethoprim, sulphamethoxazole, enrofloxacin and fosfomycin. One of the two isolates was resistant to erythromycin (Table 5.11). Though the number of isolates does not allow for significant extrapolation from these results, the results suggest that human enterococcal isolates in this country carry resistance to fosfomycin, an antimicrobial not registered for human use in South Africa.

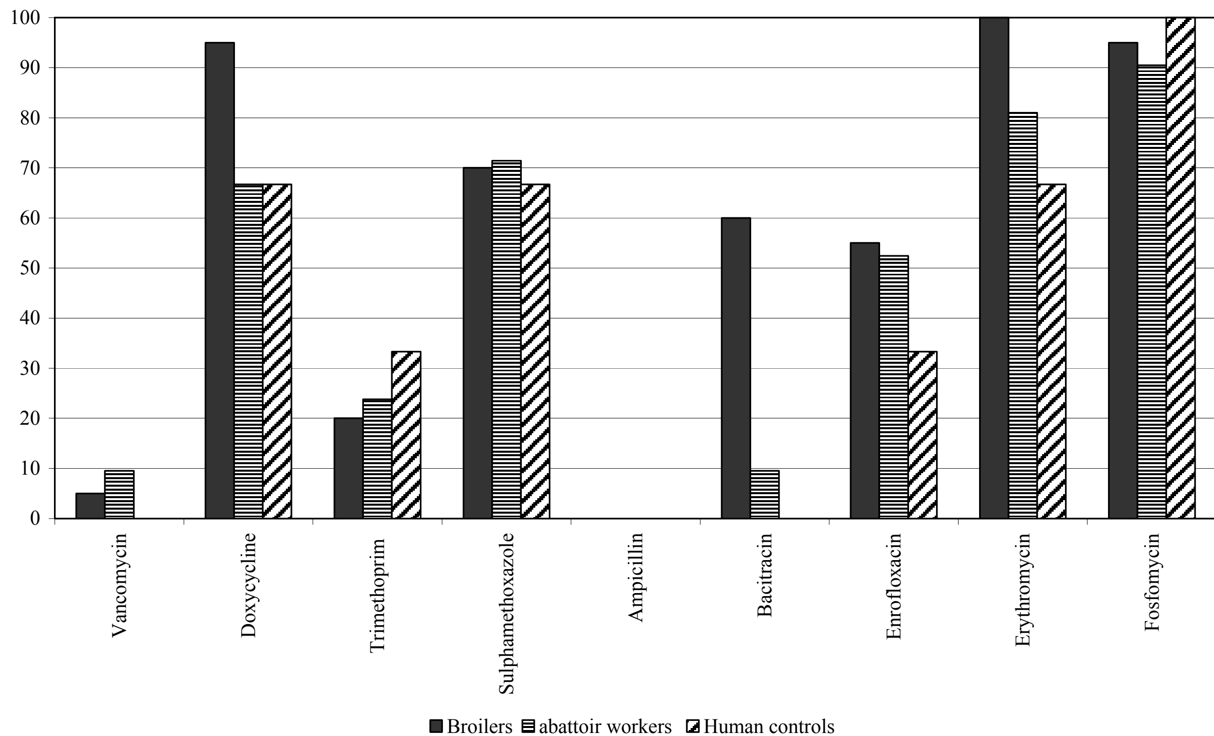

Figure 5.2: Percentage resistance of *E. faecalis* from broilers (n = 20), abattoir workers (n=21) and human controls (n=3) to antimicrobials

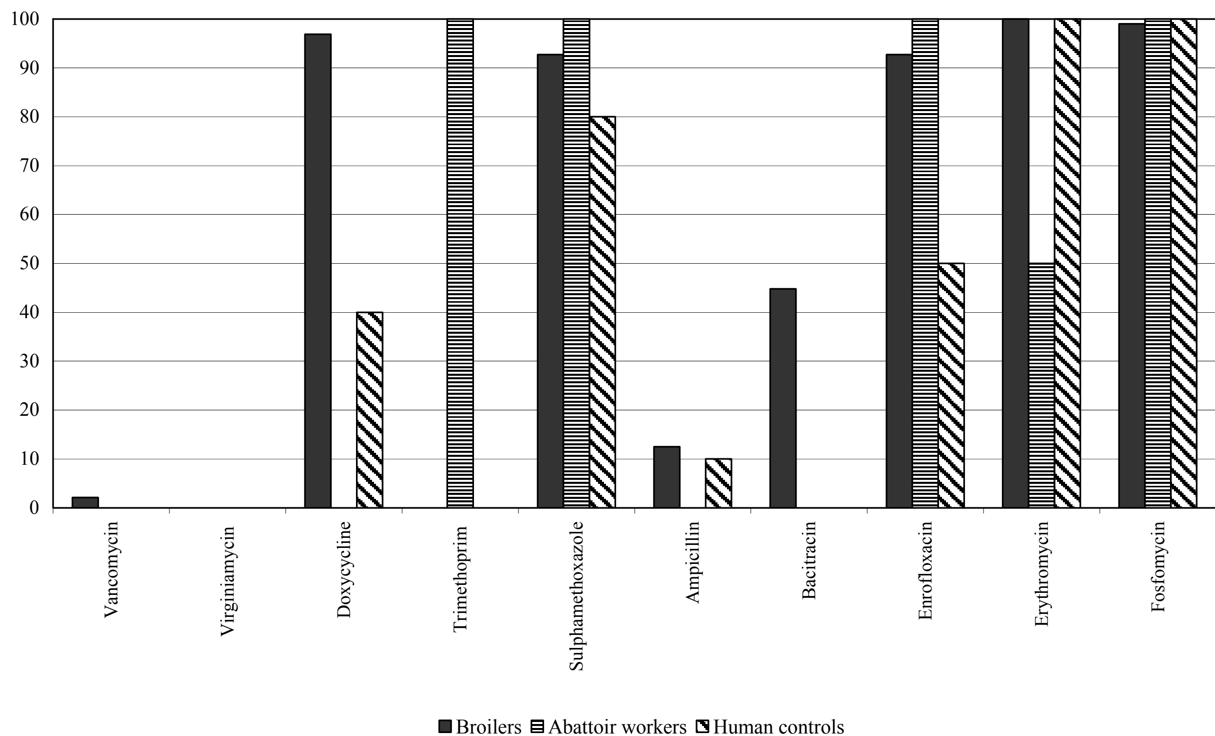

Figure 5.3: Percentage resistance of *E. faecium* from broilers (n = 96), abattoir workers (n=2) and human controls (n=10) to antimicrobials

Figure 5.2 is a presentation of a comparison of the level of resistance among *E. faecalis* isolates from broilers, abattoir workers and the control group. Apart from potentiated sulphonamides and fosfomycin, it is clear that broilers carry high levels of resistance compared to humans not associated with the poultry industry to antimicrobials tested. Worthy of note, is that the level of resistance from the broilers and the humans does not suggest wide differences in the rate of resistance. However, as observed for *E. coli* isolates, abattoir workers carried a higher level of resistance as compared to the control group to some antimicrobials, the exception being doxycycline, ampicillin and fosfomycin.

When the level of resistance observed among *E. faecalis* isolates from abattoir workers and broilers was compared, a significant difference in the level of resistance was noted for doxycycline ($p = 0.05$) and bacitracin ($p < 0.01$) indicating that these are two separate populations and that transfer of antimicrobial resistance was less likely. These findings contrast with what was observed when the prevalence of resistance among *E. coli* isolates from the same groups were compared (Figure 5.1). Figure 5.2 shows that for

sulphamethoxazole, enrofloxacin and fosfomycin, the difference in the level of resistance between *E. faecalis* isolates from broilers and abattoir workers was minimal. This was not expected given that the selection pressure in the broiler isolates is higher than that in the isolates from abattoir workers.

A low isolation rate of *E. faecalis* (n =3) from the control group hindered extrapolation from the results obtained from the study. This explains why when comparing the prevalence of resistance among *E. faecalis* isolates from the control group and those from the broilers (Figure 5.2), significant differences in the level of resistance was observed for only doxycycline and enrofloxacin, suggesting that the *E. faecalis* isolates from the control group carried a level of resistance that was not significantly different from that observed in broiler isolates to most antimicrobials (including growth enhancers) tested.

A resistance rate of 9.5% for *E. faecalis* to vancomycin observed among abattoir workers (Table 5.10) compared to 0% for control group (Table 5.11) is a concern as this antimicrobial is considered the last line of defence in the treatment of *Enterococcus* infections in human medicine. Meaning that the selection pressure for vancomycin in the two populations is expected to be low, and so the levels of resistance than observed here. This too could be attributed to the low numbers of *E. faecalis* (n =3) from the control that was used in the comparison. Due to the small sample size, it is not possible to draw meaningful conclusions from these findings. The *Enterococcus species* tended to have low MIC levels (< 128 µg/ml), suggesting the absence of *van*A genes that code for high level resistance among VRE in both populations.

There were no significant differences in the levels of resistance for doxycycline, sulphamethoxazole, bacitracin and fosfomycin among *E. faecalis* isolates from the abattoir workers and the control group (Tables 5.5, 5.10 and 5.11). Once again the slightly higher level of resistance observed in the *E. faecalis* isolates from abattoir workers as compared to the control group (Table 5.10 and 5.11) to sulphamethoxazole, enrofloxacin, and erythromycin indicates the people working in abattoirs carry an elevated level of resistance than the public not associated with animals fed feed with antimicrobials.

The 100% resistance to fosfomycin observed in all enterococci isolates from the control group (Table 5.11 and 5.12) though not conclusive due to the low numbers of *E. faecium* (n = 2) from the abattoir workers and *E. faecalis* (n = 3) from the control group, also confirms that there is resistance among isolates from humans not associated with poultry fed AMGP to fosfomycin.

Though it was expected that the difference in the prevalence of resistance among *E. faecium* isolates from the abattoir workers and broilers would be significant, this was not true for vancomycin ($p > 0.05$), sulphamethoxazole ($p > 0.05$), ampicillin ($p > 0.05$), fosfomycin ($p > 0.05$). For vancomycin this result was expected given that vancomycin is a third line drug in human medicine and will have therefore not been used that frequently among the abattoir workers, neither is its analogue avoparcin available for use in poultry in South Africa. Meaning that in both populations, the selection pressure for vancomycin resistance is very low. For the other antimicrobials the small sample size of *E. faecium* used in the comparison could have led to failure to notice a significant difference despite the knowledge that the selection pressure in broilers is different in the two populations. As demonstrated in Figure 5.3, a meaningful comparison of the MIC values for *E. faecium* from the three populations was not possible.

Against virginiamycin and vancomycin, no resistance was observed for *E. faecium* from the control group. Since the vancomycin analogue avoparcin is not available for use in poultry in this country, a very low level of resistance was therefore anticipated. However, this cannot be said of virginiamycin, due to lack of knowledge on the part of the writer about the pattern of usage of this antimicrobial in poultry in South Africa. The 50% of the *E. faecium* isolates from the control group that had MIC's above the cut-off point for enrofloxacin (a drug commonly used in poultry) is suggestive of a high level of resistance among human enterococci isolates to antimicrobials used in poultry. This is supported by the 100% resistance observed among the two *E. faecium* isolates from abattoir workers to enrofloxacin (Table 5.9).

Table 5.13: Median MIC of *E. coli* isolates from eviscerators and packers (n = 28)

Drug	Eviscerators (n = 20)	Packers (n = 8)	*p*-value
Doxycycline	20	3	0.277
Trimethoprim	**<0.2**	**>32**	**0.002**
Sulphamethoxazole	>2048	>2048	0.506
Ampicillin	**1**	**>32**	**0.041**
Enrofloxacin	<0.06	<0.06	0.977
Fosfomycin	>128	12	0.100
Ceftriaxone	<1	<1	0.256
Nalidixic acid	**2**	**<1**	**0.022**

Results of the statistical analysis of the median MIC's of *E. coli* from the two groups of abattoir workers (eviscerators and packers) are summarised in Table 5.13. Statistically significant differences were observed for the following antimicrobials tested against *E. coli*: trimethoprim ($p = 0.002$), ampicillin ($p = 0.041$) and nalidixic acid ($p = 0.022$).

Table 5.14: Median MIC of enterococci (*E. faecalis* & *faecium*) isolates from eviscerators and packers (n = 23)

Drug	Eviscerators (n = 17)	Packers (n = 6)	*p*-value
Vancomycin	<2	<2	0.390
Doxycycline	32	4	0.450
Trimethoprim	**<0.2**	**16**	**0.003**
Sulphamethoxazole	**1024**	**>2048**	**0.013**
Ampicillin	<0.25	<0.25	0.522
Bacitracin	5	<0.78	0.942
Enrofloxacin	**0.25**	**>8**	**0.001**
Erythromycin	32	32	0.749
Fosfomycin	>128	>128	0.785

As shown in Table 5.14, a comparison of the median MIC's for the enterococci isolates from eviscerators and packers likewise showed a difference in the median MIC's. Between these two groups, statistically significant differences in the median MIC's were observed for the following antimicrobials: trimethoprim (p = 0.003), sulphamethoxazole (p = 0.013), enrofloxacin (p = 0.001).

Based on the fact that significant differences were observed between the two groups of abattoir workers for certain antimicrobials, and that for the same antimicrobials (except nalidixic acid) the tendency was for the packers to have higher median MIC values, it was decided that the experimental group be split and the comparative study based on the median MIC's of isolates from packers (who have a much closer contact with the enteric organisms from the broilers as compared to the eviscerators) and the control group.

Table 5.15: Median MIC of *E. faecalis* isolates from control group and packers

Drug	Control (n = 3)	Packers (n = 4)	*p*-value
Vancomycin	<2	<2	
Doxycycline	16	32	0.711
Trimethoprim	<0.2	16	0.168
Sulphamethoxazole	2048	>2048	0.078
Ampicillin	0.5	<0.25	0.078
Bacitracin	3.13	12.5	0.708
Enrofloxacin	**0.25**	**>8**	**0.019**
Erythromycin	8	>256	0.266
Fosfomycin	>128	>128	0.180

Analysis of the median MIC's of *E. faecalis* isolates from the control group and the packers (Table 5.15) revealed a statistically significant difference for enrofloxacin (p = 0.019) only. For this antimicrobial, packers had a higher median MIC as compared to the control group. Enrofloxacin is a "second line" antimicrobial in humans, and hence not prescribed that regularly. While it is true that the numbers involved here are small, these results suggest that abattoir workers are at an increased risk of picking up resistance to enrofloxacin among their enterococci.

Table 5.16: Median MIC of *E. faecium* isolates from control group and packers

Drug	Control (n = 10)	Packers (n = 2)	*p*-value
Vancomycin	<2	<2	>0.05
Virginiamycin	1	2	0.247
Doxycycline	<1	4	0.810
Trimethoprim	**<0.2**	**32**	**0.010**
Sulphamethoxazole	>2048	>2048	0.230
Ampicillin	<0.25	0.25	0.903
Bacitracin	3.13	2.34	0.662
Enrofloxacin	**<0.06**	**>8**	**0.029**
Erythromycin	**>256**	**6**	**0.030**
Fosfomycin	>128	>128	0.655

When MIC's for *E. faecium* from packers and the control group (Table 5.16) were compared, antimicrobials for which a significant difference was noticed are: trimethoprim (p =0.01), enrofloxacin (p = 0.029) and erythromycin (p = 0.03), none of which is a growth promoter. Again, there was no significant difference observed in antimicrobials (fosfomycin and bacitracin) to which abattoir workers would have been expected to carry a much higher level of resistance than the control group given that they are widely used in poultry compared to human medicine. Especially that the packers are presumed to have been exposed to poultry isolates (carrying high levels of resistance) more frequently than the control group. Trimethoprim is regularly used in human medicine and so conclusions cannot be made as to the cause of the significant difference observed. However, for erythromycin, this is an expensive drug compared to others like penicillins that are also regularly used in human medicine. It is therefore possible that the difference in the usage pattern between the control and packers is responsible for this difference. The higher median MIC observed in the two *E.*

faecium isolates from the control group compared to the 10 from packers, not withstanding the small numbers involved, suggests that the selection pressure is higher in the control group. As for enrofloxacin, the significant difference when the median MIC's were compared also confirms as indicated above for *E. faecalis* that abattoir workers are at an increased risk of picking resistance to enrofloxacin.

Table 5.17: Median MIC for *E. coli* isolates from control group and packers (n = 34)

Drug	Control (n = 26)	Packers (n = 8)	*p*-value
Doxycycline	4	3	0.837
Trimethoprim	**0**	**>32**	**0.012**
Sulphamethoxazole	128	>2048	0.102
Ampicillin	**1**	**>32**	**0.036**
Enrofloxacin	<0.06	<0.06	0.838
Fosfomycin	16	12	0.403
Ceftriaxone	<1	<1	0.426
Nalidixic acid	2	<1	0.069

Higher levels of median MIC's were recorded for the *E. coli* isolates from the control group compared to the packers for doxycycline, fosfomycin and nalidixic acid (Table 5.17), contrary to what was expected. However, significant differences between the two groups (control and packers) were observed for the following antimicrobials; trimethoprim ($p = 0.012$) and ampicillin ($p = 0.036$). For these two antimicrobials, isolates from packers had higher median MIC's. This is consistent with reports that abattoir workers carry a higher level of resistance compared to people not associated with the abattoir. Unlike enterococci isolates, a significant

difference was not observed for enrofloxacin when the median MIC's for *E. coli* from the two populations were compared.

The rank correlation co-efficient was determined for isolates from abattoir workers and the broilers, as demonstrated in Figure 5.4 below. No correlation was observed among *E. coli* isolates (Spearman's r = 0.16, $p = 0.68$).

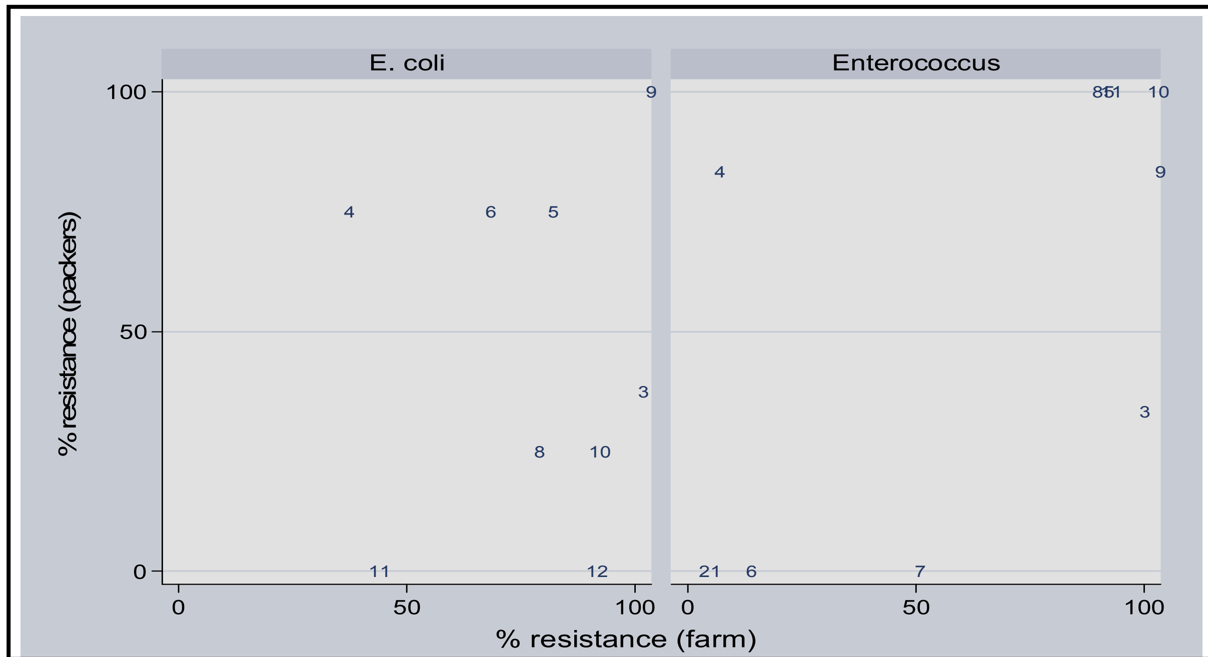

Figure 5.4: Scatter plot for % resistant isolates from packers and broilers for each antimicrobial drug*

*** 1 = vancomycin; 2 = Virginiamycin; 3 = doxycyciline; 4 = trimethoprim; 5 = sulphamethoxazole; 6 = ampicillin; 7 = bacitracin; 8 = enrofloxacin; 9 = erythromycin; 10 = fosfomycin; 11 = Ceftriaxone and 12 = nalidixic acid.**

For example, antimicrobials 3, 8, 12 and 10 had high levels of resistance among broiler isolates, but low levels of resistance among the packers. However, when the rank correlation coefficient was determined for enterococci, a correlation was observed (Spearman's r = 0.62, $p = 0.043$). That is to say, if resistance was high to a certain antimicrobial among the broiler isolates, it would also be high among isolates from the packers, and vice versa. For example, the level of resistance to antimicrobials 1, 2 and 6, was low in isolates from the two groups, while for antimicrobials 8, 5, 11, 10 and 9 the level of resistance was high in isolates from

both groups. This indicates that antimicrobial drug resistance is more likely to occur between humans and abattoir workers by way of enterococci rather than *E. coli*.

5.3 ANTIMICROBIAL USAGE PATTERNS

Only tetracycline and fosfomycin were used as feed additives over this period. Alternation between these two was based on antimicrobial susceptibility testing using the disc diffusion test during this period, with tetracycline used for the first eight months of 2005 and fosfomycin brought in from September to the end of 2005. According to the completed questionnaire, at about the time of sampling, oxytetracycline was included in the feed for prophylactic purposes (to prevent outbreaks of sinusitis due to *Ornithobactrium rhinotracheale* infection).

Antimicrobials used for metaphylaxis (for disease control following an outbreak) during this same period included the following: fosfomycin, fluoroquinolones, amoxycillin and potentiated sulphonamides. However, it was not clear from the questionnaire as to the types of potentiated sulphonamides and AMGP that had been used in the flocks. A second questionnaire conducted telephonically was completed, which established that the only AMGP that had been used in the flocks for the duration of the sampling was bacitracin, while the potentiated sulphonamide used was the registered sulfa plus trimethoprim combination.

Therefore the results presented above showing high levels of resistance against the antimicrobials mentioned in the questionnaire were expected given that antimicrobial usage is accepted as one single most important factor responsible for increase in resistance (20, 43). This was true for both therapeutics and AMGP. These results concur with the observation by Ishihara *et al.* (43), amongst others, that resistance profiles of animal isolates reflect antimicrobial substances used to treat the animals. For example, drugs like virginiamycin and vancomycin that were not used and to which no known drug that could cause cross resistance was used had low levels of resistance. This was not the case with erythromycin and nalidixic acid. The explanation for this irregularity is cited above. For antimicrobials that are registered and hence available for use in the flocks studied, it is known that once antimicrobials are introduced for use in veterinary medicine, there is a corresponding increase

in resistance among faecal flora (9, 77, 84,). A number of studies involving different methods show that after the introduction of an antimicrobial in veterinary practice, resistance in pathogenic bacteria and/or faecal flora increases (4, 17, 28, 42, 46, 54, 57, 59, 84, 88, 92).

Fosfomycin was used both as a feed additive and for metaphylaxis purposes. This implies that the selection pressure for resistance against fosfomycin was particularly high, hence the high levels of resistance observed among all the three species of bacteria from broilers that were tested. Though it was indicated on the questionnaire that amoxicillin had been used in the flocks studied, the levels of resistance observed among enterococci isolates to ampicillin against which amoxicillin would cause cross resistance was very low. This is contrary to what was observed with *E. coli*, against which high levels (75%) were observed (Table 5.2). The implication of is that there is a difference in the development of resistance between the two species (enterococci and *E. coli*), with enterococci (particularly *E. faecalis* and to a lesser extent *E. faecium*) remaining susceptible while *E, coli* develops resistance.

An assessment of the level of resistance of *E. coli* to sulphamethoxazole and trimethoprim showed a wide level of resistance between the two drugs (sulphamethoxazole 78.6% and trimethoprim 33.9%). The possible explanation for this observation is that resistance to trimethoprim develops much slower than for the sulpha component.

CHAPTER 6

CONCLUSIONS, RECOMMENDATIONS AND QUESTIONS ARISING

6.1 CONCLUSIONS AND RECOMMENDATIONS

The prevalence of the *E. faecalis* and *E. faecium* in the flocks studied differed from that reported in Canary Islands study, but agreed with what was observed in Denmark. However, given the limited scope of this study, before a reliable conclusion could be made as to which of the two species is more prevalent in the poultry flocks in South Africa, a wider study is necessary to assess the prevalence of the two species. This is relevant in the light of the fact that a difference in species distribution between countries has been suggested by Kuhn *et al* (48). What is important however is that the two *Enterococcus* spp. isolated in this study, did not occur in equal proportions in broilers, and both species could easily be cultivated from the intestinal tract of broilers.

Since the dominant *Enterococcus* species among isolates from the two populations (abattoir workers and broilers) was different, it indicates that movement of *Enterococcus* species from broiler intestinal tract to abattoir workers is minimal. In view of this, strategies like the one employed at the abattoir studied, where workers do not get into direct contact with the bacteria from the intestines of chickens should be encouraged at all times to prevent or minimise colonisation of human gastro intestinal tract with enterococci from broilers.

It was not possible to evaluate the antimicrobial susceptibility of zoonotic *Salmonella* as the farms under study had at the time of the study been able to control *Salmonella* infection. However, there are farms in the country from which multi-resistant salmonellae have been cultured (69). Thus a study making use of these farms and farm workers can be used to determine the role of *Salmonella* in the transfer of resistance. Strategies like the one employed on the farms studied to control *Salmonella* in poultry should be extended to other

micro-organisms to reduce the need for antimicrobial usage, as a way to control development of resistance.

Failure to isolate *Cl. perfringens* that lead to the suspension of culturing of the organisms mid-sampling is attributed to usage of antimicrobials, performance enhancers and coccidiostats in the flocks under study. However, a broader study involving larger samples than used in this study is necessary to assess the level of resistance among *Cl. perfringens* to antimicrobials like the β-lactams that form the "first line" of treatment for *Cl. perfringens* infections.

It can be assumed that the level of resistance seen here is a reflection of what could be happening in the enteric population of both the Gram-positive and Gram-negative bacteria. However, given that the isolation rate for *E. faecalis* and *E. faecium* were very low as in some instances, broader studies to assess and monitor the general level of resistance among commensal bacteria in poultry in South Africa are needed. Thus it is recommended that the South African National Veterinary Surveillance and Monitoring programme for resistance to Antimicrobial Drugs (SANVAD) receive the full support of government, veterinarians and the farming community. The importance of this is appreciated when consideration is given to the fact that emergence of antimicrobial resistance phenotypes among food-borne bacteria (22, 89), implies a likelihood of failure of empiric treatment of food associated diseases (22).

Antimicrobial usage patterns in the farms studied appear to favour the development of resistance among poultry isolates as there was a high level of resistance to antimicrobials commonly used in the poultry industry namely, tetracyclines, fluoroquinolones, fosfomycin, suphonamides and macrolides. Thus it is recommended that the poultry industry and in particular the farms in this study adopt a prudent antimicrobial usage policy or even consider moving to a high health status with minimum antimicrobial usage. The latter programme has been successful in some European countries where there was no marked loss in production (3, 6, 94, 42, 91, 92). The company that owned or had farms under contact has subsequently converted many of these farms into high health status farms and antimicrobials are only administered for therapeutic purposes. The effect of this change has not yet been studied.

The use of bacitracin as a performance enhancer certainly resulted in the increase of bacitracin resistance among the enterococci of chicken origin, but not in the human enterococci. Thus there is no indication that bacitracin-resistant enterococci are transferred to the enteric tract of humans. As expected no resistance to virginiamycin was observed, providing further proof that when a specific antimicrobial is not used, resistance levels tend to be low or even absent. It cannot be positively concluded from this study that packers who work in sections where they handle isolates with a high level of antimicrobial drug resistance places them at an increased risk of acquiring resistance among their enteric organisms to AMGP compared to the general public.

The low level of rate of resistance to vancomycin observed among poultry isolates in this study is an indication that resistance genes built up during the time when avoparcin was extensively used in this country has not completely disappeared. Although 5 *E. faecalis* and 2 *E. faecium* isolates from broilers were resistant to vancomycin, the MIC values were $\leq$ 128 µg/ml. This indicates that the vanA gene, which confers a high level of resistance was not present. There is a possibility however, that other genes such as *van*B, *van*C and *van*E may be present. These usually confer low-level resistance to vancomycin. Thus it can be concluded that vancomycin resistance is not a problem in the poultry farms tested. However, the genetic basis of the resistance should be further investigated. None the less, based on what was observed in this study, it is advisable that glycopeptide analogues not be reintroduced for use as performance enhancers in this country's poultry flocks, as they would give a competitive edge to VRE leading to wide spread occurrence of the same. It is reported that as a result of co-selection, even after the specific selection pressure (like in the case where avoparcin that selects for vancomycin resistance) was removed, use of other antimicrobials could continue to select for vancomycin resistance (1, 39). Persistence of GRE in production animals as a consequence of co-selection by the continued use of tylosin for growth promotion has been reported (1). It can therefore be concluded that the rate of resistance observed in this study is being sustained by the use of tylosin in the poultry industry in this country. However, further studies involving farms where tylosin is being used extensively are necessary to establish the rate of resistance on such farms as compared to what was observed in this study.

Based on the results reported here there is a species difference between *E. coli* and enterococci in developing resistance to ampicillin when the same selection pressure is exerted. This could be attributed to intrinsic bacterial species differences. In view of this it is recommended that future antimicrobial drug resistance surveillance studies include both species to determine the extent of antimicrobial drug resistance among Gram-positive and Gram-negatives.

While these results confirmed that abattoir workers generally carried higher levels of resistance, Statistical analysis did not show significant difference in the level of resistance between the two populations (abattoir workers and control group) for all antimicrobials used. This was true for both AMGP and classes of antimicrobials (e.g. fosfomycin) used exclusively in poultry.

The observation of the median MICs of the enterococci to enrofloxacin being significantly higher in the abattoir workers when compared to the control group, suggests that there could be transfer of enrofloxacin resistance to the workers. The fact that ciprofloxacin is used as a "second" or "third line" antimicrobial in human medicine, and therefore a high selection pressure and consequently a higher level of resistance among abattoir was not expected, could account for this. However, these findings need to be further verified by studies where new employees are regularly monitored for the development of antimicrobial drug resistance from the time they start working at the abattoir.

While no association between the antimicrobial resistance patterns of *E. coli* in the chickens and abattoir workers was observed, an association between the resistance patterns of the enterococci in both groups was recorded. This means that antimicrobial drug resistance transfer between broiler offals and abattoir workers is more likely to occur in the enterococcal species as opposed to *E. coli*.

Thus it is recommended that the poultry industry in South Africa review the way they use therapeutic antimicrobials so as to minimise antimicrobial drug resistance in the chickens and hence possible transfer of resistance to humans. It is highly recommended that this industry re-examine the oral use of antimicrobials where resistance is highest and even consider

withdrawing those antimicrobials for use as growth promoters which would be similar to the stance taken by the EU and Australia. The other recommendation is that all antimicrobials should be prescription drugs. Particularly, there is a need for the continued use of fosfomycin as a feed additive to be re-evaluated. In South Africa fosfomycin is not registered for use in human medicine and so the high levels of resistance observed among human isolates is a concern, with animals suspected as the likely source of the observed resistance.

6.2 QUESTIONS ARISING

Large numbers of humans (5000) die, get hospitalised (325, 000) or become ill (approximately 76 million) per year due to food associated diseases in the USA alone (89). Although not recorded, it is generally believed that there is a higher prevalence of these diseases in South Africa. It is known that in the more serious cases, antimicrobials are needed in the treatment of these diseases (29, 33, 79, 89). Thus antimicrobial drug resistance should be considered a serious veterinary public health problem not only from a food safety perspective (5, 17, 28, 30, 35, 72), but also as an occupational health hazard. Even with this limited study that did not incorporate farm workers, who are considered to be at higher risk of obtaining antimicrobial resistance from bacteria of animal origin than abattoir workers, it is clear that there is some risk. In view of this, is a risk analysis study including both antimicrobial resistances as a food safety issue as well as an occupational hazard not long overdue in South Africa?

The abattoir where the broilers sampled in this study are slaughtered employs Hazard Analysis and Critical Control Point (HACCP) system for hygiene and quality control purposes. Therefore the question that arises here is whether this could explain the minimal transfer of resistance from broiler offals and the abattoir workers suggested in this study.

It is acknowledged that the use of antimicrobials in livestock is both legitimate and vital, and in most cases it leads to considerable economic advantage to the extent that producers cannot simply afford not to include antimicrobials in animals' diet (31, 68, 79, 82). In view of this, the use of antimicrobials as AMGP may not be done away with in the near future (82), despite the high level of resistance from poultry isolates observed in this study. This lack of will to

do away with AMGP is enhanced by lack of suitable alternatives e.g. vaccines (82). Therefore there has to be a strong case justifying the existence of a link between the use of antimicrobials in animals and development and the amplification of resistant micro-organisms if the poultry industry in this country is to embrace abolishment of AMGP. The question that arises here is whether enough is being done in South Africa to find alternatives to AMGP.

Below follows questions that this study has not fully addressed and is pertinent in the South African context, and from a VPH point of view:

i. Are resistant organisms present in animals receiving the relevant antimicrobial?
ii. Are resistant organisms more common in animals and farming areas in South Africa where the relevant antimicrobial has been used, but absent or near absent in areas where it has not been used?
iii. Are resistant organisms detectable in food products from animals fed the relevant antimicrobial?
iv. Are resistant organisms found in the general community in people who have, or are likely to have, consumed these products?

Researchers at the University of Illinois Urban-Campaign found antimicrobial resistant bacteria as far as 250 meters down stream from lagoons where waste from pig farms was dumped. These same researchers also found antimicrobial resistance genes not only in intestinal bacteria from pigs that had survived in the soil, but also in “typical soil inhabitants,” micro-organisms that originate from the soil itself (92). Therefore the question that arises here is; if the level of resistance observed among poultry isolates is a reflection of the situation in the country, how is this affecting bacteria flora in areas where chicken litter that is used in the growing of broilers and faecal waste from the abattoir is dumped or disposed off. Could this have an effect on the resistance profiles of enteric organisms of bovines fed on poultry litter from broilers fed AMGP?

ANNEXURE I: Pilot study disc diffusion results

Enterococci																				
Isolate	Sulph/Tri		Lincospectin		Fosbac		Gentamicin		Vancomycin		Ampicillin		Enrofloxacin		Neomycin		Doxycycline		Lincomycin	
	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R	mm	S/R
B20 [E1]	25.62	S	17.01	R	0	R	16.37	S	13.75	R	22.79	S	19.03	I	16.82	R	7.83	R	0	R
B35[E2]	24.33	S	0	R	22.42	S	15.16	S	15.29	I	22.97	S	19.45	I	14.11	R	9.23	R	0	R
B79[E1]	25.28	S	17.19	R	21.89	S	13.93	S	19.34	S	21.89	S	16.65	R	16.45	R	9.1	R	0	R
B18[E2]	24	S	14.07	R	15.63	R	10.74	R	18.99	S	25.07	S	10.9	R	12.86	R	8.77	R	0	R
B42[E2]	25.35	S	13.59	R	21.48	S	15.58	S	14.59	I	24.92	S	17.13	I	15.79	R	0	R	0	R
B70[VRE1]	24.68	S	19.09	R	0	R	17.08	S	15.64	I	27.04	S	18.85	I	0	R	8.3	R	0	R
B74	24.72	S	16.56	R	20.08	S	15.71	S	16.41	I	20.5	S	20.08	S	0	R	10.46	R	0	R
B30[E1]	25.68	S	16.69	R	20.86	S	15.27	S	17.82	S	23.77	S	13.85	R	15.78	R	8.86	R	0	R
B7[E2]	25.06	S	15.84	R	21.3	S	17.75	S	13.55	R	23.26	S	21.67	S	15.99	R	9.73	R	0	R
B14[VRE1]	24.58	S	0	R	21.49	S	16.88	S	15.72	I	24.71	S	18.25	I	15.63	R	9.13	R	0	R
B75	25.74	S	16.11	R	15.46	R	14.95	I	17.05	S	25.56	S	12	R	15.51	R	8.42	R	0	R
B78[E2]	22.96	S	16.23	R	0	R	15.32	S	16.04	I	21.04	S	15.54	R	16.6	R	0	R	0	R
B16[E1]	24.73	S	15.1	R	0	R	17.64	S	18.7	S	22.16	S	17.45	I	17.04	S	8.02	R	0	R
B37[E2]	19.19	S	0	R	20.79	S	13.94	I	19.35	S	28.11	S	15.97	R	14.16	R	8.89	R	0	R
B19[E2]	24.78	S	15.77	R	22.92	S	14.95	I	19.14	S	28.64	S	13.61	R	15.6	R	8.2	R	0	R

ANNEXURE I: Cont.

E. coli																		
Isolate	**Sulph/Tri**		**Lincospectin**		**Fosbac**		**Gentamicin**		**Ampicillin**		**Enrofloxacin**		**Neomycin**		**Doxycycline**		**Lincomycin**
	mm	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**	**S/R**	**mm**
BH 20	0	R	12.2	R	17.98	S	20.6	S	18.17	S	16.1	R	10.86	R	11.89	R	0
BH 40	24.43	S	6	R	21.66	S	15.45	R	15.6	S	15.9	R	12	R	24.43	S	0
BH 14	20.1	S	7.8	R	7.3	R	17.01	R	0	R	16.21	R	12.84	R	7.37	R	0
BH 55	0	R	0	R	7.3	R	18.65	S	13.52	R	11.54	R	10.36	R	7.8	R	0
BH 50	0	R	0	R	0	R	17.6	S	13.7	R	12.75	R	8.8	R	7.6	R	0
BH 6	28.64	S	7.9	R	11	R	15.3	R	12.9	R	11.97	R	10.72	R	0	R	0
BH 21	23.48	S	11.7	R	7	R	15.7	R	15.65	S	12.65	R	14.07	R	7	R	0
BH 54	16.17	S	0	R	0	R	16.8	I	14.2	S	13.28	R	9.8	R	7	R	0
BH 26	23.59	S	20.2	S	5	R	7	R	13.17	R	14.9	R	12.3	R	7	R	0
BH 1	29.17	R	15.08	R	6.7	R	18.5	S	12.3	R	15.44	R	16.1	I	7.22	R	0

ANNEXURE II: Volunteer information leaflet and informed consent

Title of Study

The occurrence of antimicrobial drug resistance in enteric bacteria isolated from faecal samples from broilers fed antimicrobial growth enhancers and exposed poultry abattoir workers.

Introduction

You are invited to volunteer for a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. You may at any time withdraw from this study.

The Nature and Purpose of this Study

This study is to test if the handling of chicken "mala" or intestines by people working in the abattoir could be dangerous to their health. It might make germs in their body too used to the medicine used in the chicken feed and that medicine will not work for the people if they get sick from that germ. Information will be collected and compared to that from other people who do not handle chicken "mala" or intestines when they work. This information will also be compared with that from chickens that have been fed with the medicine in their food.

Explanation of what Procedures will be followed

A stool (faecal) sample is needed to test if the germs in it will be killed or not by the medicines. This sample will be collected from you if you have not been on antimicrobials for at least three months prior to sampling. You will be asked to volunteer to collect a stool sample from yourself, which will be submitted for testing (bacterial screening) by an expert.

Discomfort Involved

You will not be hurt and you will not have to drink or swallow a medicine. The only possible problem for you is to collect your own stool sample into the specimen container provided. The procedure of doing this will be explained to you at the time when the sample bottles are issued to you.

Benefits of this Study

The study will provide essential information on:

If germs in the stool of people working with "mala" more protected from medicine (resistant) than germs in the stool of people not working with "mala"?

Can the protection from medicine in germs from people working with "mala" be linked to the protection from medicine the germs get in chickens?

Information

If you have any questions concerning this study, you should contact:
Professor Veary at telephone: 529 8015 or cell: 083 680 8285.

Has the Trial Received Ethical Approval?

The Protocol for this research was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, and that committee has granted written approval.

What are my Rights as a Participant in this Trial?

Your participation in this trial is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to medical care.

Confidentiality

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a volunteer in this project. Results will be published or presented in such a fashion that you remain unidentifiable.

Any information uncovered regarding your test results or state of health as a result of your participation in this project will be held in strict confidence.

Consent to Participate in this Study

You must confirm that you have read or have had read to you in a language that you understand the above information before signing this consent form. You must confirm that you have had the content and meaning of this information explained to you. You must confirm that you have been given opportunity to ask questions and are satisfied that they have been answered satisfactorily. You hereby volunteer to take part in this study.

....	....
Volunteer signature	**Date**
....	....
Person obtaining informed consent	**Date**
....	....
Witness	**Date**

VERBAL VOLUNTEER INFORMED CONSENT

(Applicable when Volunteers cannot read or write)

I, the undersigned, Dr James Wabwire Oguttu, have read and have explained fully to the Volunteer (named) ……………….. and /or his/her relative the attached Volunteer information leaflet, which has indicated the nature and purpose of the trial in which I have asked the Volunteer to participate. The explanation I have given has mentioned both the possible risks and benefits of the trial and the alternative treatments available for his/her illness. The Volunteer indicated that he/she understands that he/she will be free to withdraw from the trial at any time for any reason and without jeopardising his/her subsequent injury attributable to the drug(s) used in the clinical trial, to which he/she agrees.

I hereby certify that the Volunteer has agreed to participate in this trial.

Volunteer's Name ______________________

(Please print)

Investigator's Name ______________________

(Please print)

Investigator's Signature ______________________ **Date** _____________

Witness's Name ______________________

(Please print)

Witness's Signature ______________________ **Date** _____________

ANNEXURE III: Questionnaire

The objective of this questionnaire is to source for information on the types and patterns of antimicrobial usage patterns, and the amounts of antimicrobials used on some private and company farms studied over the period 2004 to 2005. This information will be used to relate the patterns and amounts of antimicrobial use to antimicrobial resistance profiles of bacterial isolates obtained in the first phase of this study.

A. A STATEMENT OF CONFIDENTIALITY

As agreed from the on set of this project, we reiterate our pledge to keep any information you provide in this questionnaire confidential and that it will not be used in any way that could be detrimental to the running of your farm (s) and or company. The respondent and the farms will be given a code number to keep them anonymous, and section A and B of the questionnaire will be kept separately from your answers during any analysis. Client confidentiality will also be maintained.

B. CONTACT PERSON'S PARTICULARS

Names	
Designation/Position held in the company	
Physical address	Code
Postal address	Code
Tel. no	
Cell number	
Code number	0001

C. FLOCK MANAGEMENT

1. Please provide information of the following farms by filling in the table below

Name of farm	Indicate category of farm (private=p or company=c)	No of houses on the farm	Floor area per house	Number of birds reared per house	Stocking density at placing	Stocking density at end of grow out period	Grow out period	Are antimicrobials included in the feed?		Company supplying feed to the farm	Turn around stocking period
								No	Yes		
01											
02											
03											
04											
05											
06											

2. Indicate what best describes the production system and disinfection methods for each of these farms for the period 2004 -2005

Name of farm	Type of housing on farm: O= open & C = closed		All in all out	Multi-age	Disinfection methods employed after cleaning			Name two chemical disinfectants used during this period
	O	C			chemical	physical	Other (specify)	
01								
02								
03								
04								
05								
06								

3. How is the effectiveness of cleaning and disinfection monitored?

All houses are sampled every time after washing/disinfection & sample sent to laboratory	
Few houses randomly sampled after washing/disinfection & samples sent to laboratory	
Other (specify)	

D FEED ADDITIVES

1. Where antimicrobials were included as growth enhancers, provide the following information on the different antimicrobials/antimicrobials that were used as additives in the feed for the following farms during the period 2004 -2005)

i) 01

Period (year and month)	**month**	**Type of antimicrobial used (e.g. Tetracycline)**	**Trade name of additive**	**To what feed is it added? (Starter-S Grower –G, Finisher- F)**			**Amount per ton of feed additive (kg/ton)**	**Total amount of antimicrobials (kg) used**
				S	**G**	**F**		
2005	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
2004	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

ii) 02

Period (year and month)	month	Type of antimicrobial used (e.g. Tetracycline)	Trade name of additive	To what feed is it added? (Starter-S Grower –G, Finisher- F) S	G	F	Amount per ton of feed additive (kg/ton)	Total amount of antimicrobials (kg) used
2005	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
2004	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

iii) 03

Period (year and month)	**month**	**Type of antimicrobial used (e.g. Tetracycline)**	**Trade name of additive**	**To what feed is it added? (Starter-S Grower –G, Finisher- F)**			**Amount per ton of feed additive (kg/ton)**	**Total amount of antimicrobials (kg) used**
				S	**G**	**F**		
	Jan							
	Feb							
	March							
	April							
	May							
	June							
2005	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
	Jan							
	Feb							
	March							
	April							
	May							
	June							
2004	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

(iv) 04

Period (year and month)	month	Type of antimicrobial used (e.g. Tetracycline)	Trade name of additive	To what feed is it added? (Starter-S Grower –G, Finisher- F) S	G	F	Amount per ton of feed additive (kg/ton)	Total amount of antimicrobials (kg) used
2005	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
2004	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

v) 05

Period (year and month)	**month**	**Type of antimicrobial used (e.g. Tetracycline)**	**Trade name of additive**	**To what feed is it added? (Starter-S Grower –G, Finisher- F)** S	G	F	**Amount per ton of feed additive (kg/ton)**	**Total amount of antimicrobials (kg) used**
2005	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
2004	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

vi) 06

Period (year and month)	month	Type of antimicrobial used (e.g. Tetracycline)	Trade name of additive	To what feed is it added? (Starter-S Grower –G, Finisher- F) S	G	F	Amount per ton of feed additive (kg/ton)	Total amount of antimicrobials (kg) used
2005	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							
2004	Jan							
	Feb							
	March							
	April							
	May							
	June							
	July							
	August							
	Sept							
	Oct							
	Nov							
	Dec							

2. Please indicate the rotation pattern/scheme of antimicrobial feed additives?

i) On company farms:

Every six months	
According to seasons	
Once a year	
Other (specify)	

ii) On private farms:

Every six months	
According to seasons	
Once a year	
Other (specify)	

E. HEALTH MANAGEMENT

1. Name of person who attends to the health problems of flock?

Private farm	Company farms

2. For each of the disease (s) problems requiring the use of antimicrobials on the farms indicated in the table below, what was your choice of antimicrobial used for the period 2004 to 2005?

Antimicrobials used for therapeutic purposes

Name of farm	Dates on which disease problems occurred	Disease (s)	Choice of antimicrobial used	Dose (mg/kg) and route	Total amount of antimicrobial used (volume)	For how long were the birds on treatment? (days)
05						
04						
06						
03						
01						
02						

3. Have you had to change over the last 3 years the choice of antimicrobial used for any of the problems named in (E2) above?

Yes	
No	

4. If yes, give reasons for this change, and indicate which antimicrobial you stopped using and the one you adopted in its place.

5. Do you include antimicrobials in the feed specifically for purposes of preventing disease out breaks?

Yes	
No	

6. If yes, please list the disease and the antimicrobial used for the period 2004 and 2005 in the table below.

Antimicrobials used for prophylaxis

Name of farm	Period	Disease (s) controlled	Antimicrobial (s) used	Duration of treatment
05	2004			
	2005			
06	2004			
	2005			
03	2004			
	2005			
01	2004			
	2005			
04	2004			
	2005			
02	2004			
	2005			

7. In event of an out break of a bacterial or viral disease on a farm, do you use antimicrobials to control the disease?

Yes	
No	
Not always(specify)	

8. If yes, which antimicrobials did you used in the period 2004 -2005 for such disease outbreaks?

Antimicrobials used for metaphylaxis

Disease	Farm on which disease was controlled	Trade name of antimicrobial used	Amount of antimicrobial used	Date when antimicrobial was used
	05			
	06			
	03			
	01			
	04			
	02			

9. If no, or where antimicrobials were not used, explain how these diseases were controlled

____

____

____

____

10. Is there any information that you think we have not asked regarding antimicrobial usage on the farms listed below over the period 2004 to 2005? Please feel free to make any comments in this regard in the tale below.

Name of Farm	Comments
05	
06	
03	
01	
04	
02	

Thank you for your cooperation.

ANNEXURE IV: Panel for determining MIC for research project

50µl / well contained the following concentrations of antimicrobials

Species: _____________ **Isolate:** _________________ **date:** ______________

	1	2	3	4	5	6	7	8	9	10	11	12
	Van	**Vi**	**Dox**	**Tri**	**Su**	**Amp**	**Ba(u)**	**Enf**	**Ery**	**Fos**	**Cf**	**Na**
A	256	64	128	32	2048	32	100	8	256	128	128	128
B	128	32	64	16	1024	16	50	4	128	64	64	64
C	64	16	32	8	512	8	25	2	64	32	32	32
D	32	8	16	4	256	4	12,5	1	32	16	16	16
E	16	4	8	2	128	2	6,25	0,5	16	8	8	8
F	8	2	4	1	64	1	3,13	0,25	8	4	4	4
G	4	1	2	0,5	32	0,5	1,56	0,13	4	2	2	2
H	2	0,5	1	0,2	16	0,25	0,78	0,06	2	1	1	1

Van = vancomycin; Vi = Virginiamycin; Dox = doxycyciline; Tri = trimethoprim; Su = sulphamethoxazole; Amp = ampicillin; Ba = bacitracin; Enf = enrofloxacin; Ery = erythromycin; Fos = fosfomycin; Cf =Ceftriaxone and Na = nalidixic acid.

CHAPTER 7

References

1. **Aarestrup, F. M., 2000.** Characterisation of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: Genetic evidence that persistence of GRE in pig herds is associated with co-selection by resistance to macrolides. *Journal of Clinical Microbiology* 38 (7): 2774-2777

2. **Aarestrup, F. M., Bager, F., Jensen, N. E., Madsen, M., Meyling, A., Wegener, H. C., 1998.** Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. APMIS; 106(6): 606-22. Also available online at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=9 (visited on 2006-09-01)

3. **Aarestrup, F. M., Seyforth, A. M., Emborg, H. D., Pedersen, K., Hendriksen, R., Bager, F., 2001.** Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in faecal enterococci from food animals in Denmark. *Antimicrobial Agents and Chemotherapy* 45: 2054-2059

4. **Ahmed, E. I. Amin., 2006.** Study: Antimicrobial ban reduces incidence of drug resistance. FoodProductionDaily-USA.com. Available at: http://www.foodproductiondaily.com/news/ng.asp?n=67114-antimicrobial-campylobacter (visited on 2006-08-04)

5. **American College of Physicians, 2001.** Antimicrobial Resistance from drug use in livestock: FDA'S approach to Risk Management. Available online at: http://www.acponline.org/ear/vas2001/livestock.htm (visited on 2006-09-01)

6. **Anderson, A. D., Nelson, J. M., Rossiter, S., Angulo, F. J., 2003.** Public health consequences of use of antimicrobial agents in food animals in the United States. *Microbial Drug Resistance* 9 (4): 373-379

7. **Anonymous, 2005.** IVS desk reference volume 8. CTP Book Printers, Cape Town

8. **Anonymous, 1984.** Animal Diseases Act No 35. available online at: http://www.nda.agric.za/vetweb/Regulate/R_Animal_Diseases_Act_No_35.htm (Visited on 2006/10/06)

9. **Antunes, P., Ren, C., Sousa, J. C., Peixe, L., Pestana, N., 2002.** Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. *International Journal of Food Microbiology Agents* 82: 97-103

10. Alliance for the Prudent Use of Antimicrobials, **2005.** Antimicrobial abuse. Editorial, the Boston Globe. Available online at: http://www.tufts.edu/med/apua/News/Animalfeed.html

11. **Arakawa, Y., Ike, Y., Nagasawa, M., Shibata, N., Doi, Y., Shibayama, K., Yagi, T., Kurata, T., 2000.** Trends in Antimicrobial-Drug Resistance in Japan. CDC. Available on line at: http://www.cdc.gov/ncidod/eid/vol6no6/arakawa.htm visited on 20006-08-10

12. **Archibald, L. K., Reller, B. K., 2001,** Clinical Microbiology in Developing Countries. *Emerging Infectious Diseases* 7 (2): 302-305
Available at: http://www.cdc.gov/ncidod/eid/vol7no2/archibald.htm

13. **Bager, F., Aarestrup, F. M., Madsen, M., Wegener, H. C., 1999.** Glycopeptide Resistance in *Enterococcus faecium* from broilers and pigs following discontinued use of Avoparcin. *Microbology Drug Resistance* 5 (1): 53-56

14. **Bates, J., 1997.** Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. *Journal of Hospital Infection* 37: 89-101

15. **Bennig, V. R., Mathers, J. J., 1999.** Comparison of agar dilution and Broth micro-dilution methods of anaerobic antimicrobial susceptibility testing using several veterinary antimicrobials against *Clostridium perfringens* strains originating from porcine and avian sources. *Anaerobes* 5: 561-569

16. **Bok, E. H., Holzapfel, W. H., Odendaal, E. S., van der Linde, J. H., 1986.** Incidence of food borne pathogens on retail broilers. *International Journal of Food Microbiology* 3: 273-285

17. **Bonten, M. J. M., Willems, R., Weinstein, R. A., 2001.** Vancomycin-resistant enterococci: why are they here, and where do they come from? *Infectious Diseases* 1: 314-325

18. **Bren, L., 2001.** Antimicrobial resistance from down on the chicken farm. *FDA Consumer* 35(1):10-11.

19. **Butaye, P., Devries, L. A., Haesebrouck, F., 2001.** Differences in antimicrobial resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* Strains isolated from farm and pet animals. *Antimicrobial Agents Chemotherapy* 45(5): 1374-1378

20. **Byarugaba, D.K., 2004,** Antimicrobial resistance in developing countries and responsible risk factors. *International Journal of Antimicrobial Agents* 24(2): 105 - 110

21. **Catry, B., Leavens, H., Devriese, L. A., Opsmer, G., de Kruif, A., 2003.** Review article: antimicrobial resistance in livestock. *Journal of Veterinary Pharmacology and therapeutics* 26: 81

22. **CDC, 2002.** Outbreak of multidrug resistant *Salmonella* Newport---United States. MMWR 51 (25): 545-548
Available at http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5125a1.htm (visited 2006-08-08)

23. **Chen, H. Y., Robert, L. R. H., Kirk, M., Casewell, M. W., Beigton, D., 2002.** Differential antimicrobial susceptibility between human and chicken isolates of vancomycin resistance and sensitive *Enterococcus faecium*. *International Journal of Antimicrobial Agents* 19: 39-46

24. **Collignon, P. J., 1999.** Vancomycin resistant enterococci and use of avoparcin in animal feed: is there a link? MJA, 171: 144-146

25. **Craven, S. E., Cox, N. A., Stern, N. J., Mauldin, J. M., 2001.** Prevalence of *Clostridium perfringens* in commercial broiler hatcheries. *Avian Diseases* 45: 1050-1053

26. **Crespo, R., Fisher, D. J., Shivaprasad, H. L., Fernandez-Miyakawa, M. E., Uzal, F. A., 2007.** Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. *Journal of Veterinary Diagnostic Investigation* 19(3): 329-333

27. **Dahiya J. P., Hoehler D., Wilkie D. C., Van Kessel A G, Drew M D 2005** Dietary glycine concentration affects intestinal *Clostridium perfringens* and lactobacilli populations in broiler chickens. *Poultry Science* 84(12): 1875-1885

28. **De Oliveira, D. S., Flores, F. S., dos Santos, L. R., Brandelli, A., 2004.** Antimicrobial resistance in *Salmonella* Enteritidis strains isolated from broiler carcasses, food, human and poultry-related samples. *International Journal of Food Microbiology* 97(3): 297-305. Available online at www.sciencedirect.com

29. **Essack, S. Y., 2000.** Laboratory detection of extended-spectrum β-lactamases (ESBLs): The need for a reliable, reproducible method. *Diagnostic Microbiology and Infectious Diseases* 37: 293-295

30. **Fidler, D. P., 1998.** Legal issues associated with antimicrobial drug resistance. *Emerging Infectious Diseases* 4 (2): 169-177 Available at: http://www.cdc.gov/ncidod/eid/vol4no2/fidler.htm (visited on 2006-08-10)

31. **Florea, N. F., Nightingale, C. H., 2004.** Review of the pharmaco-dynamics of antimicrobial use in animal food production. *Diagnostic Microbiology and Infectious Disease* 49: 105-108

32. **Frediani-Wolf, V. R. S., 2003.** Resistance patterns of *Campylobacter* spp. strains isolated from poultry carcasses in a big Swiss poultry slaughterhouse. *International Journal of Food Microbiology* 89 (23): 233-240

33. **Freeman, A., 1970.** The Swann Report. *Journal of American Veterinary Medical Association* 157(1): 13-16

34. **Gholamiandekhordi, A. R., Ducatelle, R., Heyndrickx, M., Haesebrouck, F., Van Immerseel, F., 2006.** Molecular and phenotypical characterisation of *Clostridium perfringens* isolates from poultry flocks with different disease status. *Veterinary Microbiology* 113(1-2): 143-52

35. **Glynn, K. M., Bopp, C., Dewitt, W., Dabney, P., Mokhtar, M., Angulo, F. J., 1998.** Emergence of Multidrug-Resistant *Salmonella enterica* Serotype Typhimurium DT 104 Infections in the United States. *The New England Journal of Medicine* (338): 1333-1339

36. **Goodyear, K. L., 2002.** Veterinary surveillance for antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* 50: 612-614

37. **Gouws, P. A., Brozel, V. S., 2000.** Antimicrobial Resistance of *Salmonella* isolates associated with retail chicken and poultry abattoir. *South African Journal of Science* 96: 254-256

38. **Grave, K., Kaldhusdal, M., Kruse, H., Fevang, L. M., Knut, H., Flatland, S. M. O., 2004.** What has happened in Norway after the ban of avoparcin? Consumption of antimicrobials by poultry. *Preventive Veterinary Medicine* 62: 59-72

39. **Gray, J. T., Shryock, T. R., 2005.** Antimicrobial susceptibility testing of bacteria isolated from animals. *Clinical Microbiology Newsletter* 27 (17): 131-135

40. **Hong-Zhou Lu, Xin-Hua Weng, Haijing Li, You-Kuan Yin, Mao-Yin Pang, Yi-Wei Tang, 2002.** *Enterococcus faecium*-related outbreak with molecular evidence of transmission from pigs to humans. *Journal of Clinical Microbiology* 40(3): 913-917

41. **Ike, Y., Tanimoto, K., Ozawa, Y., Nomura, T., Fujimoto, S., Tomita, H., 1999.** Vancomycin resistant enterococci in imported chickens in Japan. *The Lancet* 353 (9167): 1854

42. **Iovine, N. M., Blaser, M. J., 2004.** Antimicrobials in animal feed and spread of resistant *Campylobacter* from poultry to humans. *Emerging Infectious Diseases*: 10(6): 1158-1159. Available online from: http://www.cdc.gov/ncidod/EID/vol10no6/04-0403.htm

43. **Ishihara, K., Kira,T., Ogikubo, K., Morioka, A., Kojima, A., Kijiama-Tanaka, M., Takahashi, T., Tamura, Y., 2001.** Antimicrobial susceptibilities of *Campylobacter* isolated from food producing animals on farms 1999-2001: results from the Japanese Veterinary Antimicrobial Monitoring Program. *International Journal of Antimicrobial Agents* 24: 63-69

44. **ISO, 2006.** Clinical laboratory testing and *in vitro* diagnostic test systems – susceptibility testing of infectious agents and evaluation of performance antimicrobial susceptibility devices. International standard ISO/FDIS 20776 – 1

45. **Iversen, A., Khun, I., Rahman, M., Franklin, A., Burman, L. G., Olsson-Liljequist, B., Torell, E., Mollby, R., 2004.** Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. *Environmental Microbiology*, 6(1): 55-59

46. **Iversen, A., Khun, I., Franklin, A., Mollby, R., 2002.** High prevalence of vancomycin-resistant enterococci in Swedish Sewage. *Applied Environmental Microbiology* 68(6): 2838-2842

47. **Kalender, H., Ertas, H. B., 2005.** Isolation of *Clostridium perfringens* from chickens and detection of the alpha toxin gene by polymerase chain reaction. *Turkish Journal Veterinary Animal Science* 29: 847-851

48. **Khun, I., Iversen, A., Burman, L. G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A. M., Blanch, A. R., Vilanova, X., Taylor, H., Caplin, J., Moreno, M. A., Dominguez, L., Herrero, I. A., Mollby, R., 2003.** Comparison of enterococcal populations in animals, humans, and the environment-a European study. *International Journal of Food Microbiology* 88 (2-3): 133-45

49. **Khun, I., Iversen, A., Finn, M., Greko, C., Burman, L. G., Blanch, A. R., Vilanova, X., Manero, A., Taylor, H., Caplin, J., Dominguez, L., Herrero, I. A., Moreno, M. A., Mollby, R., 2005.** Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. *Applied Environmental Microbiology* 71(9): 5383-90

50. **Klein, G., 2003.** Taxonomy, ecology and antimicrobial resistance of *Enterococci* from food and the gastro-intestinal tract. *International Journal of Food Microbiology* 88: 123-131

51. **Leavis, H. L., Willems, R. J. L., Top, J., Spalburg, E., Mascini, E. M., Fluit, A., Hoepelman, A., de Neeling, A. J., Bonten, M. J. M., 2003.** Epidemic and none epidemic multidrug-resistant *Enterococcus faecium*. *Emerging Infectious Diseases* 9(9): 1108-1115

52. **Lees, P., Aliabadi, F. S., 2002.** Rational dosing of antimicrobial drugs: Animals versus humans. *International Journal of Antimicrobial Agents* 19: 269-284

53. **Manie, T., Khan, S., Brozel, V. S., Veith, W. J., Gouws, P. A., 1998.** Antimicrobial resistance of bacteria isolated from slaughtered and retail chickens in South Africa. *Letters in Applied Microbiology* 26: 253-258

54. **Mayrhofer, S., Paulsen, P., Smulders, F. J. M., Hilbert, F., 2004.** Antimicrobial resistance profile of five major food borne pathogens isolated from beef, pork and poultry: *International Journal of Food Microbiology*, 97: 23-29

55. **McCormick, J. B., 1998.** Epidemiology of emerging/re-emerging antimicrobial resistant bacterial pathogens. *Current Opinions in Microbiology* 1: *125-129*

56. **McDermott, P., Zhao, S., Wagner, D., Simjee, S., Walker, R., White, D., 2002.** The food safety perspective of antimicrobial resistance. *Animal Biotechnology* 13(1): 71-84

57. **Ministry of Health and Forestry, Department of Food and Health,** 1999 Bacterial resistance to antimicrobial agents in Finland. FINRES.

58. **NCCLS, 1994.** Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; proposed standard. NCCL document M31-P (ISBN 1-56238-258-6). NCCL, 771 East Lancaster Avenue, Villanova, Pennsylvania 1985

59. **Neely, A. N., Holder, I. A., 1999.** Antimicrobial resistance. *Burns* 25(1): 17-24

60. **Nel, H., 2002.** The establishment and standardization of a veterinary antimicrobial resistance surveillance program in South Africa. MSc thesis, University of Pretoria

61. **North, M. O., Bell, D. D., 1990.** Commercial Chicken Production Manual (4TH Edition). Pages 767 -774. Van Nostrand Reinhold, 115 Fifth Avenue, New York, N.Y. 10003. ISBN 0-442-31881-2

62. **Nys, S., Okeke, I. N., Kariuki, S., Dinant, G. J., Driessen, C., Stobberingh, E. E., 2004.** Antimicrobial resistance of faecal *E. coli* from healthy volunteers from eight developing countries. *Journal of antimicrobial chemotherapy* 54 (5): 952-955

63. **Okeke, I. N., Fayinka, S. T., Lamikanra, A., 2000.** Antimicrobial resistance in *Escherichia coli* from Nigerian students, 1986-1998. *CDC: Emerging Infectious Diseases* 6(4): 393-396. also available at: http://www.cdc.gov/ncidod/eid/vol6no4/okeke.htm (visited on 2006-08-07)

64. **Okeke, I. N., Adebayo, 2003.** Export of antimicrobial drugs by West African travellers. *Journal of Travel Medicine*: 10:133-135

65. **Okeke, I., 2005.** The antimicrobial rebellion: trends and containment of antimicrobial resistance in Africa. Africa Conference: African Health and Illness Available on line: http://www.utexas.edu/conferences/africa/2005/panels/okeke.html
(visited on 2006-08-07)

66. **Prescott, J. F., 1999.** Antimicrobial therapy. In Dwight C Hirsh, Yaun Chung Zee (eds) *Veterinary Microbiology Blackwell Science* USA 28-45

67. **Richet, H. M., Mohammed, J., McDonald, C. l., Jarvis, W. R., INSPEAR, 2001.** Building communication networks: International Network for the study and prevention of emerging antimicrobial resistance. Emerging infectious diseases 7(2): 319 - 322

68. **Salisbury, J. G., Nicholls, T. J., Lammerding, A. M., Turnidge, J., Nunn, M. J., 2002.** A risk analysis framework for the long-term management of antimicrobial resistance in food–producing animals. *International Journal of Antimicrobial Agents* 20 (3): 153-164

69. **SANVAD, 2007**. South African National Veterinary Surveillance and Monitoring Programme for Resistance to antimicrobial Drugs. ISBN: 978-1-86854-673

70. **Sasaki, Y., Yamamoto, K., Tamura, Y., Takahashi, T., 2001.** Tetracycline-resistance genes of *Clostridium perfringens*, *Clostridium septicum* and *Clostridium sordellii* isolated from cattle affected with malignant oedema. *Veterinary Microbiology* 33: 61 -69

71. **Saunders, P., Gnanou, J. C., 1999.** Antimicrobial Resistance in Bacteria from Animal origin: Objectives of the concerted action. In WHO Report on the informal meeting on antimicrobial resistance surveillance in food borne pathogens Geneva 13-17 of 31

72. **Sischo, W. M., 2006.** Stakeholders position paper: Dairy producer. *Preventive Veterinary Medicine* 73: 203-208

73. **Skov, M. N., Andersen, J. S., Aabo, S., Ethelberg, S., Aarestrup, F. M., Sorensen, A, H., Sorensen. G., Pedersen, K., Nordentoft, S., Olsen, K. E. P., Gerner-Smidt, P., Baggesen, D. L., 2007.** Antimicrobial drug resistance of *Salmonella* isolates from meat and humans, Denmark. Emerging *Infectious Diseases*: 13(4): 638-641

74. **Sorensen, T. L., Blom, M., Monnet, D. L., Frimodt-Møller, N., Poulsen, R. L., Espersen, F., 2001.** Transient intestinal carriage after ingestion of antimicrobial – resistant *E. faecium* from chicken and pork. The *New English Journal of Medicine*; 345(16): 1161-66

75. **Steven, M., Mackie, R. A., Lawson, G. H, K., 1995.** Antimicrobial susceptibility of ileal symbiont intracellularis isolated from pigs with proliferative entropathy. *Journal of Clinical Microbiology* 33(5), 1314-1317

76. **Stevens, D. L., Maier, K. A., Mitten, J. E., 1987.** Effect of antimicrobials on toxin production and viability of *Clostridium perfringens*. *Antimicrobial agents and chemotherapy* 31(2): 213-218

77. **Swann, M. M., 1969.** Joint Committee on the use of antimicrobials in animal Husbandry and Veterinary Medicine. Her Majesty's Stationary Office, London

78. **Tejedor-Junco, M. T., Alfonse-Rodriguez, O., Martin-Barras, J. L., Gonzalez-Martin, M., 2005.** Antimicrobial susceptibility of *Enterococcus* strains isolated from poultry faeces. *Research in Veterinary Science* 78: 33-38

79. **Threlfall, J., 2002.** Antimicrobial drug resistance in *Salmonella*: Problems and perspectives in food and water borne infections. *FEMS Microbiology Reviews* 26: 141-148

80. **Tollefson, L., Flynn, W. T., 2002.** Impact of Antimicrobial Resistance on Regulatory Policies in Veterinary Medicine: Status Report

81. **Tollefson, L., Fedorka-Cray, P., Marano, N., Angulo, F., 1999.** USA National Antimicrobial Resistance Monitoring System for Enteric Bacteria. In WHO Report on the informal meeting on antimicrobial resistance surveillance in food borne pathogens Geneva 10-13 of 31

82. **Ungemach, F. R., Müller-Bahrdt, D., Abraham, G., 2006,** Guidelines for prudent use of antimicrobials and their implications on antimicrobial usage in veterinary medicine. *International Journal of Medical Microbiology* 296 (S2): 33-38

83. **Väänänen, H. M., Pietilä, K., Airaksinen, M., 2006,** Self-medication with antimicrobials—Does it really happen in Europe? *Health policy* 77: 166-167

84. **Van den Bogaard, A. E., Stobberingh, E. E., 2000.** Epidemiology of resistance to antimicrobials: link between animals and humans. *International Journal of antimicrobial agents* 14: 327-335

85. **Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R., 2004.** *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian pathology* 33(6): 537-549

86. **Von Baum, H., Marre, R., 2005.** Antimicrobial resistance of *Escherichia Coli* and therapeutic implications. *International Journal of Medical Microbiology* 295: 503-511

87. **Von Gottberg, A., 2004.** Patterns of antimicrobial susceptibility among bacterial pathogens in South Africa. *CME* 22 : 189-192

88. **Wegener, H. C., Aarestrup, F. M., Jensen, L. B., Hammerum, A. M., Bager, F., 1999.** Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerging infectious diseases* 5(3): 329-335

89. **White, D. G., Zhao, S., Simjee, S., Wagner, D. D., McDermott, P. F., 2002.** Antimicrobial resistance of food borne pathogens. *Microbes and infection*. 4: 405-412

90. **White, D. G., Zhao, S., Sudle, R., *et al*, 2001.** The Isolation of antimicrobial resistant *Salmonella* from retail ground meats. *The New English Journal of Medicine* 345: 1147-54

91. **Wierup, M., 2001.** The experience of reducing antimicrobials used in animal production in the Nordic countries. *International Journal of Antimicrobial agents*. 18: 287-290

92. **Williams, S., 2005.** Antimicrobial resistance: not just for people anymore. *Journal of Young Investigators* 6. available at: http://www.jyi.org/features/ft.php?id=524 (visited on 2006-08-04)

93. **World Health Organisation, 2002.** Antimicrobial resistance. Geneva.

94. **World Health Organisation, 2000.** Drug resistance threatens to reverse medical progress. Information Office- Press release. WHO/41 12 June