A STUDY OF TETRACYCLINE RESISTANT *ESCHERICHIA COLI* IN IMPALA (*AEPYCEROS MELAMPUS*) AND THEIR WATER SOURCES

by

VALERIA MARIANO

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

Date submitted: December 2007

© University of Pretoria

ACKNOWLEDGEMENTS

This work involved participation of many persons, whose assistance has made this project possible. My sincere appreciation goes to:

- My supervisor *Prof C M E McCrindle* for her valuable guidance, patience and generosity. Without her support this study would not have been possible.
- My co-supervisor *Dr J Picard* for her interest, encouragement and friendly support throughout this study.
- *Kruger National Park (KNP) Scientific Services* for the opportunity to conduct this research.
- My KNP supervisor *Dr L M de Klerk* for her assistance.
- *Prof. B Gummow* for his kind epidemiological advice.
- *Prof J Boomker* for his friendly and constant advice.
- *Ms J Greyling* for her laboratory technical support.
- *Mr J Venter* and *Mr B du Plessis* for the sharing of their precious knowledge.
- *Dr M Quan* for his forthcoming GIS-software technical advice and assistance.
- *Prof. E Venter, Dr A Bosman* and *Dr H van Heerden* for PCR technical advice.
- The *KNP game capture team* for their welcoming friendship and the marvelous adventures they let me share with them.
- The *game guard* and *ranger* in the KNP for their technical guidance in the field.
- *Patricia Khoza* for her helpful arrangement of my accommodation in KNP.
- All *my friends* I met in South Africa, in particular *Nada, Sarah, Nkosi* and *Glynn*, they gave an unforgettable touch to my stay in South Africa.
- My *family* and *Carlo* for their love, patience and keen support.

DEDICATION

To researchers and all those involved in nature conservation

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AOAC	Association of Official Analytical Chemists
AOC	Assimilable organic carbon
APHA	American Public Health Association
AR	Attributable risk
ASTM	American Society for Testing and Materials
AWWA	American Water Works Association
BCIG	Bromo-cloro-indoxyl-glucuronide
BHI	Broth heart infusion
Ca	Calcium
CAMHB	Cation-adjusted Mueller-Hinton
CI	Confidence interval
Cl	Chloride
CLSI	Clinical Laboratory Standards Institute
CSIR	Council for Scientific and Industrial Research
Cumec	Cubic metre per second
DNA	Deoxyribonucleic acid
E	Enterococci
E. coli	*Escherichia coli*
e.g.	*Exemplii gratia* (for example)
EC	Conductivity
EPA	Environment Protection Agency
Et al.	*Et alii* (and others)
ET*EC*	Enterotoxic *Escherichia coli*
EU	European Union
F	Fluoride
FC	Faecal coliforms
GP	Growth promoter
i.e.	*Id est* (that is)
IBDG	Indoxyl-β-D-glucuronide
LTB	Lauril-triptose-broth
MDR-TB	Multi-drug-resistant *Mycobacterium tuberculosis*
MF	Membrane filtration
Mg	Magnesium
MIC	Minimum inhibitory concentration
MPN	Most probable number
MUG	Methyl-umbeliferyl-glucuronide
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NH_4	Ammonia
NO_3	Nitrate
OIE	World Organization for Animal Health
OR	Odds ratio

PO_4	Phosphate
RHP	River Health Programme
RNA	Ribonucleic acid
RPL	Replica plating of Lederberg
RQS	Resource quality study
RR	Relative risk
SDS	Sodium-dodecil-solfate
SO_4	Sulphate
STP	Sewage treatment plant
TDS	Total dissolved solids
TR*EC*	Tetracycline resistant *Escherichia coli*
UK	United Kingdom
UV	Ultra-violet
VCS	Voluntary Consensus Standards
WEF	Water Environment Foundation
WHO	World Health Organisation
WRC	Water Research Commission
XDR-TB	Extensively drug-resistant *Mycobacterium tuberculosis*

TABLE OF CONTENTS

ACKNOWLEDGEMENTS **II**

DEDICATION **III**

LIST OF ABBREVIATIONS **IV**

TABLE OF CONTENTS **VI**

LIST OF TABLES **X**

LIST OF FIGURES **XII**

SUMMARY **XIV**

CHAPTER 1. **INTRODUCTION** **1**

1.1 Background and motivation 1

1.2 Aim of the study 3

1.3 Problem statement 3

1.4 Hypothesis 3

1.5 Objectives 3

1.6 Work plan 4

CHAPTER 2. LITERATURE REVIEW **5**

2.1 Antimicrobial resistance and its relevance in public health 5

2.2 Use of antibiotic and selection of AMR 6

2.3 AMR mechanisms 9

2.4 Diffusion of antimicrobial resistance in the environment 11

2.5 Role of wildlife in the environmental study of AMR 12

2.6 Occurrence of AMR in the aquatic environment 14

2.6.1 Introduction of resistant bacteria into water 15

2.6.2 Antimicrobial residues in water 17

2.7 Microbial ecology of drinking water 19

2.7.1 Factors related to the presence of coliforms in water 20

2.8 Methods for detection of *E. coli* in water 22

2.9 Tetracyclines 24

2.9.1 Tetracyclines, historical aspects 24
2.9.2 Function and use of tetracyclines 25
2.9.3 Overall usage of tetracyclines in South Africa 26
2.9.4 Tetracycline resistance and its transmission 27
2.9.5 Tetracycline resistance genes in *E. coli* 28
2.10 Methods for detection of tetracycline resistance 29
2.10.1 Detection of phenotypic resistance 29
2.10.2 Detection of genotypic resistance 31
2.11 Epidemiological study designs 33
2.11.1 Selection of the most appropriate study design 33
2.11.2 The use of relative risk, attributable risk and odds ratios 35
2.11.3 Evaluation of significance 36
2.11.4 Data collection 36
2.11.5 Sampling design 36

CHAPTER 3. MATERIALS AND METHODS **38**

3.1 Model system 38
3.2 Study area 39
3.2.1 Sabie and Sand Rivers 41
3.2.2 Crocodile River 43
3.2.3 Letaba River 45
3.2.4 Olifants River 46
3.3 Experimental design and procedure 48
3.3.1 Impala as indicator 50
3.3.2 Sampling method 52
3.3.3 Estimation of minimum sampling size 53
3.4 Sampling procedures 54
3.5 Bacteriological analysis 57
3.5.1 Isolation of *E. coli* 57
3.5.2 Replica plating 58
3.5.3 Identification of *E. coli* 59
3.5.4 Phenotypic tetracycline resistance test: MIC 60
3.6 Genotypic resistance test: PCR 62
3.6.1 Optimization of the PCR method 62

3.6.2 PCR applied to the current study 65

3.6.2.2 Amplification of *tet(A)* and *tet(B)* 65

3.6.2.3 PCR analysis 66

3.7 Data analysis 67

CHAPTER 4. RESULTS **68**

4.1 Introduction 68

4.2 Determining pollution status of rivers using retrospective data 68

4.3 *Escherichia coli* cultured from water samples 72

4.4 Tetracycline resistant *E. coli* from water samples 72

4.4.1 Replica plating results from water samples 73

4.4.2 MIC results from water samples 74

4.4.3 MIC and river group formation 74

4.4.4 Distribution of MIC values between the river groups 75

4.5 *E. coli* cultured from impala faecal samples 75

4.6 Tetracycline resistant *E. coli* isolated from impala faecal samples 76

4.6.1 Replica plating results from faecal samples 76

4.6.2 Distribution of MIC values for *E. coli* in impala faecal samples 77

4.6.3 Different distribution of MIC values in faecal samples between the river groups 79

4.7 Resistance pattern related to time scale 80

4.8 Measure of association of TR*EC* between impala and their drinking water 81

4.9 Correspondence between replica plating and MIC methods 83

4.10 Genetic patterns of the isolates 84

4.10.1 DNA extraction and quantification 84

4.10.2 PCR results and electrophoresis 85

CHAPTER 5. DISCUSSION **86**

5.1 Introduction 86

5.2 Methods used for the detection of tetracycline resistance 87

5.3 Analysis of retrospective data 90

5.4 Study design considerations 94

5.5 Detection of TR*EC* in water samples 95

5.6 TR*EC* and river pollution 96

5.7 Detection of TR*EC* from faecal samples 96

5.8 The effect of season 97

5.9 Genetic pattern of the population 99

5.10 Association between TR*EC* in water and faecal samples from impala 100

5.11 AMR in water and problem associated with public health 100

CHAPTER 6. CONCLUSIONS AND RECOMMENDATIONS **102**

CHAPTER 7. REFERENCES **104**

APPENDIX 1 **117**

APPENDIX 2 **119**

APPENDIX 3 **120**

APPENDIX 4 **121**

LIST OF TABLES

Table2.1: Mobile genetic elements that confer AMR. **10**

Table 2.2: EPA proposed methods for enumerating *E. coli* (EPA 2001, 2002). **22**

Table 3.1: List of *tet(A)* and *(B)* primers and their main characteristics, as published by (Boerlin *et al.* 2005). **66**

Table 4.1: Human populations along the different rivers. **69**

Table 4.2: Stats on number of animals in the provinces of the study area (StatsOnline, 2001). **69**

Table 4.3: Characteristic of the main STPs along the studied rivers in KNP (du Plessis B., personal communication 2007). **69**

Table 4.4: Mean of water indices considered for the classification of the rivers. **71**

Table 4.5: Prevalence of water samples positive for *E. coli* from each river. **72**

Table 4.6: Distribution, per river and total, of tetracycline resistant *E. coli* isolated from water samples tested using RPL. **73**

Table 4.7: Percentage distribution of tetracycline MICs for *E. coli* from water samples (n=21) for each river. **74**

Table 4.8: Grouping of rivers into TR*EC*neg and TR*EC*pos. **75**

Table 4.9: Percentage distribution of MIC values for *E. coli* between the groups TR*EC*neg and TR*EC*pos. **75**

Table 4.10: Distribution of faecal samples between TR*EC*neg and TR*EC*pos rivers. **75**

Table 4.11: Distribution of faecal samples positive and negative for *E. coli* according to the river sampled. **76**

Table 4.12: Distribution, per river and total, of TR*EC* in faecal samples using RPL. **77**

Table 4.13: Percentage distribution of tetracycline MICs for *E. coli* isolated from impala faeces (n=191) obtained from different river zones. **77**

Table 4.14: Distribution of total water and total faecal samples time related. **80**

Table 4.15: Percentage distribution of MIC values for *E. coli* in water samples during the different collection periods. **81**

Table 4.16: Total distribution of TR*EC* for water and faecal samples in the different collection period. **81**

Table 4.17: Values considered for the OR calculation. **82**

Table 4.18: Values considered for the χ^2 calculation. **83**

Table 4.19: Comparison of RPL and MIC test results. **83**

Table 4.20: Data used for calculate Kappa. **84**

Table 4.21: Results of spectrophotometer reading for five random samples. **85**

Table A.1: Population census data for different districts on the catchments of the studied rivers (StatsOnline, 2001). **117**

LIST OF FIGURES

Figure 2.1: Mechanisms used by bacteria to avoid the effect of antibiotic (Fan sozzi, 2006). **9**

Figure 2.2: Mechanisms of horizontal gene transfer (Fan sozzi, 2006). **10**

Figure 2.3: Possible pathways of the AMR entrance in an aquatic environment (adapted from Zuccato *et al.,* 2000). **14**

Figure 3.1: Female impala drinking at a water hole. **38**

Figure 3.2: South African National Parks and main rivers (ArcGIS). **39**

Figure 3.3: Water Sampling points (ArcGIS). **40**

Figure 3.4: Ecoregion of Sabie river (WRC, 2001 b). **41**

Figure 3.5: Sabie River flow trend from 2003 to 2005 at Lower Sabie (Venter J, personal communication, 2006). **42**

Figure 3.6: Ecoregion of Crocodile River (WRC, 2001 b). **43**

Figure 3.7: Crocodile River flow trend from 2003 to 2005 at Ten Bosh (Venter J, personal communication, 2006). **44**

Figure 3.8: The Letaba River catchments and its ecosystems (WRC, 2001a). **45**

Figure 3.9: Letaba River flow trend from 2003 to 2005 at Letaba Ranch (Venter J, personal communication 2006). **46**

Figure 3.10: The Olifants River catchment area and its ecosystems (WRC, 2001 b). **47**

Figure 3.11: Olifants River flow trend from 2003 to 2005 at Mamba (Venter J, personal communication, 2006). **48**

Figure 3.12: Collection of impala faecal samples in KNP. **50**

Figure 3.13: Map of impala distribution in KNP according with the 2006 census (J Kruger, personal communication 2006). **51**

Figure 3.14: Water sampling. **54**

Figure 3.15: Water sampling procedures (DWAF, 2001). **55**

Figure 3.16: Impala faeces sampling. **56**

Figure 3.17: Measurement of the distance between the water source and impala faeces (red line on map) measured using Mapsource (Garmin®). **56**

Figure 3.18: Water filtration system. **57**

Figure 3.19: MIC: microtitre plate (AEC Amersham, South Africa). **61**

Figure 4.1: Location of principal water collection site for RHP (blue), camps (brown) and waste water discharge points (red) (ArcGIS). **70**

Figure 4.2: Pie chart showing percentage of tetracycline resistant and percentage of tetracycline susceptible *E. coli* in faecal samples per river according to the MIC method. **78**

Figure 4.3: Pie chart showing proportion of faecal TR*EC* among the different rivers. **78**

Figure 4.4: Histogram showing the distribution of MIC values in the control and exposed groups. **79**

Figure 4.5: Pie chart showing percentage of tetracycline resistant and sensitive *E. coli* in faecal samples per group, using the MIC method. **80**

Figure 4.6: PCR results of the 23 TR*EC* samples examined for *tet(A)* and *tet(B)* and their controls. **85**

Figure A.1: Census districts in the study area (ArcGIS). **118**

SUMMARY

A STUDY OF TETRACYCLINE RESISTANT *ESCHERICHIA COLI* IN IMPALA (*AEPYCEROS MELAMPUS*) AND THEIR WATER SOURCES

By

VALERIA MARIANO

Study Supervisor : Prof. C M E McCrindle

Co-Supervisor : Dr J Picard

Department : Paraclinical Science

Degree : *Magister Sciences* (Veterinary Science)

Institution : University of Pretoria

Key words:

Escherichia coli, *Aepyceros melampus*, tetracycline resistance, replica plating method, minimum inhibitory concentration method, PCR, wildlife, impala, KNP rivers.

Abstract:

A case control study was performed in the conservancy area of the Kruger National Park (KNP), South Africa to find out whether the faeces of impala (*Aepyceros melampus*) were more likely to contain tetracycline resistant *Escherichia coli* (TR*EC*) when they drank from rivers that contained these bacteria, compared to rivers that were uncontaminated with TR*EC*.

Five perennial rivers (Crocodile, Letaba, Olifants, Sabie and Sand) were selected. A total of 11 points in these rivers were sampled on three separate occasions and cultured for *E. coli*. Impala herds within 5 kilometres of each water collection site were identified and between 5 and 10 fresh faeces were collected for each collection period (n=209 faecal specimens). Selective culturing of *E. coli* was done and the resulting colonies were screened for tetracycline resistance by using the Lederberg Replica Plating (LRP) method. Resistant colonies were those that grew in the presence of 4 mg/L of tetracycline. Both a resistant and/or a susceptible isolate were then selected from each specimen, and subjected to the minimum inhibitory concentration (MIC) micro-broth dilution test for tetracyclines. The breakpoint for the MIC method was considered ≥ 8 mg/L (which is higher than for the LRP method).

Twenty one of the 33 water specimens examined were found to be contaminated by *E. coli*. Among them (n=21), 76.19% (n=16) were resistant to tetracycline using the LRP method, although using the MIC method only 19.05% (n=4) were resistant. All of the resistant strains originated from the Letaba, Olifants and Crocodile rivers (TR*EC*pos rivers). Among the 209 impala faeces sampled, 191 were positive for the presence of *E. coli* (91.38%). Within these (n=191), 36.64% (n=70) showed TR*EC* using the RPL method, while using the MIC, 9.95% (n=19) were found to be TR*EC*. The RPL and MIC methods showed a concordance of only 48%. Resistant isolates screened by PCR for *tet(A)* and *tet(B)* genes were found to be negative and it was concluded that other resistant genes were responsible.

The odds ratios (OR) showed that impala drinking from TR*EC*pos rivers were 19.3 (2.63-141.68) times more likely to be infected with TR*EC* than unexposed impala.

CHAPTER 1. INTRODUCTION

1.1 Background and motivation

Antimicrobials are used to treat and prevent microbial infections in humans and animals. They are also used as growth promoters (GP) in livestock. Since the discovery of antibacterials in 1871, numerous lives have been saved. The high mortality rate of women with puerperal problems or gas gangrene, which was common in war victims, became a thing of the past. Farmers could for the first time intensively rear food animals that were free of zoonotic diseases. This has lead to antimicrobials being used extensively in both humans and animals, even for non-life threatening diseases. With the misuse and/or overuse of antimicrobials, the number of antimicrobial resistant (AMR) bacteria in the environment has consequently increased exponentially as a result of selection pressure. Consequently, AMR in bacteria and other parasites is now considered by the World Organization for Animal Health (OIE) and the World Health Organisation (WHO) to be a major threat to the well-being of humans and animals (Chopra & Roberts, 2001; WHO, 2001).

It is acknowledged that the overuse of antimicrobials in humans plays an important role in the development of resistance in human pathogens. However, it is also believed, and in some instances proven, that resistant bacteria in animals and the presence of antimicrobial residues in animal products may contribute to problems with the treatment of bacterial diseases in humans, in particular, zoonotic salmonellosis, enterococcal and *Escherichia coli* infections (Swartz, 2002; van de Bogaard, 1997 b). Programmes for veterinary surveillance of AMR have been initiated in many countries of the world, including South Africa. They are used in decision making, as well as to monitor the effect of the removal of specific antimicrobials (Aarestrup *et al.*, 2001; Caprioli *et al.*, 2000; Franklin & van Vuuren, 2001; Nel *et al.*, 2004).

Tetracyclines are the most common class of antimicrobials sold for the treatment of livestock in South Africa, as they are essential for the treatment of common tick-borne diseases such anaplasmosis and heartwater. Furthermore, they are registered for oral use and are used liberally to treat and prevent a variety of diseases in all animal species, including humans. It has also been shown that tetracycline resistant *E. coli* (TR*EC*) is highly prevalent in food animals in South Africa (van Vuuren *et al.*, 2007; Picard & Sinthumule, 2002). Recent studies have shown that the various *tet* genes coding for tetracycline resistance, can be exchanged between bacteria, including enteric bacteria, in many ecosystems (Bryan *et al.*, 2004). Thus, the potential for the dissemination of tetracycline resistance is high. Although there are several studies assessing AMR in *E. coli* populations of animal origin, not much work has been done on molecular ecology and epidemiology – linking the type of resistance to the environment and the animal host (Boerlin *et al.*, 2005). The knowledge of how tetracycline resistance moves within animals and their habitat would assist in determining a risk management policy for tetracyclines. Moreover TR*EC* can be used as a model to study the dissemination of AMR bacteria in the environment.

Wildlife populations within large nature reserves in South Africa are useful in the study of the dissemination of AMR in nature, as they have never been treated with antimicrobials, have no direct contact or access to the faeces of domesticated animals or humans and often live in areas with low population densities. Therefore, the commensal bacterial populations in their intestinal tracts should have low levels of resistance to antimicrobials such as tetracyclines. They can thus be used to study the role that environmental pollution, in particular polluted water, plays in the dissemination of tetracycline resistance. Impala (*Aepyceros melampus*) were chosen for this project because they are reasonably common in many different parts of South Africa, making easy to find them in different ecosystems. Although they have a wide geographical range, herds tend to remain localized in a particular area, and they are selective grazers and browsers (Skinner & Chimimba, 2005), so their source of AMR is usually limited to a particular ecosystem.

1.2 Aim of the study

The aim of this study was to investigate whether there was a relationship between tetracycline resistance shown by *E. coli* in the faeces of untreated impala and the presence of tetracycline resistant bacteria in their water source.

1.3 Problem statement

There is little information available on AMR in wildlife populations and their environment. Furthermore, there are no suitable models to study the dissemination of AMR in the environment. Evaluating the correlation between drinking water containing resistant bacteria and naïve wild antelopes (such as impala) could be a way to evaluate the risk of AMR dissemination through surface water containing resistant bacteria.

1.4 Hypothesis

Impala drinking from TR*EC* contaminated water are more likely to contain TR*EC* in their faeces compared to those drinking from uncontaminated rivers.

1.5 Objectives

The objectives of this research were to:

- determine the role that polluted rivers play in the dissemination of AMR to naïve wildlife, using TR*EC* as a model.
- assess the prevalence of *tet(A)* and *tet(B)* in TR*EC* from rivers and impala faeces;
- demonstrate that the presence TR*EC* in animals may not necessarily be associated with direct therapeutic interventions;
- generate data that can be used for further studies;
- provide information for the establishment of a risk management policy for AMR dissemination.

1.6 Work plan

- Obtain data on distribution of impala in the study area using GIS data from the Scientific Service of Skukuza, Kruger National Park.
- Obtain data on the state of the rivers in the study area to determine which are likely to be TR*EC*pos or TR*EC*neg by consulting ecologists, Department of Water Affairs and Forestry (DWAF) and the Water Research Commission.
- Select two groups of rivers in KNP: those polluted and those not polluted with TR*EC*.
- Collect three water samples at timed intervals from each of the selected water points.
- Collect approximately 105 faecal samples from each impala group within a range of 5 kilometres (km) from each water sampling point.
- Detect TR*EC* in the impala faeces and water samples using bacteriological laboratory analyses.
- Detect whether the most common *tet* genes, as *tet(A)* and *tet(B),* are present in the TR*EC* positive isolates.
- Evaluate the strength of association of TR*EC* between impala faeces and their water sources using the odds ratio.
- Analyze and interpret the data.

CHAPTER 2. LITERATURE REVIEW

2.1 Antimicrobial resistance and its relevance in public health

As mentioned in Chapter 1, antimicrobials are used to treat microbial infections in humans and animals as well as for prophylaxis and as GPs in livestock. Unfortunately nowadays the effectiveness of antibiotics is under threat (Levy, 2005). Around the world, bacteria are gaining resistance to defend themselves against drugs that once would have had destroyed them. This was not unexpected as some bacteria always showed strategies to evade antibacterials, however what is threatening is the speed at which some strains are developing resistance (Priya, 2008). Furthermore, multi-resistant AMR bacteria such *Serratia marcescens* and *Acinetobacter* species that were never considered as important pathogens are gaining importance, especially as agents of nosocomial infections (Hsueh *et al.,* 2005).

The medical consequence of the spread of AMR can be estimated from the failure of therapeutic actions against bacterial infections, which causes an increase in morbidity and mortality. It has been shown that when patients with a life-threatening bacterial disease are treated with fluoroquinolones, vancomycin or beta-lactams to which the pathogen is resistant, they are less likely to survive than those appropriately treated (Chastre, 2008; Lautenbach *et al*., 2005). The problem intensifies when resistance is gained from bacteria with susceptibility to a limited number of antibiotic classes. Regarding this, there are concerns about *Staphylococcus aureus*, which easily acquires AMR, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics, including aminoglycosides, macrolides and fluoroquinolones (Chastre, 2008; Levy, 2005; Khanna *et al.,* 2008). Other challenges to clinicians are coming from the multi-drug resistance exhibited by Gram-negative enteric organisms like *E. coli, Klebsiella pneumoniae* and *Enterobacter cloacae,* the

ubiquitous environmental bacterium, *Pseudomonas aeruginosa* as well as from *Neisseria gonorrhoeae* and *Mycobacterium tuberculosis* (Davis *et al.,* 1985; Levy, 2005). Even although deaths as a result of antimicrobial failure are at this stage limited, if nothing is done to limit the numbers, especially of multi-resistant AMR bacteria, the number of mortalities as well as economic costs is likely to escalate. Research should be addressed to better understand the mechanisms that drive the spread of resistance in order to control it (Bryan *et al*., 2004; Davis *et al.,* 1985; Levy, 2005; Okeke *et al*., 2005; Teuber, 2001).

2.2 Use of antibiotic and selection of AMR

When individuals, or a group of humans or animals, are treated, particularly with oral antimicrobials, these drugs not only attack target pathogens, but also the commensal microflora of the intestinal tract. In the presence of antimicrobials, the polymicrobial nature, as well as the presence of rich nutrients in the intestines of animals and humans, makes it an ideal community for the amplification of AMR mutants and the transfer of antimicrobial resistance genes (Licht *et al.,* 1999, van de Bogaard, 1997 b). There are many studies confirming that AMR can be exchanged between bacteria of different classes and different species (Kruse & Sorum, 1994; Sayah *et al.,* 2005; van den Braak *et al.,* 1998).

The misuse of antimicrobials is today considered the most important factor for the emergence, selection and dissemination of AMR (Bonfiglio *et al.,* 2002; Grugel, 2006). In countries with poor healthcare services and where people cannot afford the services of a private medical doctor, the proper diagnosis and treatment of disease tends to take a backseat. This leads to the cheaper antimicrobials being over-prescribed and often used for too short a time at sub-therapeutic doses. Without instruction, patients also have the tendency to stop antimicrobial therapy once they feel better (Grugel, 2006; Okeke *et al*., 2005). In some developing countries antibiotics can be sold illegally at a cheaper price and these counterfeit drugs often contain a small amount of the active ingredient or are expired, which is not enough to kill the bacteria but is enough for them to develop resistance (Okeke *et. al.,* 2005). In developed countries there is a tendency to use expensive, broad-spectrum antimicrobials, such as penicillins plus beta lactamase inhibitors,

fluoroquinolones and third generation cephalosporines, even for non life-threatening diseases (Cadieux *et al.,* 2007; Pulcini *et al.,* 2007; Vaccheri *et al.*, 2008). Thus resistance to these second- and third-line antimicrobials is not only found in the hospital setting but also in the community. Of particular worldwide concern is the diffusion of extensively-resistant *Mycobacterium tuberculosis* strains (XDR-TB) which are resistant to an increasing number of second-line drugs used to treat multi-drug-resistant tuberculosis (MDR-TB) (Shah *et al.,* 2007; Zager & McNerney, 2008).

Antimicrobial resistance not only develops in humans. In intensive farming systems, over and above the therapeutic use of antimicrobials, the continuous ingestion of sub-therapeutic levels of antimicrobials when used as GP or at therapeutic levels for prophylaxis, has led to resistance in enteric bacteria. For example, in a prospective study, it was determined that tetracycline resistance among *E. coli* in faecal samples from a chicken farm increased within a week of the introduction of tetracycline-supplemented feed to the flock (Levy *et al.*, 1976). In the USA, where virginiamycin is widely used as a GP, resistance is common in *Enterococcus faecium* of animal origin, whereas for avoparcin, that is not being used, the acquired resistance to glycopeptides is virtually non-existent in enterococci isolated from animals (Phillips *et al.,* 2004). It has been shown in Hungary that the resistance levels of *Enterococcus* species to vancomycin in broilers, reduced from 72.7% in 1995 to 5.8% in 2000, after avoparacin was banned for use in food animals (Kaszanytzky *et al.*, 2006).

It has been estimated that discontinuing the use of antimicrobials as GPs would reduce the total amount of the usage of antibiotics used in production animals by at least 50% (Kaszanytzky *et al.*, 2006). However after the GP ban in the EU, it was documented that, in countries like Denmark and Sweden, there was a slight increase in the total amount of antibiotic used for therapeutic applications (DANMAP, 2003; Philips *et al.*, 2004). The Swedish monitoring report indicated an increased use of fluoroquinolones, cephalosporins, sulphonamides-trimethoprim as well as tetracyclines for treatment of individual animals (SWARM, 2006). This increased use of antibiotics for therapeutic interventions was not followed by an increase of AMR in production animals, but instead by a general decrease in the

amount of AMR, perhaps because the total amount of antibiotic sold decreased (SWARM, 2006). The decreased use of GPs did not lead to an appreciable decrease in production or in animal welfare problems (van de Bogaard, 1997 a).

It is generally accepted that the greatest driver of multi-resistance in bacteria of human origin, is the treatment of humans with antimicrobials. However, there are examples that show linkages between the usage of antimicrobials in food animals and development of resistant infections in humans, indicating that transfer of resistant bacteria from animal to human could occur (Chopra & Roberts, 2001; You *et al.,* 2006). In fact trends in antimicrobial resistance for the zoonotic S*almonella* isolates and *Campylobacter jejuni* in animals tend to be similar in humans (Swartz, 2002). Persons working with livestock have been found to carry a higher level of resistant bacteria. A good example is that of methicillin-resistant *Staphylococcus aureus,* which is highly prevalent in the pharynx of pigs as well as in the throats of farm workers (Khanna *et al.*, 2008). Although there is epidemiological evidence of transfer of these agents to humans, there is no experimental proof of direct transmission between food animals and humans (Phillips *et al.,* 2004).

The public health threat resulting from the use of antimicrobials as GPs has been the subject of an intense debate since 1960, when the Swann Committee, in the UK, concluded that antimicrobials used in human chemotherapy, or those that promote cross-resistance to other therapeutic agents, should not be used as GPs in animals (Chopra & Roberts, 2001; Swann report, 1969; Turnidge, 2004; WHO, 2001). The concern about this issue caused the EU to ban most antibiotics, with the exception of coccidiostats, as GPs in animals for precautionary reasons in 1996. Recently the World Health Organisation (WHO) has supported this decision (Steinfield *et al.*, 2006).

Although most of the research is aimed at providing data on AMR, as well as finding ways to decrease the input-level effect on the environment, there is a need to understand the mechanisms by which AMR disseminates and possibly amplifies in the environment.

2.3 AMR mechanisms

Bacteria make use of a variety of mechanisms to avoid the effect of antimicrobials (Figure 2.1). These mechanisms include:

- enzymatic inactivation of antibiotics;
- active expulsion of the drug by the cell efflux pump;
- alteration in target receptors;
- prevention of antimicrobials from entering the bacterial cell (Chopra & Roberts, 2001).

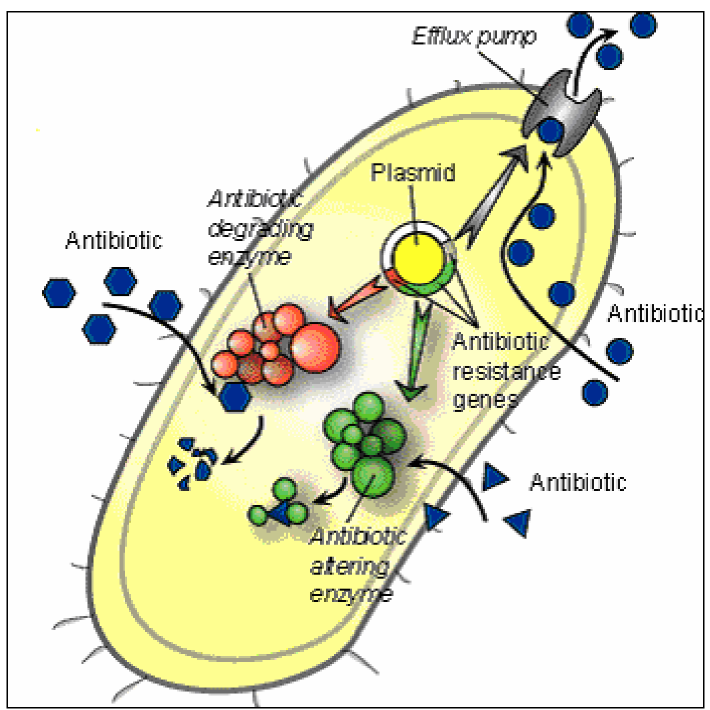

Figure 2.1: Mechanisms used by bacteria to avoid the effect of antibiotics (Fan sozzi, 2006).

Antimicrobial resistance is mainly gained though various mobile elements, such as AMR mutations in plasmids, transposons, and integrons (see Table 2.1) (Romanian, 2002; Sayah *et al.,* 2005). Originally it was believed that the acquisition of genetic resistance, which codify for the above cited mechanisms, was acquired only through spontaneous mutation. Later it was discovered that an error in DNA synthesis during replication and concomitant failure in the DNA repair systems, results in a spontaneous mutation frequency for an individual base pair of about 10^{-7}-10^{-8}. This means that mutations are very rare events.

Table2.1: Mobile genetic elements that confer AMR.

Plasmids	Circular, double strand of DNA that replicate within a cell independently of the chromosomal DNA.
Transposons	Types of transposable elements which comprise large segments of DNA capable of moving from one chromosome site to a new location. The ability of transposable elements to insert into plasmids or bacteriophages, which are transmissible from one organism to another, allows for their rapid spread.
Integrons	Gene capture systems found in plasmids, chromosomes and transposons

Furthermore the multiple mutations necessary to acquire a genetic pattern for resistance are even less frequent (Yim, 2006). The frequency of the AMR spread related to the acquisition of new genes, can thus often be explained by horizontal transfer of genes between bacteria (Figure 2.2), rather than by the sequential modification of gene functions by accumulation of point mutations (Davison, 1999).

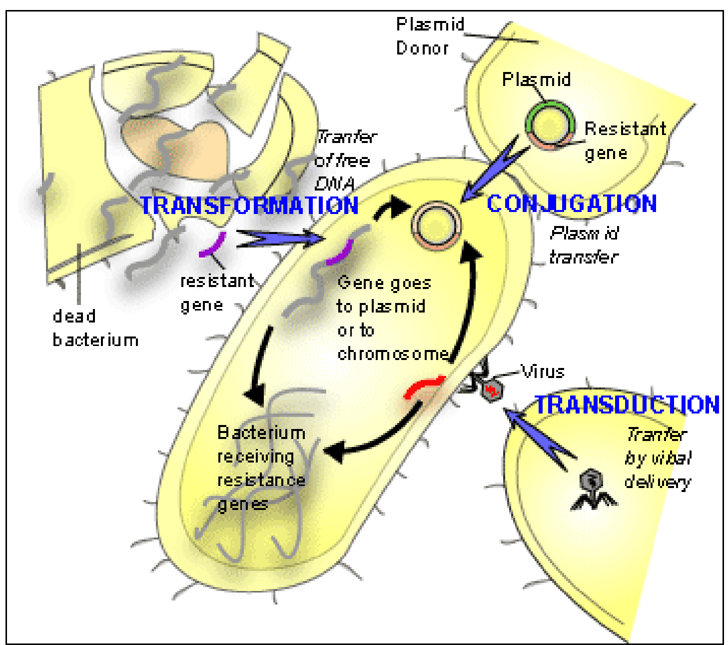

Figure 2.2: Mechanisms of horizontal gene transfer (Fan sozzi, 2006).

There are three identified mechanisms for horizontal gene transfer in bacteria (Davison, 1999):

- transformation: uptake and incorporation of naked DNA;
- conjugation: cell contact-dependent DNA transfer mechanism, found to be very common in bacteria;

- transduction: host DNA is moved from a bacteria to another by a bacterial virus (bacteriophage).

2.4 Diffusion of antimicrobial resistance in the environment

Although the spread of AMR in the environment is being studied worldwide, it is not yet fully understood (Aarestrup *et al.,* 2000; Boerlin *et al.*, 2005; Gomez *et al.*, 2004; Kümmerer, 2004; Lanz *et al.*, 2003; Lillehaug *et al.*, 2005; Poeta *et al.*, 2005 a). Essential to this, is an understanding of the persistence, survival, competition, nutrition, stress and physiological state of the different bacterial populations in an ecosystem in order to better understand the interactions between them (Davison, 1999; Kümmerer, 2004; Rysz & Alvarez, 2004).

Baird *et al.* (1996) has suggested other limitations in the current understanding of antibacterial resistance and difficulties in interpreting environmental resistance as follows:

- cross-resistance: the use of one antibacterial agent can increase levels of resistance not only to that specific drug, but also to others, even if they use different mechanisms of action;
- unpredictable onset of resistance: bacteria do not respond all in the same way to the presence of antimicrobials in the environment;
- lack of knowledge on how long bacteria maintain resistance in the absence of continued selective pressure for that resistance.

It has been demonstrated that conjugation and transfer of resistant plasmids is a phenomenon that belongs to the environment and can occur between bacterial strains of different species in different habitats (Kruse & Sorum*,* 1994; Teuber, 2001; Rysz & Alvarez, 2004). Since antimicrobial resistance has spread into different natural compartments such as water, soil, plants and animals, it has become interesting to investigate whether the major role in its diffusion is the input of antibiotics into the environment or, instead, its transmission and amplification. Even if it is the input of antibiotics that plays the main role, the molecular epidemiology of the diffusion of AMR has yet to be fully unravelled. Understanding

the mechanisms of how AMR spreads into the environment is not an easy task, due to the interconnections between different compartments.

2.5 Role of wildlife in the environmental study of AMR

The study of the dynamics in naïve systems, where no antimicrobials are used, could be a useful way to evaluate the transmission of AMR through the environment and to understand the role of the various input sources. There have been few studies in the past on the prevalence of AMR in natural ecosystems, but recent research is starting to focus on the role of wildlife in the transmission of AMR, as can be seen from the increasing numbers of papers being published (Boerlin *et al.*, 2005; Goldberg *et al.*, 2007; Gomez *et al.*, 2004; Lillehaug *et al.*, 2005; Ostebland *et al.,* 2001; Poeta *et al.*, 2005 a, b). Although there is doubt whether wildlife species can be considered as a reservoir for resistant bacteria, their role as sentinels for the spread of AMR into the environment could be of interest.

The prevalence of AMR in wildlife differs in different ecosystems. A study on characterization of AMR enterococci genes in wild animals, done in Portugal, showed that 31.4% of the isolates were susceptible to all the antibiotics tested (vancomycin, teicoplanin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, erythromycin, quinupristin-dalfopristin and ciprofloxacin). Tetracycline and erythromycin resistance were shown in 28.6% and 20.1 % of the isolates respectively (Poeta *et al.,* 2005 a). A study on quinolone resistance in *E. coli* on wild birds, done in Spain, found that all nine *E. coli* strains isolated from wild birds with septicaemia, were resistant to nalidixic acid (Gomez *et al.,* 2004).

In the Stelvio National Park, in Italy, a study of 80 faecal samples from wild ruminants (red deer (*Cervus alaphus)*, roe deer (*Capreolus capreolus)* and chamois (*Rupicapra rupicapra*)) showed that 16 of them had AMR *E. coli* strains (Pagano *et al.,* 1985). In Norway, AMR resistance was found in 13% of the 179 *E. coli* isolates from wild cervids. Eight of them were resistant to only one type of antimicrobial, while the 19 samples from which enterococci were isolated, were 100% positive for AMR, 84% against one kind of antibiotic and 16% for more than

one (Lillehaug *et al.,* 2005). In Minnesota, USA, 4% of *E. coli* obtained from wild animals were resistant to tetracycline (MIC>10 μg/mL) (Bryan *et al.,* 2004). These findings may indicate that there was direct or indirect wildlife contact with treated domestic animals or an environmental source.

Although studies on AMR prevalence in wildlife are now being found in the literature, there is still a lack of epidemiological and ecological links between AMR observed in different species and their surrounding environment (Boerlin *et al.,* 2005). A study done in Uganda, revealed that there were similarities in resistance of *E. coli* isolated in chimpanzees (that had never been treated with antibiotics), and the most common drugs sold in the area for human therapy. A high percentage of *E. coli* isolated in faecal samples from humans and animals, sharing the same environment, were resistant to ampicillin, tetracycline, sulfisoxazole, and trimethoprim. These are the main, cheap oral antibiotics sold in the area. Only a low percentage of *E. coli* of human origin was found to be resistant to the more expensive and rarely used ceftiofur and ciprofloxacin (Goldberg *et al*., 2007).

The results of a recent environmental study in the USA done in the Cedar River area in Michigan are very interesting. The study was conducted to determine patterns of AMR in *E. coli* originating from human sewage, wildlife, domestic animals, farm environments and surface water. It reported that, in general, *E. coli* isolated from domestic animals showed resistance to the highest number of antimicrobials in comparison to isolates from human sewage, surface water and wildlife. It was also demonstrated in this study, that although AMR in bacteria isolated from wildlife was very low, it was still present. Of the *E. coli* isolated from 34 deer, 2.94% were resistant to tetracycline and 11.76% to cephalothin, while in 54 wild waterfowls sampled, 1.85% of the *E. coli* were resistant to tetracycline and 11.11% to cephalothin. An interesting result of this research was the similarities found in AMR patterns from animal faecal and farm environment samples. Most interesting was that the levels of cephalothin resistance in the environment were higher than those in the faecal samples. Although that research did not show a great deal of AMR in bacteria from water samples, apart from cephalothin, it still showed that many different environmental compartments such as soil, water, human and animals are interconnected (Sayah *et al.,* 2005).

2.6 Occurrence of AMR in the aquatic environment

Water is a critical resource in all ecosystems and the interconnectivity of many water bodies implies that it provides the route by which an event such as pollution in one ecosystem is easily transferred to another, even a distant, ecosystem. Although it is believed that antimicrobials or AMR bacteria present in pollutants such as animal and human waste could play a large role in the presence of AMR bacteria in water, further studies must be done to determine the extent of this and whether other factors play a role (Kümmerer, 2004; Webster *et al.,* 2004). Different possible pathways for the arrival of AMR in water are shown in the organogram in Figure 2.3 and explained in the following section.

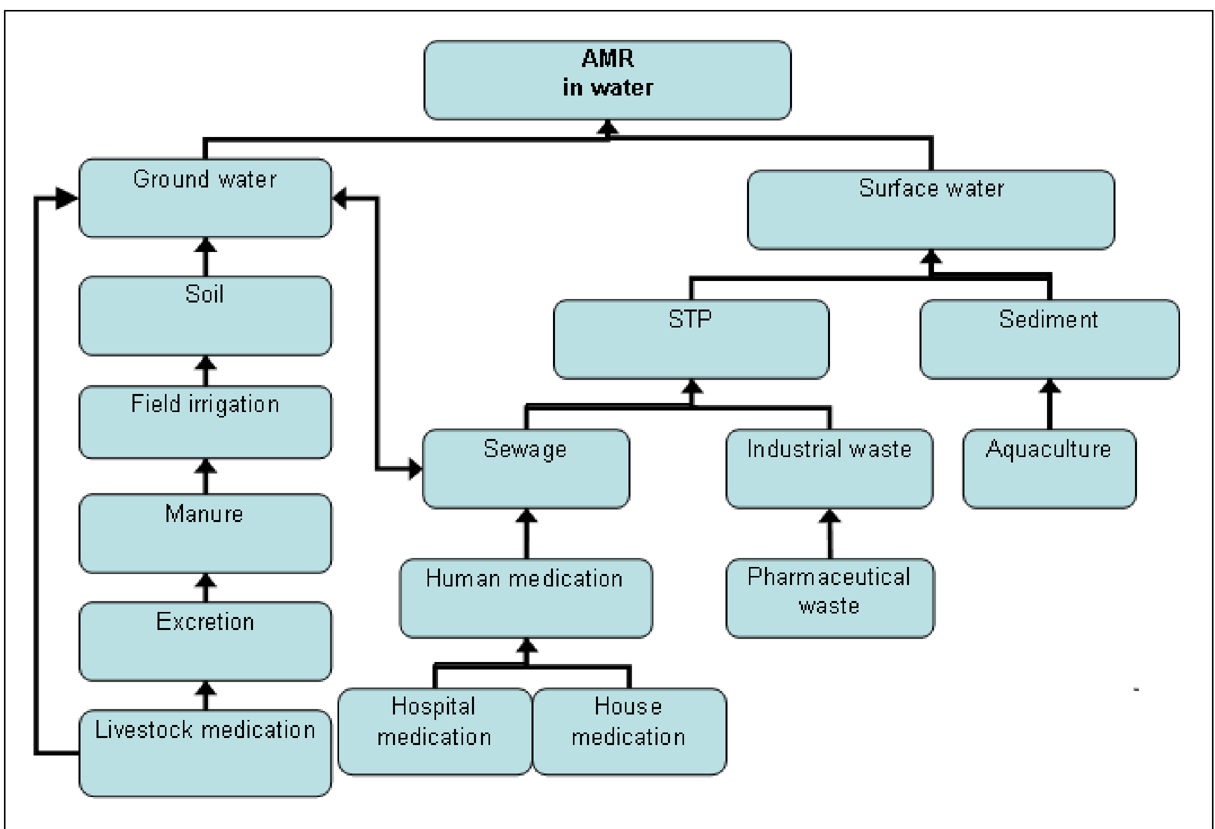

Figure 2.3: Possible pathways of the AMR entrance in an aquatic environment (adapted from Zuccato *et al.,* 2000).

Pollution origins are mainly classified into point source and non point-source. Point source pollution is an observable, specific and confined discharge of pollutants into the water body *e.g.* sewage input. Non-point source pollution is characterized

by a diffuse discharge of pollutants, generally over large areas *e.g.* contaminated riverine soil for animals using a river as a drinking source (Steinfield *et al.,* 2006). In either case, the sources of antimicrobial resistance pollution in water can be postulated as:

- the introduction of resistant bacteria in pollutants;
- the presence of antibiotic residues in pollutants.

2.6.1 Introduction of resistant bacteria into water

The introduction of resistant bacteria into water is mainly through faecal contamination of water from either human or animal sources. It can be seen from Figure 2.3 that there are many different possible sources of resistant bacteria. Tracing these pathways could be very difficult due to the many sequential steps in the processes.

2.6.1.1 Role of humans in water pollution

The sources of AMR pollution resulting from human activities can be classified as hospital effluent, municipal sewage and activated sludge from sewage treatment plants (STP) (Zuccato *et al.,* 2000; Kümmerer, 2004). Assuming, in accordance with the study of Feuerpfeil & Stelzer (1992), that 80.5% of the faeces of healthy persons contain bacteria resistant to antimicrobials and that 98% of these bacteria are *E. coli* (Feuerpfeil & Stelzer, 1992), a large amount of resistant *E. coli* reach the sewage every day. In a study, done in Austria, it was evaluated that the amount of *E. coli* arriving in the STP was between 2×10^4 and 6.1×10^4 CFU/mL, even if, after treatment, there was a reduction of 200-fold, more than 10^2 CFU *E. coli*/mL reached the environment (Reinthaler *et al.*, 2003).

It is often assumed that hospital effluents are the most important source of resistant bacteria in municipal waste water. This belief stems from the fact that as a result of the heavy use of biocides and antimicrobials, hospitals tend to harbour highly resistant bacteria. Biocides that may be implicated in the development of AMR include bisphenol (phenylether) and triclosan as well as the cationic

chlorhexidine salts and quaternary ammonium compounds (Russell, 2002). For example, triclosan, a common biocide, has been shown to select for a low-level of antimicrobial resistance in *E. coli* and a high level of ciprofloxacin resistance in *Pseudomonas aeruginosa* (Chuanchuen *et al*., 2001). Multi-resistant bacteria other than *Pseudomonas aeruginosa,* such as *Serratia marcescens*, and methicillin-resistant *Staphylococcus aureus,* are but a few of the well described examples of the increasing level of multi-resistant bacteria in the hospital environment (Gad *et al*., 2007; Levy, 2005; Oteo *et al.,* 2004; Pagani *et al.,* 2005; Weigel *et al.,* 2004). In Austria, the high level of resistance selected in the hospital environment was reflected in sewage that contained a higher level of resistant *E. coli,* compared with other two municipal sewage sites. Also, the effluent from the sewage coming from the hospital was the only one to contain resistance against gyrase inhibitors, such as norfloxacin, ciprofloxacin and ofloxacin (Reinthaler *et al*., 2003).

However, taking into consideration that hospital effluents contribute less than one percent of the total amount of municipal sewage and that the numbers of resistant bacteria found in the effluent of an hospital intensive care unit were in the same range as those found for the effluent of municipal STP, hospitals are not the sole source for AMR in municipal sewage. It is probable that the general community also plays an important role *e.g.* when antimicrobials are used on an outpatient basis, as well as the high use of biocides in households and communities (Kümmerer, 2004). There are many studies that reveal the presence of resistant bacteria in municipal sewage that did not contain hospital effluent (Gonzalo *et al.,* 2002; Schwartz *et al.,* 2003; Kümmerer, 2004). The study of Reinthaler *et al.* (2003), described bacteria carrying AMR to penicillins, cephalosporins, tetracycline and sulfamethoxazole/trimethoprim in sewage not receiving hospital waste (Reinthaler *et al*., 2003).

2.6.1.2 Role of livestock in water pollution

There are many ways in which livestock contributes to the increase in water pollution. Water contamination from excrement of livestock can be direct, through the run-off from farm buildings, losses through the failure of storage facilities,

direct deposition of faecal material into freshwater sources and deep percolation and transport through soil layers via drainage waters at farm level. It can be also indirect through non-point source pollution from surface run-off and overland flow from grazing areas and manured fields (Smalla *et al.,* 2000; Steinfield *et al.,* 2006). The use of manure as fertilizer is known to spread zoonotic pathogens such as *Salmonella* typhimurium to humans through edible plants (Lemunier *et al*., 2005). Studies have been conducted on the effect upon bacteria of the waste storage time, before using it as fertilizer (Duriez & Topp*,* 2007; Lemunier *et al*., 2005). A recent publication noted that, although in a four months storage period there was a decline of bacteria in stored manure (from 1.4×10^7 to 1.2×10^3 *E. coli*/mL), there was not a concurrent decrease in their genetic resistance patterns (Duriez & Topp*,* 2007).

The excrement of livestock can contain a high level of AMR bacteria and disseminate it to the environment (Koike *et al*., 2007; Sengelov *et al.,* 2003 a; Smalla *et al.,* 2000). This is especially true where intensive farming is practiced and antimicrobials are used liberally as GPs, as well as for prophylaxis and metaphylaxis. At two swine confinement facilities, tetracycline resistance was found in the waste water of the nearest lagoon. Furthermore, the novel *tet*(W) gene, isolated from pig farms, was also recovered in the nearest ground water, indicating that there is a high potential that AMR can reach potable water (Koike *et al.,* 2007). At least another way in which livestock can contribute to the dissemination of AMR is that their organic wastes contain a large amount of nitrogen and phosphorus. These substances act as nutrient surpluses that not only stimulate eutrophication but also furnish nutrients for bacterial growth, increasing their multiplication (Hirsch *et al.,* 1999; Steinfield *et al.,* 2006).

2.6.2 Antimicrobial residues in water

Antimicrobial residues in water originate from their use in humans, animals and aquaculture, as accidental leakage or due to inappropriate dumping from industrial laboratories. The latter, however, represents a point pollution source affecting a limited area and is unlikely to represent a main source of AMR (Diaz-Cruz *et al.,* 2003; Hirsch *et al.,* 1999).

Generally, after intake, drugs are absorbed by the animal or human and many are metabolized to inactive products. Nevertheless, a significant amount of the original substance will leave the animal or human unmetabolized via urine and faeces and is discharged into the sewage system (Hirsch *et al.,* 1999). As most of the antimicrobials are not fully eliminated during the sewage purification process, they end up in surface waters (Castiglioni *et al*., 2004; Kümmerer, 2004). The chemical characteristics of the compound dictate whether or not antimicrobials are broken down in the sewage system, for instance, polar antimicrobials such as tetracyclines, may not be eliminated effectively, as a large part of elimination is achieved by absorption on activated sludge, which is partially mediated by hydrophobic interactions (Ternes, 1998). It was found that 44% of the original amount of tetracyclines in the environment persisted for at least 30 days at 30℃. At a temperature of 4-20℃, 88-100% of the original amount persisted over the same period (Halling-Sorensen *et al.,* 1998). Furthermore, most of the antimicrobials administered to poultry and pigs are administered in the feed or drinking water, which infers that active antimicrobials can enter the water system directly through the farm waste (Kemper, 2008; Kümmerer, 2004).

Another source of antimicrobials in surface water is their usage in aquaculture (Giraud *et al.,* 2006). This usage has increased in parallel with the growth of aquaculture over the last 50 years (WHO *et al*., 2006). The main groups of antimicrobials used are tetracyclines, sulphonamides and chloramphenicol (Hirsch *et al.,* 1999). These are applied through the food or by addition to the water (WHO *et al*., 2006). Most of the unused drugs, as well as excessive feed and fish excrement, end up in the sediments of the aquatic environment (Hirsch *et al.,* 1999).

The concentration of antimicrobials in the environment is normally considerably less than that required for therapeutic use (Hirsch *et al.,* 1999). It is conceivable that AMR could arise in much the same way when sub-therapeutic doses are used as GPs in animals as with improper therapeutic use in human and veterinary medicine. The exposure of bacteria to low concentrations of antimicrobials is known to increase the speed with which resistant bacteria strains are selected.

However there have not been enough studies to determine the role of these antimicrobial residues in water, related to the development, transfer and maintenance of AMR (Kümmerer, 2004; Watkinson *et al.,* 2007 b).

2.7 Microbial ecology of drinking water

The supply of pathogen-free drinking water is a major public health milestone in many developed countries (Berry *et al.,* 2006). However, in rapidly developing countries with large population densities, sewage systems are often overloaded and many resource-poor communities do not have access to purified drinking water. Natural disasters such as typhoons, tsunamis, earthquakes and floods also overwhelm sewage systems resulting in people being forced to drink sewage-contaminated waters. Evidence for this is an associated increase in water-borne diseases in humans such as cholera and typhoid (Huq *et al.,* 2005). Unfortunately even the potable water in an industrialized country can carry enteric bacteria (Gannon & Busse, 1989; LeChevallier *et al.,* 1996; Messi *et al.*, 2005).

Researchers are studying the microbial ecology of water distribution systems, in order to design effective control strategies that will ensure safe and high-quality drinking water (Berry *et al.,* 2006). It is known that the conventional approach to biological control in distribution systems is often ineffective at controlling microbial growth. Under normal circumstances, coliforms are found in water distribution systems, after purification, for the following reasons:

- unfiltered water systems with a lack of water quality control;
- systems with sub-optimal disinfectant concentrations *i.e.* chlorine levels <0.2 mg/l or monochloramine levels <0.5 mg/l;
- high assimilable organic carbon (AOC) levels (<100 µg/L) which act as a good nutrient for bacteria;
- temperatures above 15℃ are related to an increase in microbial activity;
- low ozone concentrations can result in increased microbial growth;
- corrosion of iron pipe surfaces;
- turbidity deposits within systems that could accelerate residual chlorine depletion and provide a habitat for bacteria growth;

- engineering factors such as a large proportion of storage tanks in the system or inappropriate flushing of the system or uncovered water reservoirs (LeChevallier *et al.,* 1996).

2.7.1 Factors related to the presence of coliforms in water

Traditionally, commensal *E. coli* has been viewed as an indicator of faecal water pollution, as it is a common inhabitant of the lower bowel of humans and animals (DWAF, 2001 b; Whitlock *et al.,* 2002). It can be also considered as an indicator of AMR, because *E. coli* is easily able to acquire AMR, either via other resistant bacteria or through mutation (Levy, 2005).

As part of the normal flora, *E. coli* is advantageous to the host because it may prevent potential infections with pathogenic bacteria (Young-Ju *et al.,* 2005). Nevertheless it is also the aetiological agent for various types of human and animal infections. *Escherichia coli* is one of the most common bacteria causing diarrhoea, septicaemia, peritonitis, cystitis, other infections of the urinary tract and pyogenic wound infections. There are also certain highly pathogenic strains, such as the zoonotic *E. coli* O157:H7, that can produce diarrhoea with abdominal cramping, sometimes with haemorrhagic colitis or the fatal haemolytic uraemic syndrome in humans (White *et al.,* 2002).

Escherichia coli and many coliforms have limitations as faecal indicators as they are able, in the presence of high nutrient levels and temperatures, to grow outside of the intestinal tract and to persist for long periods in water and the surrounding environment (LeChevallier *et al.,* 1996; Ogden *et al.,* 2001; Whitman *et al.,* 2006). Tropical soils, such as those in Hawaii, with ambient temperatures of around 23-25℃ and sufficient nutrients, support the prolifer ation of *E. coli* (Byappanahalli & Fujioka, 1998).

A 10 year study of Lake Michigan showed that once *E. coli* had established itself in forest soil, it could persist throughout the year, potentially acting as a continuous non-point source of *E. coli* to nearby streams. If *E. coli* is adsorbed onto the near-shore areas, the bacteria are protected from environmental stresses, such as

desiccation and solar radiation, so they can grow in warm and moist soil using the presence of nutrients such as decomposing vegetation or other organic sources (Whitman *et al.,* 2006).

On the other hand, *E. coli* suspended in open waters are low in numbers as they are stressed by biotic and abiotic factors and are diluted. A study on the die-off of *E. coli*, done in Canada using a membrane filter chamber, reports that this organism has a slow die-off and can survive for a minimum of 28 days in river water (Dutka & Kwan*,* 1990). Recently one study found that the numbers of *E. coli* declined over time in all kinds of water (lake, river, puddle and animal-drinking troughs) but it was still detected in 45% of the samples after 2 months (Avery *et al.,* 2008). Fluctuations in the die-off indexes of *E. coli* in water seem to be governed primarily by the seasonal variations of solar ultraviolet (UV) radiation and the concomitant changes in pH and in temperature (Voets & Verstraete, 1980). Also, it has been noted that *E. coli,* as well as organic nutrients, increase with the highest concentrations being found at the outfall (place where the river flows out into the sea) and the lowest near the stream origins (Whitman *et al.,* 2006). In Lake Michigan, *E. coli* was found to depths of just below the water table and up to 5 m inland of the shoreline, providing a large surface area over which *E. coli* can proliferate. Wind, especially onshore wind, has been seen to cause an increase in *E. coli* counts in water, possibly resulting from re-suspension of *E. coli* from river shores. Moreover, it has been found that rainfall increased *E. coli* counts in water and that the concentration quickly declined to the pre-rain levels within three days after the rainfall event (Whitman *et al.,* 2006). Although a difference in *E. coli* levels in rivers during the dry and rainy season could be expected, a study in a South African river showed, instead, that there were no significant differences between the survival patterns of *E. coli* during different seasons. In both July and December, a decrease from 10^{10} to 10^{7} *E. coli*/100mL of water cultured in the river using a membrane filter chamber, was observed in 10 days during either periods (de Wet *et al.,* 1995). The survival patterns and longevity of *E. coli* suggest that survival during long-distance transportation of the organism via river water is possible (Avery *et al.*, 2008).

2.8 Methods for detection of E. coli in water

Since *E. coli* detection in water is extremely important for public health reasons, many methods for its detection and enumeration have been described in the literature. The following organizations have adopted various protocols:

- The American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Foundation (WEF) jointly publish methods approved by a methods approval program, in the publication Standard Methods for the Examination of Water and Wastewater ("Standard Methods").
- The American Society for Testing and Materials (ASTM) methods are those that have met the requirements of the ASTM methods approval program .
- The Association of Official Analytical Chemists (AOAC) also publishes methods that have met the requirements of the AOAC methods approval program.
- The Environment Protection Agency (EPA) methods are those that have been developed by the United States EPA (EPA, 2001).

The main methods recognised by the EPA for enumerating *E. coli* from water are listed in Table 2.2 (EPA, 2001; 2002).

Table 2.2*:* EPA proposed methods for enumerating *E. coli* (EPA, 2001; 2002).

Main group of technique	Method
Most Probable Number (MPN)	LTB- EC-MUG ONPG-MUG CPRG-MUG
Membrane Filter (MF)	mENDO-NA-MUG LES-ENDO-NA-MUG mFC-NA-MUG mTEC agar Modified mTEC agar MI agar m-Coliblue24 broth

The Most Probable Number (MPN) method consist of mixing unfiltered water samples with specific liquid growth media, where 3, 5 or 10 repeats of 10-fold dilutions of the water samples are made. The presence or absence of growth for each dilution is then recorded and the results interpreted by the use of a statistical table known as an MPN table. In the LTB-EC-MUG test, after the sample dilutions have been incubated in lauril-tryptose-broth (LTB) tubes, methyl-umbeliferyl-glucuronide (MUG) is added to those tubes with visible growth. Those with a bright blue fuorescence under UV light are considered to be *E. coli.* The two tests ONPG-MUG and CPRG-MUG, use a chromogenic/fluorogenic enzyme substrate for the simultaneous detection of total coliforms and *E. coli.*

All the Membrane Filtration (MF) methods use a 0.45 μm diameter membrane to filter a fixed volume of sample and then the membrane is added to selective media. In particular, mENDO-NA-MUG, LES-ENDO-NA-MUG, mFC-NA-MUG are selective media containing the fluorogen MUG, so that the *E. coli* colonies will be recognised by their fluorescence under UV light. The mTEC agar method is another selective media on which the membrane is placed, incubated and transferred onto a filter pad saturated with urea and a chromogen. The modified mTEC uses a single step procedure where chromogen magenta glucuronide is added to the selective media. Other single step procedure methods, using the detection of the β-glucuronidase enzyme, are the selective MI agar and m-Coliblue24 broth, the first using the chromogen Indoxyl-β-D-Glucuronide (IBDG) and the latter the chromogen Bromo-Cloro-Indoxyl-Glucuronide (BCIG) (EPA, 2001; 2002; Hamilton *et al.,* 2005).

Although these methods simplify the enumeration of *E. coli* in water, if the detection of *E. coli* and not a coliform count is the main aim, a cheaper culturing approach can be used. After filtration, the membrane filters are incubated in a pre-enrichment broth such as peptone water, followed by selective culturing in a medium such as MacConkey agar (Hamilton *et al.,* 2005). All lactose fermenting bacteria are then further identified using morphology and biochemical tests.

2.9 Tetracyclines

Tetracyclines are broad-spectrum antibiotics that inhibit the growth of a wide variety of bacteria, protozoa and many intracellular organisms, such as mycoplasmas, chlamydiae and rickettsias (Booth & McDonald, 1995; Bryan *et al.*, 2004).

They can be divided into two groups: typical and atypical. The first group includes: tetracycline, chlortetracycline, minocycline and doxycycline (Roberts, 1996). These are bacteriostatic agents as their function is to inhibit bacterial protein synthesis by binding the 30S ribosomal subunit of microorganisms and preventing the attachment of aminoacyl-tRNA to the RNA/ribosome complex (Adams, 1999; Chopra & Roberts, 2001).

The second group includes: chelocardin, anhydrochlortetracycline, 6-Thiatetracycline and anhydrochlortetracycline. These appear to be bactericidal agents as they interfere with the membrane permeability resulting in cell damage and lysis (Roberts, 1996).

2.9.1 Tetracyclines, historical aspects

Tetracyclines were discovered in the late 1940s. They are derived from various species of *Streptomyces*. Since then, many synthetic structural modifications have been made on the molecules, to arrive at products with different pharmacokinetic properties and antimicrobial activities (Adams, 1999; Booth & McDonald, 1995; Chopra & Roberts, 2001).

The performance properties of tetracyclines were discovered in 1949, when it was observed that low levels of chlortetracycline in livestock and chicken rations had a positive effect on the rate of growth. In some countries, tetracyclines are therefore still currently used at sub-therapeutic levels as feed additives for intensive production of livestock and poultry (Chopra & Roberts, 2001).

2.9.2 Function and use of tetracyclines

Bacteria susceptible to tetracyclines include: *Bacillus* spp., *Corynebacterium* spp., *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, *Streptococcus* spp., *Actinobacillus* spp., *Bordetella* spp., *Brucella* spp., *Francisella tularensis*, *Haemophilus* spp., *Pasteurella multocida, Yersinia* spp., *Campylobacter fetus*, *Borrelia* spp., *Leptospira* spp., *Actinomyces* spp., *Fusobacterium* spp., the rickettsias, and *Mycoplasma* spp.. Bacteria which show variable susceptibility include: *Enterobacter* spp., *E. coli*, *Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Bacteroides* spp. and *Clostridium* spp. (Adams, 1999; Booth & McDonald, 1995). Relatively resistant bacteria include: *Proteus vulgaris, Pseudomonas aeruginosa*, *Aerobacter* spp., *Aerogenes* spp., *Shigella* spp., *Enterococcus faecalis* and many strains of *Staphylococcus* spp. (Adams, 1999; Booth & McDonald, 1995).

Tetracyclines also have non-antibacterial properties which include anti-inflammatory and immunosuppressive properties. These additional properties have encouraged the use of tetracycline in non-infectious conditions such as resistant rheumatoid arthritis, rosacea, pyoderma gangrenosum, prurigo pigmentosa, pleural effusions, recurrent pneumothorax, recurrent thyroid cysts and biliary-cutaneous fistula (Humbert *et al.,* 1991; Roberts, 1996).

Because of their broad-spectrum and low cost, tetracyclines have many therapeutic applications in clinical veterinary medicine and as livestock GPs (Col & O'Connor, 1987). In countries where the use of tetracyclines as feed additives is still permitted, it has been estimated that approximately 90% of tetracyclines are administered to cattle and swine at sub-therapeutic concentrations, whereas only 15% of usage in poultry reflects sub-therapeutic administration (Chopra & Roberts, 2001). They are very important in Africa in regard to food security, as they are essential to the treatment of several life-threatening tick-borne diseases such as anaplasmosis and heartwater in ruminants (IVS, 2007). Furthermore oxytetracycline is being used also to treat certain bacterial diseases which effect field crops, fruit trees and honeybees (Levy, 1992; Roberts, 2005).

Their use in human therapy has declined, possibly due to increasing AMR, although they are still widely used as the antibiotic of choice for long term acne control (Chopra & Roberts*,* 2001; Humbert *et al.,* 1991; Levy, 1992). Moreover they are used as drugs of choice as part of a triple therapy for management of gastritis and peptic ulcer disease associated with *Helicobacter pylori* and for prophylaxis and treatment (for some strains of *Plasmodium falciparum* resistant to mefloquine) of malaria. They are also effective for zoonotic diseases such as *Ehrlichia conorii* (tick fever) and psittacosis. In combination with rifampicin it is used to treat brucellosis (*B. abortus, B. suis* and *B. melitensis* infections) (Chopra & Roberts*,* 2001). Because they are cheap they are particularly attractive for use in developing nations for the treatment of a wide variety of bacterial infections (Liss & Batchelor, 1987). In fact, the HIV meeting in 2000 suggested the use of tetracyclines to reduce bacterial sexually transmitted bacterial diseases in the developing world (Chopra & Roberts*,* 2001).

2.9.3 Overall usage of tetracyclines in South Africa

In South Africa, tetracyclines are used in human and animal therapy and prophylaxis and as animal GPs. The total amount of tetracyclines sold in South Africa from 2002 to 2004 was 256 502.11 kg of active substance (Eagar H., personal communication 2007). This amount represents the total sales figure for both veterinary and human medicine and includes all the types of tetracycline formulations. It represents only an approximation of the usage of tetracyclines because it cannot be known if the entire amount sold was used. It is also known that tetracyclines represent the greatest volume of an antimicrobial class sold for use in animals. As companies are unwilling to reveal the total amount and distribution of tetracyclines sold, as well as the fact that the distribution of this antibiotic is governed by two Acts, Act 36 of 1947 and Act 101 of 1965, it is not possible to know what proportion is used parentally and as an in-water or feed medication, or the numbers and type of animals treated. Tetracyclines in fact, appear in the “Fertilizers, farm feeds, agricultural remedies and stock remedies” control Act (Act 36 of 1947) (NDA, 2007). Thus farmers have access to both chlortetracycline and oxytetracycline without a need of any prescription. Thus no control is possible over the amount of drugs sold directly to farmers. Instead Act

101 on "Medicines and related substances" classify tetracyclines together with the other antimicrobials, with the exception of topical formulation, as Schedule 4 substances. Even though the use of Act 101 of 1965 requires a veterinary prescription for Schedule 4 drugs, there is no central register and thus it is not possible to determine how much tetracycline is prescribed (DOH, 2003).

Since, tetracycline resistance in *E. coli* isolated from the intestines of healthy poultry and pigs has been shown to be high, 93.5% and 88.2% respectively (van Vuuren *et al.*, 2007), and tetracyclines form the bulk of antimicrobial imports, it can be assumed that tetracycline use in intensive farming systems is high.

2.9.4 Tetracycline resistance and its transmission

In 1953 the first case of resistance to tetracycline was reported in *Shigella dysenteriae* (Hartman *et al.,* 2003). Since that time, tetracycline resistance genes have increasing being detected in both Gram-negative and Gram-positive bacteria, mainly by conjugal transfer of plasmids and/or transposons. This dramatic increase in tetracycline resistance has led to a reduction in their efficacy and decreased use in human therapy for many diseases (Chopra & Roberts*,* 2001).

Resistance to tetracycline is conferred by thirty-eight different tetracycline resistance (*tet*) and oxytetracycline resistance (*otr*) genes. These genes have been associated with mobile plasmids, transposons and integrons (see Table 2.1). Their transmission in bacterial populations can take place by clonal spread of particular strains or horizontal transfer of resistance mainly by plasmid- or transposon-mediated conjugation (see section 2.3)(Hartman *et al.,* 2003; Roberts, 2005). These genes encode for four different mechanisms of resistance: an efflux pump; a method of ribosomal protection; a direct enzymatic inactivation of the drugs; and possibly an unknown fourth mechanism (explained below). Essentially, *tet* and *otr* genes have the same modes of action (Bryan *et al.,* 2004; Chopra & Roberts*,* 2001; Hartman *et al.,* 2003; Roberts, 2005).

Twenty-two of the *tet* genes and one of the *otr* genes code for efflux pumps. These efflux pumps consist of membrane-associated proteins which export tetracycline

from the cell. This mechanism reduces the intracellular drug concentration, exchanging a proton for a tetracycline-cation complex, against a concentration gradient, and thus protects the ribosomes from tetracyclines (Miranda *et al.,* 2003; Roberts, 2005). The efflux genes of Gram-negative bacteria are normally associated with large plasmids, most of which are conjugative. These genes are often found on the same plasmid as genes that code for resistance to other antimicrobials, heavy metal resistance and virulence factors. It is this phenomenon, known as cross-resistance, that has contributed to the huge increase in the number of multiple-drug-resistant bacteria (Chopra & Roberts*,* 2001; Hätchler *et al.,* 1991).

Ten of the *tet* genes and one of the *otr* genes (*otr*(A)) code for ribosomal protection proteins, which are cytoplasmic proteins. They protect the ribosomes in different ways from the action of tetracycline (Chopra & Roberts*,* 2001; Whittle *et al*., 2003; Roberts, 2005)

Before 2001, the *tet*(X) gene was the only one of the tetracycline resistance genes known to effect an enzymatic alteration of tetracycline. Today two more genes, *tet*(34) and *tet*(37), have been described. They code for a cytoplasmatic protein that chemically modifies tetracycline in the presence of NADPH and oxygen (Chopra & Roberts*,* 2001; Roberts, 2005; Weigel *et al.* 2004). At least one other gene *tet*(U), its action currently unknown, has been discovered recently (Roberts, 2005).

2.9.5 Tetracycline resistance genes in E. coli

In the genus *Escherichia,* eight different *tet* genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(I), *tet*(Y) and *tet(M)* have been found. All of them code for efflux pumps, except *tet(M)* that codifies for ribosomal protection (Bryan *et al.,* 2004; Chopra & Roberts*,* 2001).

In previous studies, it has emerged that *tet(A)* and *tet(B)* were the most common genes found in resistant samples of *E. coli* from both humans and animals (Bryan *et al*., 2004; Roberts, 2005). Bryan *et al.* (2004), found *tet(B)* and *tet(A)* resistant

genes, in 63% and 35%, respectively, of 1263 isolates from humans and animals (Bryan *et al.*, 2004). In Switzerland, a study on the occurrence of tetracycline determinants in 160 *TREC* isolated from swine, cattle, dogs and hens, showed that *tet(A)* was the most frequent determinant from swine, while *tet(A)* and *(B)* where observed with almost equal frequency from cattle, dogs and hens (Lanz *et al.,* 2003). In Denmark, of 100 TR*EC* isolates from pigs, cattle and poultry, *tet(A)* was found in 71% and *tet(B)* in 25%. The predominance of *tet(A)* and *(B)* applied to all three animal species and there was no difference in resistance between pathogenic and non-pathogenic *E. coli* (Sengelov *et al.*, 2003 b). Guerra *et al.* *(*2006) also reported a predominance of the *tet(A)* gene (87%) in 77 TR*EC* isolates from human and cattle in different countries, followed by *tet(B)* (19.5%). In the USA, among 32 TR*EC* isolates from cows with mastitis, *tet(A)* comprised 43.8% (Srinivasan *et al.,* 2007). Boerlin *et al.,* investigating diarrheic isolates from swine in Ontario, found that among enterotoxigenic *E. coli* (ET*EC*, n=83) the 89% had *tet(A)* and 12% *tet(*B). Among non-ET*EC* (n=67) 37% had *tet(A)* and 63% *tet(B)* genes. In 168 healthy pigs sampled, 32% had *tet(A)* and 46% *tet(B)* (Boerlin *et al.*, 2005). The others genes have been found in very low percentages (Bryan *et al.,* 2004; Lanz *et al.,* 2003; Guerra *et al.,* 2006; Sengelov *et al.*, 2003 b).

2.10 Methods for detection of tetracycline resistance

There are several methods to detect AMR in the laboratory, those based on phenotypic resistance and those on genetic determinants of resistance. Obviously, the use of both methods together can optimize the collection of information about AMR. Unfortunately, the determination of genetic resistance is a very expensive method that cannot always be routinely used.

2.10.1 Detection of phenotypic resistance

Two antimicrobial susceptibility methods are commonly used for the determination of the phenotypic resistance patterns of bacteria. These are the Kirby-Bauer disc diffusion test and the Minimal Inhibitory Concentration (MIC) method, using broth dilutions (White *et al.,* 2001).

The first is cheaper and less labour intensive than the MIC method and so it is of common use in clinical practice. Although this is a qualitative method able to divide bacteria into two groups, resistant or sensitive, to each of the antibiotics specifically tested, it is not useful to complex studies about characterization of phonotypical patterns. The cut-off values in this test are based on clinical breakpoint values (CLSI, 2002).

The MIC method, instead, is a quantitative method that enables determination of the lowest concentration of antimicrobial that inhibits the visible growth of a micro-organism after overnight incubation. This method is considered the ‘gold standard’ for laboratory phenotypic studies on the susceptibility of an organism to antimicrobials. It is able to detect low-level resistance as well as trends in resistance over time (Andrews, 2001; CLSI, 2002; Nel *et al.*, 2004).

The replica plating method is another qualitative method that can be used to detect the presence of resistant bacteria and their percentage in a mixed population (Leistevuo *et al.,* 1996; Österblad *et al.,* 1995). It is a technique in which one or more Petri plates containing different solid selective media are inoculated with the same colonies of micro-organisms from a primary plate, reproducing the original spatial order (Österblad *et al.,* 1995). There are two main techniques for replica plating. The first is the traditional method, invented by Lederberg in 1951; it consists of pressing a sterile velvet-covered disk onto a master plate and then printing it on secondary plates (Lederberg & Lederberg, 1951). The alternative method, invented by Lindstrom, uses more complex equipment than the previous. It picks up each single colony to be transferred onto a filter paper that will function as a stamp (Lindstrom, 1977). The Lederberg method may give misleading results on the percentage of resistant and sensitive colonies, because the replicator does not produce a uniform pressure over the entire surface of the print. On the other hand, it is a less expensive and time-consuming method that can be used when it is not in the aim of the study to know the percentage of resistant and sensitive isolates in the mixed plate (Österblad *et al.,* 1995).

2.10.2 Detection of genotypic resistance

Several methods are used to determine genotypic resistance, the cheapest and the more commonly used is the amplification of the target genes using the polymerase chain reaction (PCR) (Venter & Bosman, 2003). This method, invented in 1985, isolates a selected DNA fragment and amplifies it many times so that it become possible to detect the presence or the absence of the desired fragment (McPherson & Møller, 2000). Thus it can be useful to determine whether a particular gene is found in a cell DNA library.

The discovery of the PCR has opened the door to rapid and accurate diagnosis of the agents that cause disease as well as the detection of genetic mutations (Venter & Bosman, 2003). Since this method detects genetic material, it is able to detect unexpressed genes *i.e.* those that code for genetic resistance but cannot be detected using phenotypic tests. In fact many studies are found in the literature about the use of PCR for the detection of AMR genes (Bryan *et al.,* 2004; Boerlin *et al.,* 2005; Guerra *et al.,* 2006; Hartman *et al.,* 2003; Lanz *et al.,* 2003; Sengelov *et al*., 2003 b).

PCR has many variations, like reverse transcription PCR (RT-PCR) for amplification of RNA, real-time PCR which allow for quantitative measurement of DNA or RNA molecules and multiplex PCR for simultaneous detection of different genes (McPherson & Møller, 2000). As many genes govern the expression of resistance by a particular bacterium to antimicrobials, the multiplex PCR can be used to produce amplicons of varying sizes specific to different DNA sequences (Bryan *et al.,* 2004; Boerlin *et al.,* 2005; Lanz *et al.,* 2003*;* Turner *et al.,* 1997). By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primers sets must be optimized to work correctly with a single reaction. Also the amplicons size must be different enough to form distinct bands when visualized by gel electrophoresis (McPherson & Møller, 2000; Venter & Bosman, 2003).

2.10.2.1 DNA extraction methods

For any PCR it is important to extract DNA from the cells in the samples with maximum efficiency. There are several kinds of DNA extraction methods to fit the characteristics of the different cells being lysed (Venter & Bosman, 2003).
They can be divided into three different groups of techniques:

- physical methods that achieve the rupture of the cells by physical stress to the membranes (*e.g.* using high and/or low temperatures, or beads);
- organic extractions that use chemicals (*e.g.* a buffer such as sodium-dodecil-solfate (SDS) can be used to dissolve lipids in the cells, phenol/chloroform and alcohol can be used to lyse the proteic part and ethanol is used to precipitate the DNA);
- commercial kits that use resin in ionic exchange columns to extract and purify DNA. These kits represent the method of choice because they are adaptable to different kind of cells, and able to exclude proteins which may contain nucleases and inhibitory molecules. They are also specific for either DNA or RNA. Thus they are ideal when working with specimens, but due to their high cost, they are rarely used for the extraction of genetic material from pure cultures. (McPherson & Møller, 2000).

Other methods are described in the literature that combine these three basic groups of techniques. For example, Miller *et al.* (1997) described a method to extract DNA from *Mycobacterium tuberculosis* in paraffin-imbedded, formalin-fixed tissue. The tubes containing the paraffin section were centrifuged at high speed for 1 minute to compact the tissue and then 200 µl of water with 0.5% Tween 20 was added. The tubes were then boiled for 10 minutes, followed by snap freezing. This cycle was repeated and the samples were then subject to a third 10 minute boil. Without cooling the tubes were centrifuged at low speed for 20 minutes to pellet the tissue (Miller *et al.*, 1997).

After the DNA extraction, if it is necessary, some enzymes (*e.g.* proteinase K or RNase) that remove proteins and RNA contamination, can be used to purified the DNA (McPherson & Møller, 2000).

2.11 Epidemiological study designs

Epidemiological studies require the selection of an appropriate study design. Equally important are the selection of the sampling strategy and the estimation of an appropriate number of samples (Thrusfield, 2005).

2.11.1 Selection of the most appropriate study design

Epidemiological studies are categorised into experimental studies and observational studies. The first group includes clinical trials and the basic principle is that the design of the study involves deliberately changing certain parameters within a population to assess the effect of a specific treatment. Such studies are preferred because they can provide the strongest evidence about causality. However, they are often time-consuming and costly, requiring large numbers of individuals. Observational studies assume that there is no alteration to the population structures or dynamics. The researcher can only select suitable conditions for the observation of population characteristics (Pfeiffer, 2002).

As cited by Wartenberg (2000), the three most common epidemiological study designs are:

- Cohort study;
- Case-control study;
- Cross-sectional study.

Cohort studies are based on selecting two groups of non-diseased animals or people, one exposed to a factor postulated to cause a disease and another one unexposed to the factor. They are followed over time and the disease states are recorded during the study period. The cohort study is the most effective observational study for the investigation of causal hypotheses for diseases. It can

be used to study multiple outcomes and minimizes bias. Unfortunately these studies do not provide absolute proof of causality and can be expensive and time consuming. As this is a prospective study design, incidence is used to calculate probability and relative risk is used to demonstrate a causal relationship (Pfeiffer, 2002; Thrusfield, 2005).

Case-control studies are less-expensive and are more commonly used. In case-control studies animals or humans with a certain condition (cases) and without that condition (controls) are selected. The case and control groups are then compared in relation to simultaneous exposure to the agent or risk factors and statistical analysis used to estimate the strength of association of each factor with the studied outcome. The disadvantages include: they cannot provide information on the disease frequency in a population; they are not suitable for the study of rare exposures and it can be very difficult to ensure an unbiased selection of the control group. A prospective case-control study is when the disease is still to occur and incidence is used. It is the more accurate way of establishing a causal relationship than a retrospective case-control study, where both the risk factor (agent) and the disease have already occurred. For a retrospective study, prevalence is used to calculate the probability of a causal relationship and odds ratios are used (Thrusfield, 2005).

In cross-sectional studies, a random sample of individuals from a population is studied at a single point in time. Individuals included in the sample are examined for the presence or absence of disease and their status in regard to possible risk factors. These studies are useful to describe the situation at the time of data collection. They are relatively quick, but a cause-effect relationship cannot be definitely established (Pfeiffer, 2002). For a cross-sectional study, prevalence is used to calculate the probabilities. Odds ratios are used to rank associations between all possible variables in order of magnitude. Those with the highest odds are the most likely cause of disease (Thrusfield, 2005).

2.11.2 The use of relative risk, attributable risk and odds ratios

The degree of association between variables, such as agent (risk factor) and disease can be demonstrated or calculated using relative risk, attributable risk and odds ratios. These depend on the kind of study, as discussed above.

The relative risk (RR) can only really be used in a cohort study where there are exposed and unexposed animals. It is the ratio between the cumulative incidence of disease in exposed and unexposed animals. If the RR in exposed animals is 1, it means that there is no association of disease and factor. If the RR is greater than 1, there is a positive association – in other words there is a risk that animals exposed to the factor will get the disease. If the RR is less than 1, the exposure to the factor actually prevents the disease (Pfeiffer, 2002; Wartenberg, 2000).

The attributable risk (AR) is important when, as in most cases, there is not a clear cut difference between the exposed and non-exposed population. A few members of the non-exposed population could also be positive for the condition or disease. If there is an endemic condition or data indicates that there is a natural low level of exposure to all members of a population, AR can be calculated by subtracting this "background risk" from the total risk. It is often based on prevalence rather than incidence of disease and is then known as attributable proportion. In other words, the proportion of the diseased animals, where a direct link to a causal factor could be demonstrated (Thrusfield, 2005).

The odds ratio (OR) is the probability of an event occurring compared to the probability of it not occurring. Comparing and ranking OR for different factors is a way to measure the magnitude of association with a specified disease or outcome. The (OR) can only be used if the events are mutually exclusive**.** However, if the chance of disease is very low, the OR can be very close to the value of the RR (Pfeiffer, 2002; Thrusfield, 2005).

2.11.3 Evaluation of significance

Association between two variables can be tested statistically to determine if it is significant. This is usually used in epidemiology to link a disease or condition to a causal factor. The level of association between two variables as well as causal relationships, are measured using statistical significance. Testing for statistical significance is a way of measuring how unlikely it is that the association between two or more variables (events or observations) has happened by chance alone. If the association is significant, the factor is more likely to be the cause of the disease or condition (Thrusfield, 2005; Pfeiffer, 2002)

2.11.4 Data collection

Data collection for an epidemiological study can either be prospective or retrospective. Prospective data is used to determine incidence as the data is collected while the animal or human host is exposed to the agent causing the condition or disease. Use of retrospective data is considered to be less rigorous epidemiologically than use of prospective data and requires a different type of study design. However, when doing a situational analysis to characterise the agent and the likely agent – host – environment interactions (epidemiological triad) , prior to sampling, retrospective data is important (Thrusfield, 2005).

2.11.5 Sampling design

To ensure that results are reliable and meet the objectives of the study, it is critical that the sampling strategy is well planned. It is often not practically possible to study the entire population; hence enough subjects are studied to get an acceptable accuracy for the true value (Edwardes, 2001; Hopkins *et al.,* 1999). The sample should be representative of the entire population and formulae have been developed to calculate the minimum sample size that meets this criterion.

There are two main types of sampling, non-probability sampling and probability-sampling. Non-probability sampling, in which the choice of the samples is left to the investigator, includes judgment sampling, convenience sampling and purposive sampling. In judgment sampling, representative units of the population are selected by the investigator, in convenience sampling the sample is selected because it is easy to obtain and in purposive sampling the selection of units is based on known exposures (Cameron, 1999; Gummow, 2006; Thrusfield, 2005).

Probability sampling is the sampling method in which each sampling unit in a group has an equal probability to be selected; hence an individual can only be chosen once without replacement. Probability sampling includes simple random sampling, systematic random sampling, stratified random sampling, cluster sampling and multistage sampling (Thrusfield, 2005).

In simple random sampling the samples are selected randomly from the study population by using random numbers, while in systematic random sampling the samples are selected at equal intervals and only the first animal is selected randomly. In stratified sampling the study population is divided into groups (strata) and samples are selected randomly from each group. These sampling methods improve accuracy because they ensure that each group in the population is represented. When the strata are divided by geographical locations (different countries, regions, districts and villages), by different periods of sampling time or by other categories, the strata is termed a cluster. If all the animals in each cluster are sampled, the sampling method is defined as one-stage cluster sampling. If the cluster is divided into sub-units and the clusters are the primary units, while the selected members of the sub-samples are the secondary units, we talk about two-stage cluster sampling. If further stages of sampling are undertaken and in this way a higher level of sub-sampling is created, the sampling method is defined as multistage cluster sampling. Cluster sampling is often used in a study population where the number of units (size of the population) is unknown (Thrusfield, 2005).

CHAPTER 3. MATERIALS AND METHODS

3.1 Model system

A case control study was performed to find out whether the faeces of impala (*Aepyceros melampus*) were more likely to contain tetracycline resistant *Escherichia coli* (TR*EC*) when they drank from rivers that contained these bacteria compared to rivers that were uncontaminated with TR*EC*.

Figure 3.1: Female impala drinking at a water hole.

A case control study was chosen as the general water status of the rivers in the Kruger National Park are routinely monitored, making it possible to divide the impala population in two groups on the basis of different exposure to contaminated rivers. The presence of bad water quality of the river was presumed to indicate contamination and thus a higher risk of AMR. After the presence of TR*EC* was assessed in the polluted rivers, it was possible to compare the consequences for two groups exposed to different risk factors (TR*EC* in drinking water) at a pre-determined moment.

3.2 Study area

The Kruger National Park (KNP) was selected as the study area for this research. Its location in South Africa and main rivers are illustrated in Figure 3.2. A second, more close-up map of the park and its main rivers is shown in Figure 3.3.

Figure 3.2: South African National Parks and main rivers (ArcGIS)[1].

The KNP was chosen for the following reasons:

- it is a large park with a surface area of 18 989 square km;
- it has free-ranging animals that are not treated with antimicrobials;
- it is fenced all around, so animals have no contact with domesticated animals or human faeces as livestock cannot get into the park, pets are not allowed inside the park and tourists can only move around in vehicles, using only ablution facilities at designated fenced rest-camps;
- the perennial rivers have different levels of pollution.

[1] ArcGIS: www.esri.com

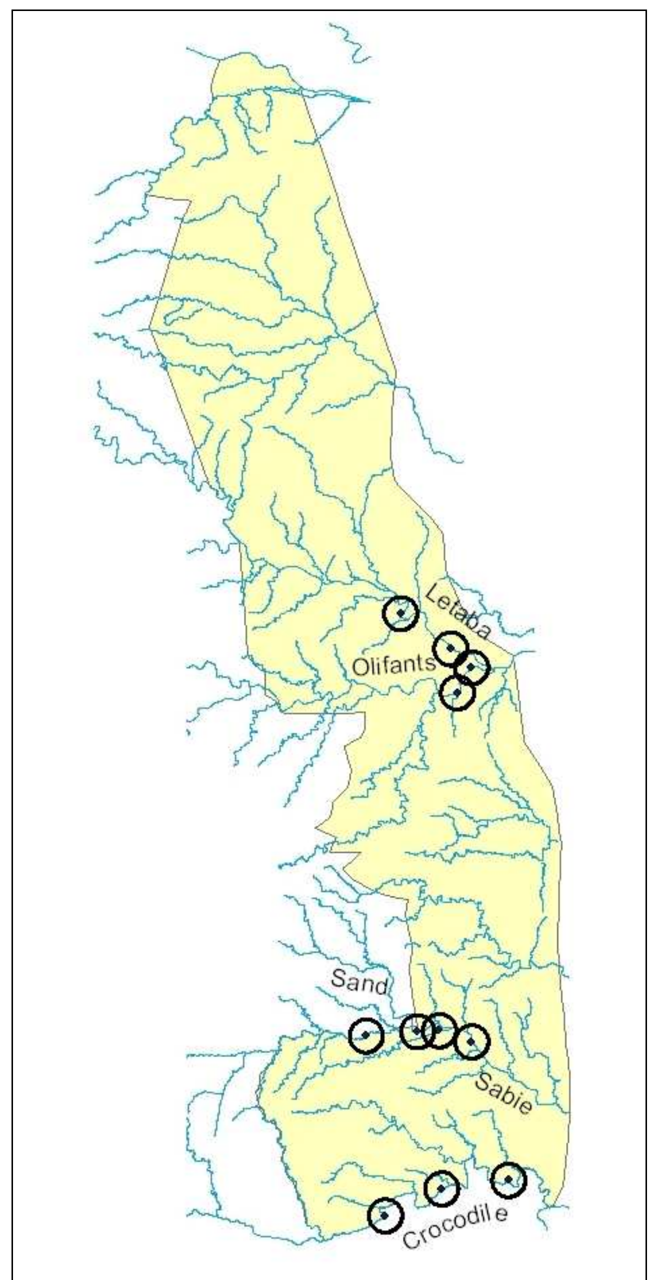

Figure 3.3: Water Sampling points (ArcGIS)[1].

In essence, the animals living in the park could be considered as a naïve population when it came to antimicrobial drug-induced AMR. Thus an association with TR*EC* in rivers used as drinking water was feasible.

The perennial rivers in the KNP that were sampled were the: Crocodile; Letaba; Olifants; Sabie; and Sand rivers (Figure 3.3). The first characteristic considered for the choice of the rivers was their location, as all of them had to be situated were there were high numbers of impala (see Figure 3.13). Secondly perennial rivers

[1] ArcGIS: www.esri.com

were considered, so that it would be possible to take samples in different periods of the year and that some would have been polluted by animal and human waste before entering the KNP. Each river will be described in more detail under separate subheadings below.

3.2.1 Sabie and Sand Rivers

The Sabie-Sand River system covers around 6 320 km^2. The Sabie River is the mainstream of the catchments, with the Sand and Marite Rivers acting as major tributaries, and the Mac Mac River being a tertiary drainage. The Sabie River has its source at 2 130 m above mean sea level in the Drakensberg Escarpment, then drops into the Lowveld and joins the Sand River inside the KNP (Figure 3.4).

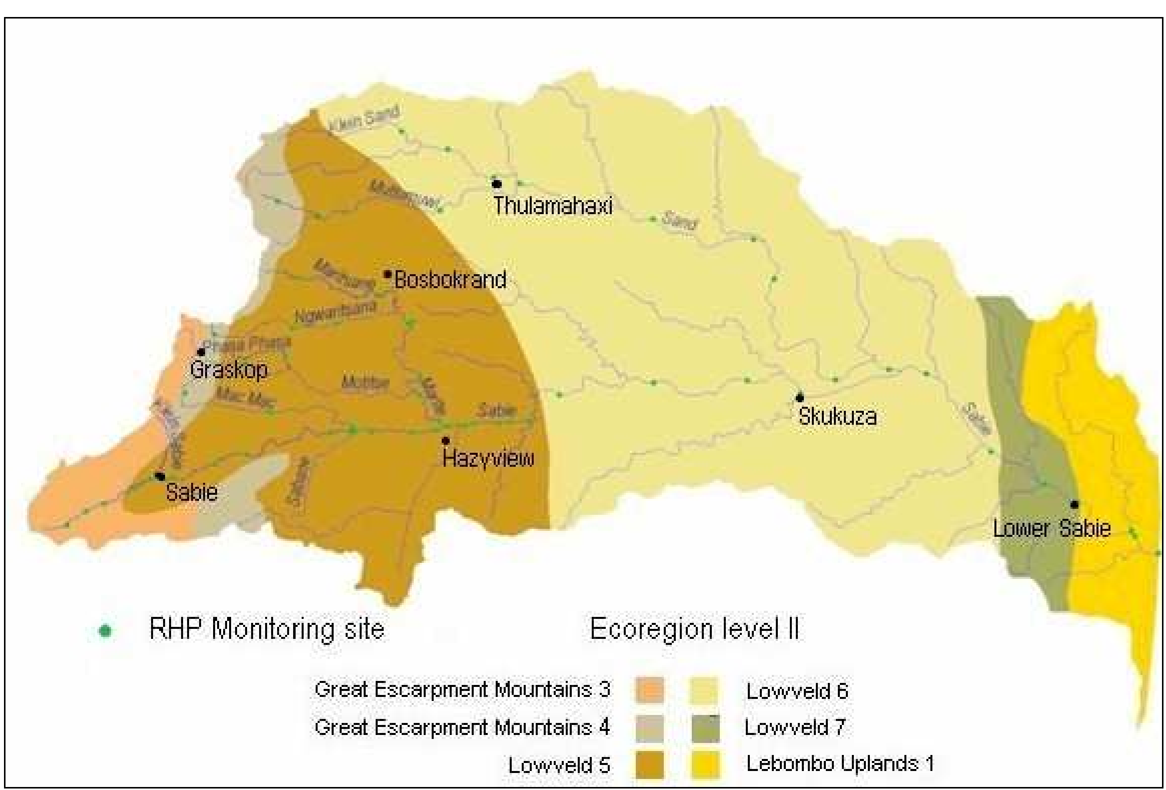

Figure 3.4: Ecoregion of Sabie river (WRC, 2001 b).

Mean annual rainfall in this system varies between 2 000 mm on the Escarpment to around 600 mm in the Lowveld. Most of this precipitate falls between November and March, in the form of tropical storms. Summer maximum temperatures are high and evaporation averages 1 700 mm per year in the Lowveld region. Mean

annual runoff in the Sabie-Sand is approximately 762 million cubic metres. Flows in the Sabie River peak in summer, and low flows are experienced at the end of the winter dry season, although no-flow conditions have never been recorded (WRC, 2001 b).

Information about the flow trend of the Sabie River at the Lower Sabie RHP collection site can be extrapolated from the graph shown in Figure 3.5 (Venter J, personal communication 2006).

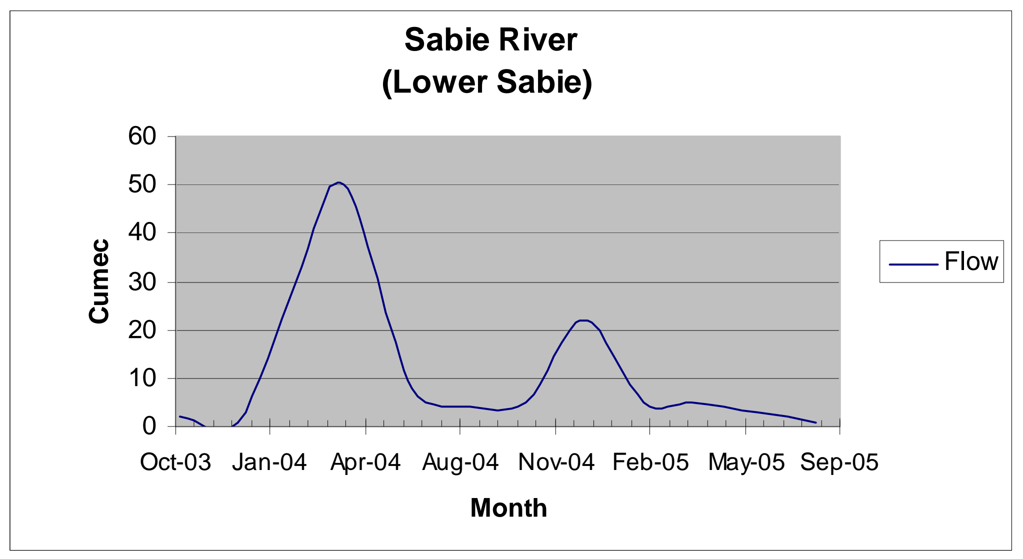

Figure 3.5: Sabie River flow trend from 2003 to 2005 at Lower Sabie (Venter J, personal communication, 2006).

The main activities upstream of the KNP that can influence pollution levels in the rivers are related to forestry and trout farming in the ecozone recognised as great escarpment mountain 3, sewage from the Sabie, Graskop, Bosbokrand and Thulamahaxi small towns and sedimentation from banana plantations in the Lowveld 5 region. The middle part of Lowveld 5 region is also rich in rural community activities such as small scale farming of livestock and fruit (Figure 3.4)(WRC, 2001 b). Inside the park the sewage from tourist camps such as Kruger Gate, Skukuza, Nkuhlu and Lower Sabie, are discharged into the river after going through sewage purification plants, to reduce to a minimum the impact of this pollution into the river (du Plessis B., personal communication, 2007).

3.2.2 Crocodile River

The Crocodile River system covers about 10 450 km^2. The mean annual runoff is 1 200 million cubic metres per year, with an estimated maximum yield (that is the maximum amount storable in dams) of 859 million cubic metres per year.

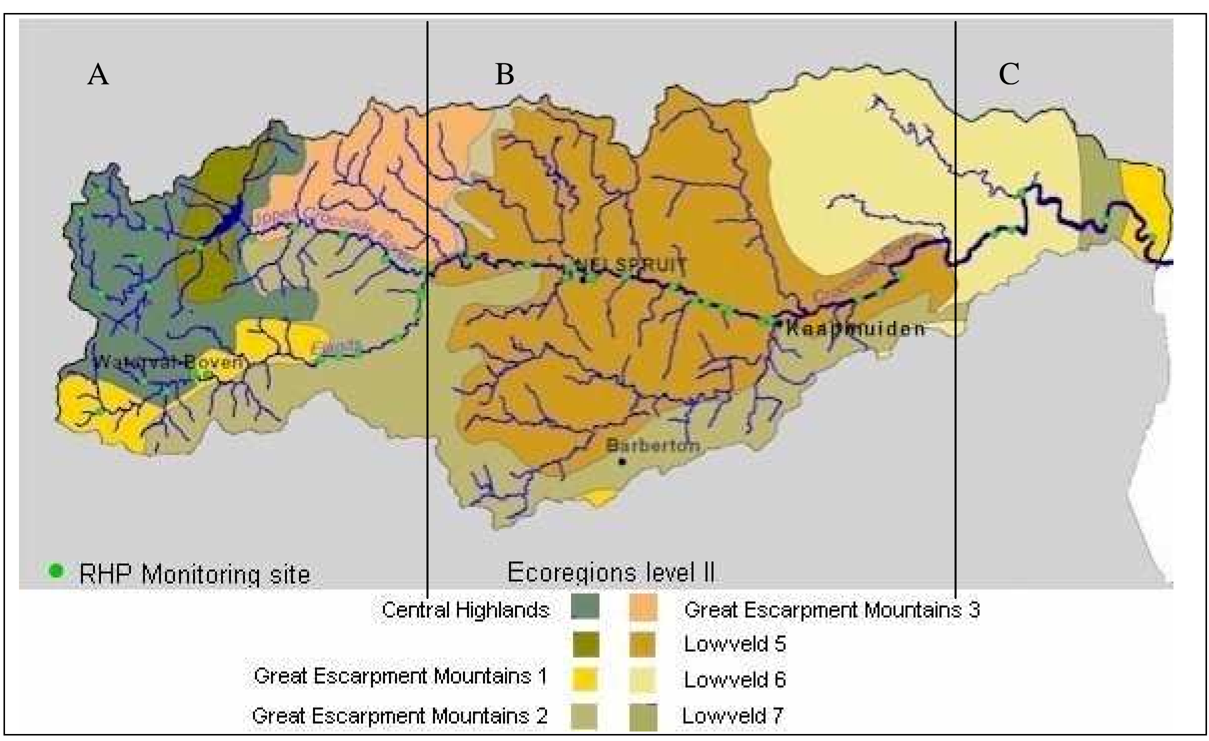

Figure 3.6: Ecoregion of Crocodile River (WRC, 2001 b).

Due to its length, the river passes through different ecosystems that, for practical reasons can be grouped into three clusters A, B and C (Figure 3.6). The ecoregion of zone A is mainly represented by the Drakensberg Mountains (1000 to 2000 m). In this region there is a great variation of the mean annual temperature (12-22℃) and of the mean annual rainfall (600 to 1200 mm). Ecoregion B is typical of the start of the Lowveld. The altitude range is around 800-1000 m. Rainfall is variable, with some zones receiving 400mm per year and others receiving 600-1000 mm. Group C is typical Lowveld (apart from the border with Mozambique where there are the Lebombo uplands), with a higher temperature (20-22℃) and lower rainfall (400-800 mm) (WRC, 2001 b). Information about the flow of the Crocodile River at the Ten Bosh collection site can be extrapolated from the graph shown in Figure 3.7 (Venter J, personal communication 2006).

The main sources of pollution in zone A are livestock, forestry and trout farming activities, as well as urban development (mainly Machadodorp and Waterval Boven villages). Zone B is polluted by intensive agricultural activities (mainly sugar cane, citrus, orchards, vegetables and tobacco) as well as industrial and urban land uses. Domestic runoff, urban and industrial waste from the highly populated city of Nelspruit has a significant impact on the health of the Crocodile River.

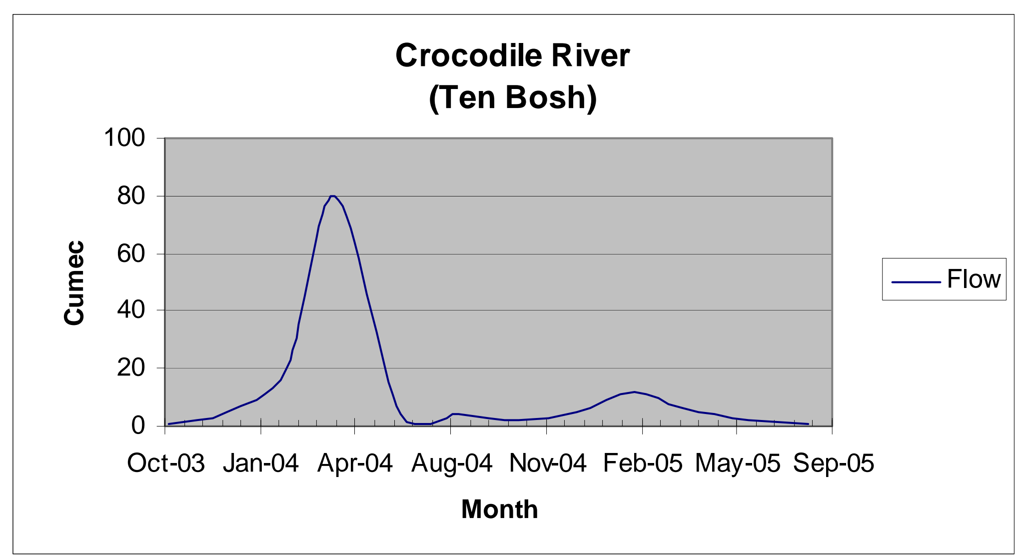

Figure 3.7: Crocodile River flow trend from 2003 to 2005 at Ten Bosh (Venter J, personal communication, 2006).

The ecoregion of zone C is protected by the KNP on the northern side, but on the southern bank is heavily utilised. Vegetation removal for citrus and sugar cane farming, as well as tourist lodges, have modified large parts of the southern bank. The water that returns to the river after irrigation is often enriched with nutrients. In addition, the cumulative effect of water abstraction, primarily for irrigation purposes, often results in lower than desired flow. Thus has a negative impact on the overall water quality (WRC, 2001 b).

On the KNP side, the sewage sources that discharge into the Crocodile come mainly from Malelane, Berg en Dal and Crocodile Bridge tourist camps (du Plessis B., personal communication, 2007).

3.2.3 Letaba River

The Letaba River arises in the Northern Drakensberg mountains and flows down in an easterly direction across the KNP where it joins the Olifants River. Its major tributaries are the Klein Letaba, the Middle Letaba, Nsama and Molotosi Rivers. In the complex, the Letaba catchments area covers about 13 670 km^2.

The mean annual precipitation is 612 mm, while the mean annual evaporation is 1 669 mm. Due to its length the river passes through different ecosystems that, for practical reasons, can be grouped into the Limpopo plains, the Central Highlands (ecogroup 2.15), the Great Escarpment Mountains (ecogroups 4.03; 4.04), the Lowveld (ecogroups 5.01; 5.02; 5.03; 5.04; 5.05; 5.07) and the Lebombo Mountains (ecogroup 6.01) (Figure 3.8).

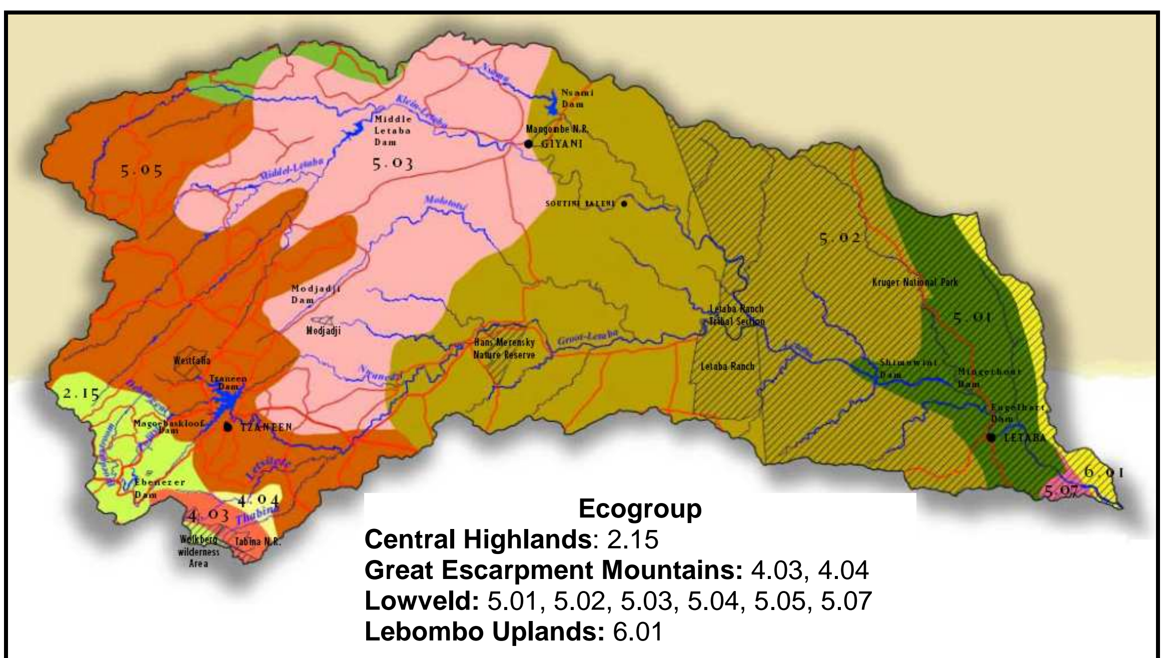

Figure 3.8: The Letaba River catchments and its ecosystems (WRC, 2001 a).

Information about the flow of the Letaba River at Letaba Ranch can be extrapolated from the graph shown in Figure 3.9 (Venter J, personal communication, 2006).

The land use and so, also, the source of pollution, differs between the ecosystems (Figure 3.8). In ecosystem 2.15 the most important are: forestry, trout farming and sand mining. In ecoregions 4.03 and 4.04, subsistence farming and commercial

farming represent, respectively, 35% and 7% of the land use. In ecoregion 5.05 forestry plantations take up 30% of the total land cover, while 36% and 22% are respectively used for subsistence and commercial farming. Subsistence farming comprises 35% of the total land-use in ecoregion 5.03, while both commercial and subsistence farming activities comprise 25% of ecoregion 5.02, in the catchment area outside of the KNP. In this last area nearly 55% of the land use consists of subsistence farming (20 800 ha) and 40% of commercial farmlands (14 300 ha), in particular subsistence farming, is the main land use in the Molototsi and Nsama river catchments. Urban settelments comprise 6,5% and 5% of the total catchment areas respectively. Agricultural activities are mainly concentrated in the Giyani area, which consists of small-scale farming by rural communities and large-scale commercial banana, papaya, pawpaw and mango plantations (WRC, 2001 a).

Inside the park, the sewage from Shimuweni and Letaba camps, after treatment, are discharged into the river (du Plessis B., personal communication, 2007).

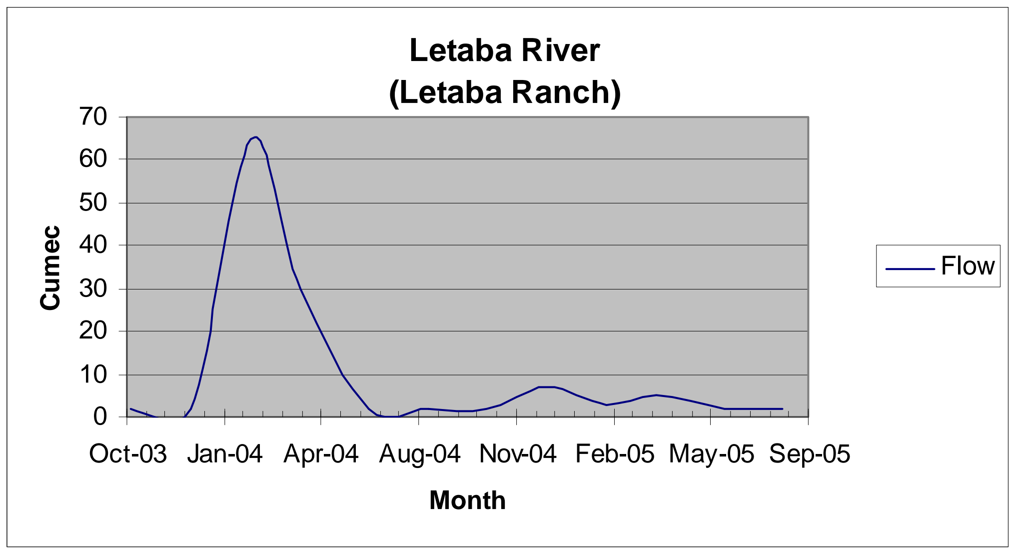

Figure 3.9: Letaba River flow trend from 2003 to 2005 at Letaba Ranch (Venter J, personal communication 2006).

3.2.4 Olifants River

The Olifants River rises in the Highveld grasslands. Overall the catchments area covers about 54 570 km^2 and is subdivided into nine secondary catchments.

The total mean annual runoff is approximately 2 400 million cubic metres per year. It passes through several different ecosystems (Figure 3.10). Information about the flow of the Olifants River at Mamba can be extrapolated from the graph in Figure 3.11 (Venter J, personal communication, 2006).

The upper part of the Olifants River catchments area up to Witbank, is characterised by mining, predominantly for coal, and other industrial activities. The in-stream conditions are impaired by poor water quality, where acid leaching from the mines is a primary contributor.

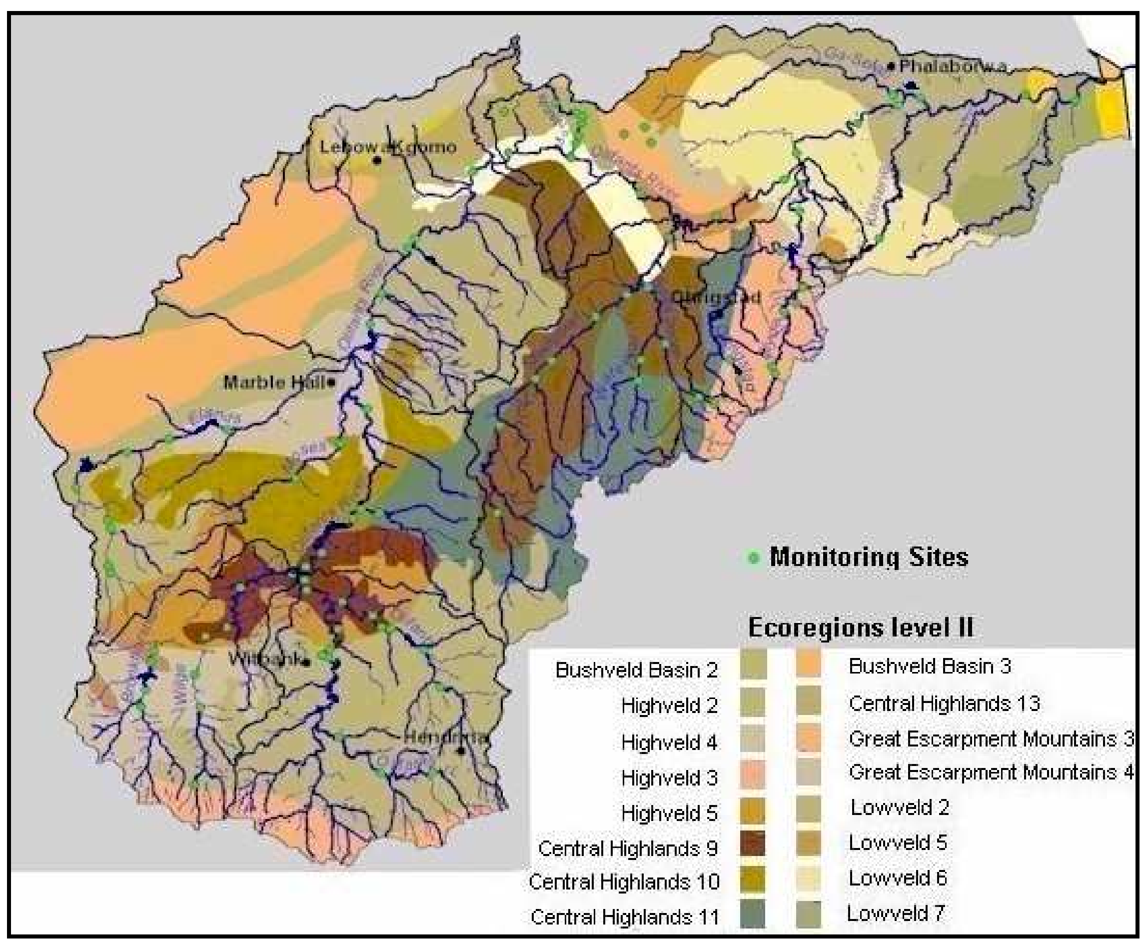

Figure 3.10: The Olifants River catchment area and its ecosystems (WRC, 2001 b).

Low pH (high acidity) and high concentrations of dissolved salts are characteristic of streams in this area. The conditions improve gradually until the central highlands where, once again, the high acidity and salt concentration from some tributaries like the Klip River, impact negatively on water quality. The conditions become unacceptable around Marble Hall where deforestation, informal

settlements and subsistence farming result in eutrophication and contamination of water. An improvement is once more visible around the Wilderness conservancy area, upstream of the Mohlapitse River. This water condition is more or less maintained until around the joint with the Spekboom and Orhrigstad rivers, where the runoff from mines and intense cultivation, lowers the water quality. The health of the Olifants River improves again downstream of the confluence with the Blyde River, which contributes water of better quality, as it belongs to the “Kruger to Canyon Biosphere Reserve”. Upstream of the KNP, the activities in the Phalaborwa area, such as mining and farming, cause severe damage to the ecosystem of the river. Furthermore an ecologically insensitive releases of water and sediment from storage dams are another major cause of environmental degradation of thr lower parts of the catchment (WRC, 2001 b).

Inside the park the sewage from the Olifants camp enters the river after treatment (du Plessis B., personal communication, 2007).

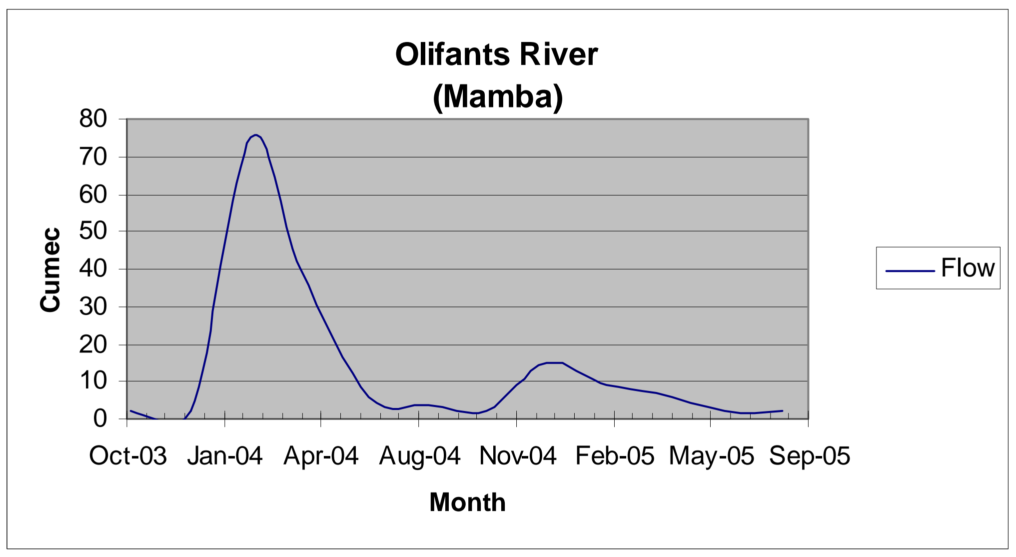

Figure 3.11: Olifants River flow trend from 2003 to 2005 at Mamba (Venter J, personal communication, 2006).

3.3 Experimental design and procedure

In the case control study performed, the risk factor considered was the presence of TR*EC* in rivers used as a drinking source by impala herds. Retrospective data about general water quality of the rivers and main figures of human population and

livestock density around the area were analysed. This to established the possibilities of the rivers to be polluted from TR*EC* and so to divide the river in the two basic groups of pollutes and unpolluted with TR*EC* (section 4.2). Data about the characteristic of the sewage of the main camps in KNP along the river studied were also collected, mainly by personal communications, in order to better understand the pollution source of these rivers.

The data about general water quality were collected mainly from the database of the River Health Programme (RHP). This programme, initiated by the DWAF, focus on the changing from “end of pipe” monitoring to an “integrated water resource management approach”. It is supported by the Water Research Commission (WRC), and the Council for Scientific and Industrial Research (CSIR) provide, from the data available in 2001, information on the state of river ecosystems in South Africa (WRC 2001 a, b). The figures about population and livestock density in the studied area have been extrapolated from the data available of the 2001 national census and can be found in Chapter 4 and Appendix 1 where the map of the human census districts can also be found (StatsOnline, 2001).

In the epidemiological study approximately two hundred impala faecal samples were collected, divided into two groups, those exposed and unexposed to TR*EC*. These would then effectively be the “case” and “control” groups. Samples would simultaneously be taken from the rivers used as drinking water by the identified impala herds. The OR was used to estimate the strength of association of the risk factor (TR*EC* in drinking water) with the selected outcome (TR*EC* in impala).

The study design and procedures for collection of impala faeces and river water within the KNP had to be approved by the *Scientific Service Committee* and from *People and Conservation Managers Committee* of KNP. The approved proposal can be found as Appendix 4. It entailed that the researcher had to go out with a game guard to track the impala herds in person and to investigate their feeding and drinking behavior in order to select the part of the river to sample. Part of the observation and tracking was to find out where the middens were so that very

fresh faeces that had just been eliminated by the impala, could be collected (Figure 3.12).

Figure 3.12: Collection of impala faecal samples in KNP.

***3.3.*1 Impala as indicator**

Impala (*Aepyceros melampus*) were chosen as the indicator animal for this project as they are widely distributed in different ecosystems and are highly social and territorial. These are gregarious animals, occurring often in small herds of 6-20 animals but also in larger congregations of 50-100 animals during the wet season. Their social organisation consists of males, which are territorial only during the rut, bachelor and breeding herds. Annual size of the home range of the herd changes with the number of individuals but Murray (1982, cited by Skinner & Chimimba, 2005), estimated that for 50 females it is about 5.16 Km^2, while for a single male it varies significantly with age, from 0.9 Km^2 at 3-4 years to 0.49 Km^2 at 5-6 years (Skinner & Chimimba, 2005). Their habitat consists of grassland and woodland edges and, they are usually found very close to water (Stuart & Stuart, 2000).

Figure 3.13: Map of impala distribution in KNP according with the 2006 census (J Kruger, personal communication 2006).

They are generally associated with *Acacia* species and mopane (*Colophespermum mopane*), but also occur in other types of woodland with *Baikeaea, Combretum* and *Terminalia* spp.. While they often avoid open grassland and floodplains, they are sometimes found in these habitats when there is green grass. Usually they are absent on mountains, but they were observed in KwaZulu-Natal up to an altitude of 1400 m. Their social organization allows them to adapt to prevailing environmental conditions and they are so adaptable that they are found

from southern Africa to the northern limits of East Africa (Skinner & Chimimba, 2005). In the recent wildlife census done in 2006 in Kruger National Park, the estimated number of impala was 98 003 (J Kruger, personal communication 2006). Their distribution in the KNP is shown in Figure 3.13.

3.3.2 Sampling method

Non-probability sampling methods were used for water and faecal sampling.
A three stage cluster sampling method was used to collect water samples. The first stage was to select purposively a perennial river, so that the sampling could have been done all the year long and that the river would be transporting upstream pollutants. The Letaba, Olifants, Crocodile, Sabie and Sand Rivers were selected. The second stage was the division of the rivers in two groups on the bases of the possibility of finding TR*EC.* They were initially selected on the likelihood of being polluted or not (section 4.2) and thereafter on the presence of TR*EC*, which was determined by laboratory methods (section 3.5).

The last stage was the selection of the sampling points. A total of 11 points in these rivers were sampled on 3 separate occasions: three points in the Crocodile, two in the Letaba, two in the Olifants, three in the Sabie Rivers and one in the Sand River. The river sampling points were selected on the basis of impala distribution, so that it could be possible to collect more or less the same amount of faeces from the two groups of impala (case and control). Although special attention was given to selecting points that had a minimum distance between them of 10km or a physical barrier, thus avoiding mixing between the impala herds.

A three stage cluster sampling method was also used to select fresh faecal samples of impala. The first stage was the division of the impala into two populations: those drinking from TR*EC* containing rivers (exposed) and those that were not (non-exposed). The second stage was the selection of the herds (clusters) within each group. A convenience sampling method was used whereby the first impala herds spotted within a range of 5km from the water sampling point was selected. The range of 5 km was selected considering the impala home range distribution (see previous section). The third stage was the selection of single

faecal samples from the herds. Fresh faecal pellets were purposively selected, as they were more likely to have the highest number of viable *E. coli*.

3.3.3 Estimation of minimum sampling size

The estimation of the minimum amount of number of faecal samples needed for the case and the control groups was found by applying the following equation (Cobb, 2004):

$$n=[(r+1)/r]/\{[\ \bar{p}\ (1\bar{p}-)(\ Z_{\beta}+Z_{\alpha/2})^{2}]\ (p_{1-}\ p_{2}\ )\}$$

[equation 1]

Where:

n= estimation of sample size in the case group

r= ratio of controls to cases

$\bar{p}$= average prevalence of exposed $((p_1+ p_2)/2)$

p_1= estimated prevalence of cases

p_2= estimated prevalence of controls

$Z_{\alpha/2}$= normal standard deviation for type error II

Z_{β}= normal standard deviation for type error I

The estimated prevalence of cases is derived from the formula:

$$p_1=(OR\ p_2\ )/[p_2\,(OR-1)+1]$$

[equation 2]

Where:

OR= expected Odds Ratio

P_2= estimated prevalence of controls

Assuming an expected maximum prevalence of TR*EC* in the control group of 5%; and a minimum Odds Ratio of 5, the prevalence, the formula for case prevalence [equation 2] becomes:

$$p_1=5(0,5)/[(0,05)(5-1)+1]=0,25/1,2=0,21$$

Considering a collection of an equal number of cases and controls (r=1) and assuming a power of 80% and a significance level of 0.05 ($Z_{\alpha/2}$=0.84 and Z_{β}=1.96), the formula [equation 1] is substituted as:

$$n=0,13(1-0,13)(0,84+1,96)^2/(0,21-0,05)^2=89$$

Taking into consideration that most of the parameters in the equation were assumed and that the larger the sample size the closer is the possibility to get a true estimate, about 15 more samples were collected per group.

3.4 Sampling procedures

Each water point was sampled at three different times during the year (March, May and June). To make it possible to collect samples from the same site, Geographical Positioning Systems (GPS) coordinates were recorded using e-Trex ® (Garmin)[1] and transferred on the software ®Mapsource (Garmin)[1]. Each time, 500mL of flowing water was collected into sterile glass bottles (Lasec®)[2].

Figure 3.14: Water sampling.

---

[1] Mapsource Garmin: www.garmin.com
[2] Lasec SA (Pty) Ltd., PO Box 1296, South Africa.

A 5 step sampling technique was used as suggested by the Department of Water Affairs and Forestry (DWAF 2001 a). The technique is illustrated in Figures 3.14 and 3.15.

Step 1	Step 2	Step 3	Step 4	Step 5
Wear gloves and remove the cap from the bottle without contaminate the inner surface of the cap or the neck of the bottle.	Take the sample by holding the bottle near the base. Plunge the sample bottle with the neck downward, below the water surface .	Turn the bottle until the neck points upward and mouth is directed toward the current.	Replace the cap immediately paying attention to leave at least 2.5 cm of air space in the bottle (to facilitate mixing before examination).	Label the bottle and complete the sample schedule

Figure 3.15: Water sampling procedures (DWAF, 2001).

Within a distance of 5 km from each water sampling point, about 10 grams of faecal pellets were collected from the impala middens. Special care was given to select fresh faecal samples and to pick them up from the top of faecal middens (see Figure 3.15). This way avoided contamination by soil bacteria. Sterile latex gloves were used for each sample, to avoid any cross-contamination. Five faecal samples were collected per water sampling point from all the rivers, with the exception of the Sabie River, where 10 were collected to reach an equivalent amount of faecal samples from polluted and unpolluted rivers. The samples were stored in sterile plastic sample bottles (PlastPro)[1], in a cooler bag until their arrival at the laboratory. The GPS coordinates for each sampling point were recorded on a Table (Appendix 2) using e-Trex® (Garmin)[2]. The distance of each water

[1] PlastPro, Ltd South Africa
[2] Garmin ®: www.garmin.com

sampling point (Figure 3.17) from each corresponding faecal sampling point was controlled by Mapsource® (Garmin)[1].

Figure 3.16: Impala faeces sampling

Figure 3.17: Measurement of the distance between the water source and impala faeces (red line on map) measured using Mapsource (Garmin)[1].

---

[1] Garmin ®: www.garmin.com

3.5 Bacteriological analysis

All the following procedures were executed in the Bacteriology Laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria and in the Infectious Diseases Laboratory, Department of Wildlife Services, Skukuza, Kruger National Park. The samples were processed within 24 hours of collection.

3.5.1 Isolation of E. coli

Once the faecal samples arrived at the laboratory, they were mixed thoroughly and 1gr was put into a sterile bottle with 10mL of physiological saline solution (BR 53, Oxoid)[1]. Six serial 10-fold dilutions were made using physiological saline and 0,1mL of the last three (10^{-7}, 10^{-6}, and 10^{-5}) were spread-plated onto MacConkey agar (CM0507-CM7b Oxoid)[1] and incubated overnight at 37℃.

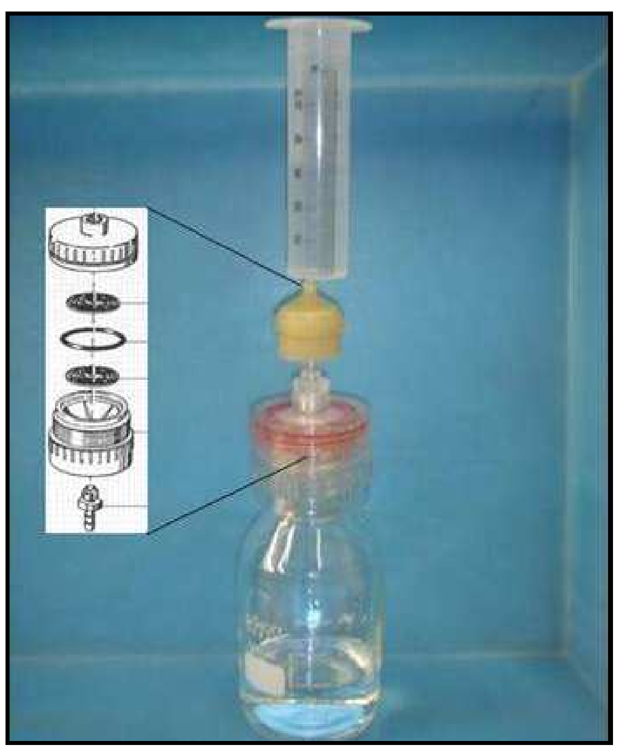

Figure 3.18: Water filtration system.

[1] Oxoid Pty Ltd., Basingstoke, England, UK

The water samples were pre-filtered using a paper filter (Munktell 1336/2)[1] and then filtered through a 47mm sterile cellulose nitrate filter of 45±2 µm pore diameter (Sartorius)[1] (Figure 3.18). Each membrane was placed into 10mL of peptone water (Oxoid)[2] and incubated overnight at 37℃. The next day seven serial 10–fold dilutions were made of the broth culture and 0,1mL aliquots of the last three (10^{-7}, 10^{-6}, and 10^{-5}) were spread-plated onto MacConkey agar plates (Oxoid)[2].

3.5.2 Replica plating

One MacConkey plate for each sample that showed the growth of a good number (30-50) of separate colonies with the morphology of *E. coli* was copied, following the replica plating method of Lederberg (Lederberg & Lederberg, 1951). In brief, a sterile velvet-covered disk, the size of the plate, was pressed onto the chosen MacConkey plate, removed and printed onto an unused MacConkey agar plate, to which 4µg/mL of doxycycline powder (p13177, Medpet)[3] was added.

The preparation of the MacConkey plates containing 4 µg/mL of doxycycline was done in the following way:

The amount of antibiotic to be added to the stock solution was calculated using the following formula [equation 3] (Andrews, 2001):

$$W = (1000/P) \times V \times C \quad \text{[equation 3]}$$

Where: W= Weight of antibiotic powder to be dissolved in V.

P= Potency of drug given from the manufacturer in µg/mg.

V= Final solution volume required in mL.

C= Final concentration in µg/mL.

[1] Sartorius Stedim Biotec, S.A. http://www.sartorius.com
[2] Oxoid Pty Ltd., Basingstoke, England, UK
[3] MedPet Pty., Ltd., http//www.medpet.co.za ,Box 27239,Beurose 2011,Gauteng, S.A.

In this study:

$$mg_{\text{antibiotic powder}} = (1000 / 980) \times 250mL \times 4 \times 10^3 \mu g/mL$$
$$mg=1020{,}4mg$$

The above calculated amount of doxycycline powder was dissolved in 250mL of sterile water. The solution was then stored in the freezer at -18℃, in obscured 20mL bottles.

The working solution was made up as required, using the following formula [equation 4]:

$$C_1V_1= C_2V_2 \quad \text{[equation 4]}$$

Where C_1= Concentration of stock solution.

V_1= Volume of stock solution.

C_2= Desired concentration antibiotic in the MacConkey agar.

V_2= Volume of MacConkey agar.

In this study:

$$4mg/L \times V_1= 4\mu g/mL \times 500mL$$
$$V_1= 0{,}5mL$$

Following the above calculation, 0,5mL of the stock solution was filtered by a 0,2µg pore sized cellulose acetate filter (Sartorius)[1] and added to 500mL of MacConkey mixture, a few seconds before plating.

3.5.3 Identification of <u>E. coli</u>

After incubation and where possible, three lactose fermenting colonies showing tetracycline resistance and three lactose-fermenting colonies not showing tetracycline resistance, were selected from each MacConkey plate and streaked onto 5% horse blood Columbia agar (CM0331-Columbia agar base, Oxoid)[2] and incubated at 37℃ overnight, for identification pur poses.

[1] Sartorius Stedim Biotec, S.A. http://www.sartorius.com
[2] Oxoid Pty Ltd., Basingstoke, England, UK

Pure cultures were identified as *E. coli* when they were Gram-negative short rods, oxidase-negative, catalase-positive, spot indole positive, motile and citrate-negative. Once each *E. coli* had been isolated and identified from each sample, it was stored in 2.2mL NUNC cryotubes®[1] containing 1mL brain-heart infusion broth (BHI) (Oxoid)[1] and 50% glycerol (Merck)[3] in the ultra cool freezer (Forma Scientific)[4] at - 86ºC.

3.5.4 Phenotypic tetracycline resistance test: MIC

Once *E. coli* has been obtained and stored from all the samples collected, phenotypic antimicrobial resistance patterns were determined. Susceptibility to tetracycline was determined using the minimum inhibitory concentration (MIC) technique. The guidelines of the Clinical Laboratory Standards Institute (CLSI, 2002) were used as the reference method for preparing the varying dilutions and determining the MIC. The method is briefly described below.

Material from 1 to 3 colonies of *E. coli* from a 24 hour MacConkey plate, were inoculated into 2mL of sterile deionized/distilled water containing 0.02% Tween 80, until a turbidity of 0,5 MacFarland standard was reached. After it was vortexed well, 10µL of the mixture was transferred into 12mL of cation-adjusted Mueller-Hinton broth (CAMHB) (Oxoid)[1], to obtain a concentration of approximately 5×10^5 CFU/mL. The CAMHB solution added with the inoculum was dispensed into sterile plastic Petri dishes (PlastPro)[5] to facilitate the work of dispensing the solution into the sterile, round-bottomed, 96-well microtitre plates (AEC Amersham)[6].

The wells in the microtitre plate (AEC Amersham)[6]. (Figure 3.19) were prepared in two-fold dilutions so as to have a final tetracycline concentration in the wells of 32, 16, 8, 4, 2, 1, 0.5 and 0.25µg/mL. This was achieved by serial transfer of 100µL of

[1] NUNC A/5, Ros-Kilde, Denmark.
[3] Merck (Pty) Ltd; Box 1998, Halfway House, 1865,S.A.
[4] Forma Scientific, Supplied by Labotec: http//www.Labotec.co.za
[5] PlastPro, Ltd South Africa
[6] AEC Amersham, www.aecam.co.za

a 64µg/mL doxycycline working solution, to 100µl sterile water and discarding the last 100µL.

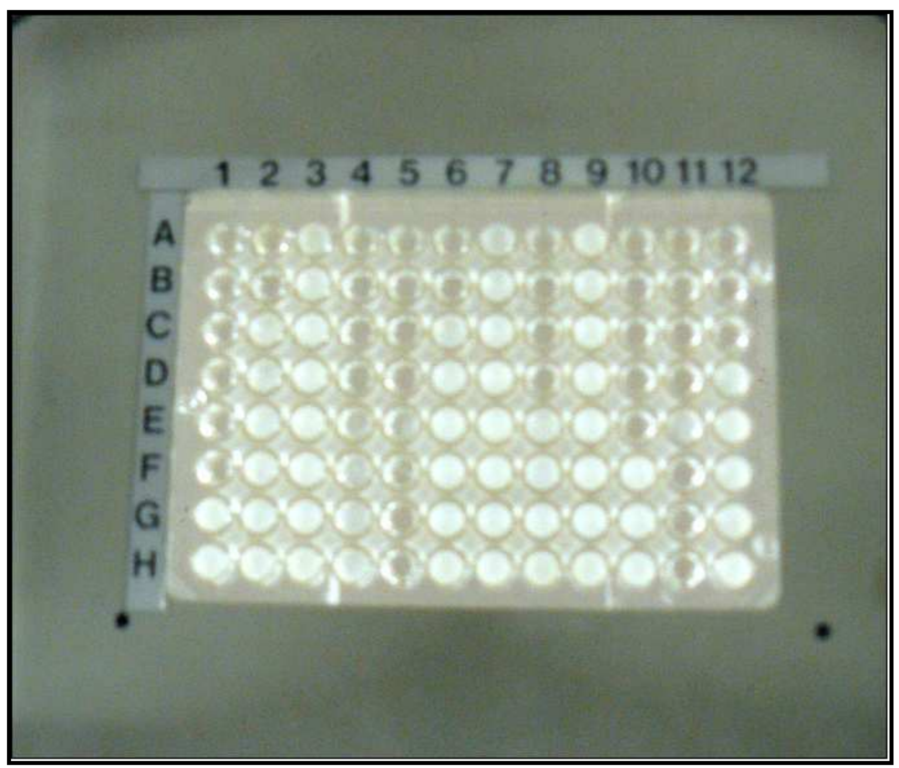

Figure 3.19: MIC: microtitre plate (AEC Amersham, South Africa)[1].

Each well of the microtitre plates was inoculated with 100µL of the bacterial suspension using a multi-channel automatic pipette (Eppendorf)[2]. Each plate was sealed with a transparent cover (AEC Amersham)[1] to avoid drying out of the wells. From the inoculum, 10µL was streaked onto Columbia blood agar (Oxoid)[3] to check for purity and inoculum size.

The plates were incubated at 37℃ for 18-24 hours. After incubation, the inoculum control was checked for purity and for the number of colonies (around 30-50 per plate). The results were read by a viewer mirror and the MIC was recorded on a Table (Appendix 3) as the lowest concentration inhibiting visible growth.

---

[1] AEC Amersham, www.aecam.co.za
[2] Eppendorf ®; supplied by Scientifica Group, Box 4261, Randburg 2125,S.A.
[3] Oxoid (Pty.) Ltd. Basing stoke, UK

3.6 Genotypic resistance test: PCR

All the isolates phenotypically positive for tetracycline resistance were examined for the two most common *tet* genes in *E. coli* namely *tet*(A) and *tet*(B) using the PCR method.

3.6.1 Optimization of the PCR method

Before applying PCR techniques to the resistant samples obtained from the current study, a pilot study was done to optimize the method and as well to find laboratory controls for further study. Four cultures of highly tetracycline resistant bacteria from different species were used to detect resistant genes.

3.6.1.1 Optimization of DNA extraction

The method of Boerlin *et al.* (2005) was first used to extract DNA from the isolates. DNA was extracted from *E. coli* by resuspending a loopful of bacteria into 500µL of water and heating the homogenous suspension at 95℃ for 10 minutes. After being cooled to room temperature it was centrifuged at maximum speed for 3 minutes.

The validity of the extraction was evaluated by electrophoresis of the obtained supernatant on an agarose gel (Fermentas, Inquaba)[1] (2% in 1 x Tris-Acetate-EDTA buffer (Invitrogen, The Scientific Group)[2], stained with 1µL ethidium bromide (10mg/mL) (Sigma Aldrich)[3], and visualized under UV illumination using a transilluminator (UVP Dual density transilluminator, The Scientific Group)[2].
As this method did not give satisfactory results it was then combined with the method of Miller *et al.* (1997). A loopful of bacteria from a fresh overnight culture on a MacConkey agar plate was resuspended homogeneously in 0.6mL centrifuge

[1] Inqaba Biotechnical Industries (PTY)Ltd PO Box 14356 Hatfield South Africa
[2] The Scientific Group, Box 4261, Randburg 2125,S.A.
[3] Sigma-Aldrich SA PTY LTD PO Box 10434, Aston Manor 1630, South Africa.
.

tubes (Plastpro)[1] containing 200μL of 0.5% Tween 20 (Sigma Aldrich)[2] in sterile water. The tubes were boiled for 10 min at 95℃ in a water bath (Labotec)[3]. They were then immediately transferred into a liquid nitrogen freezer (K series, Cryostorage system, Labotec)[3]. After 5 minutes, the tubes were transported on ice to the water bath where they were boiled again for a further 10 minutes. They were then centrifuged (Eppendorf centrifuge 5417C)[4] for 3 min. at 13000rpm and the supernatant was transferred into new sterile tubes. Once the electrophoresis was run and was confirmed the presence of DNA, it was quantified using the spectrophotometer machine (Beckman Coulter DU 530)[5]. The amount of DNA in the extract was then used to calculate the quantity of DNA template to add to the PCR mix, so to have an approximate amount of 3.2pMol.

3.6.1.2 Optimization of the multiplex PCR

Initially the amount of DNA template for the PCR reactions was optimised using three different reaction mixes. The reactions were separately tested for both the primers *tet(A)* or *tet(B),* to check if the primers were working properly. The quantity of DNA template added to the master mix was 2.5μL, 1μL and 1:10 dilution of 2.5μL respectively.

The master mixes were prepared as follows:

- 12.5μL of Platinum® Quantitative PCR Super-Mix-UDG (Invitrogen, The Scientific Group)[6] containing: Platinum® *Taq* polymerase, Mg^{++}, uracil DNA glycosylase (UDG), proprietary stabilizers, and deoxyribonucleotide triphosphates with UDP instead of dTTP.
- 1μL forward and 1μL reverse primer of 32pmol solutions of *tet(A)* or *tet(B).* The primers used are indicated in Table 3.1. Primers were synthesized at Inquaba[7].

---

[1] PlastPro, Ltd South Africa
[2] Sigma-Aldrich SA PTY LTD PO box 10434, Aston Manor 1630, South Africa
[3] Labotec Group PO Box 6553 Halfway House 1685 South Africa
[4] Eppendorf, supplied by Merck, PO Box 1998 Halfway House, South Africa
[5] Beckman, PO Box 13119, South Africa
[6] The Scientific Group, Box 4261, Randburg 2125,S.A.
[7] Inqaba Biotechnical Industries (PTY)Ltd PO Box 14356 Hatfield South Africa

Sterile water was added to the reaction mixes so as to reach a total amount of 25µL.

The DNA was amplified using a thermocycler PE2700/2600 (Applied Biosystems)[1] as described by Boerlin *et al.* (2005) as follows:

- 1 cycle of 4 min at 95℃;
- 35 cycles each consisting of 1 min at 94℃, followed by annealing at a 64℃ and elongation at 72℃ for 1 min
- 1 cycle of 7 min at 72℃.

After amplification, PCR products were separated by electrophoresis as described in section 3.6.1.1 and then visualized under UV illumination. The size of the PCR products were determined by comparison of the migration to a standard 100bp molecular marker (GeneRuler™ Fermentas, Inquaba)[2] and it was determined whether isolates had *tet(A)* or *tet(B)* genes.

Apart from the 2.5µL concentration of template which was too high, the other two concentrations gave good results. A light white shadow could be noted for all the samples, showing that the amount of primer was more than sufficient.

Another PCR reaction was carried to check whether the primers would work well together in a multiplex PCR and so two samples, labelled B4691/03/poultry and B234/2004/equine, which were found to have respectively *tet(A)* and *tet(B)* genes during the initial reaction, were chosen and mixed in equal parts to give 1µl of template for the new reaction. They were tested once with a reaction mix containing only *tet(A),* once with only *tet(B)* and once with the same amount of primer divided in equal parts between *tet(A)* and *tet(B).*

The DNA was amplified and the products were read as described in section 3.6.1.1. This reaction gave good results and was then used to test the TR*EC* isolates obtained from the study.

---

[1] Applied Biosystem, supplied by PE Biosystem SA (Pty) Ltd, PO Box 48453, South Africa
[2] Inqaba Biotechnical Industries (PTY)Ltd PO Box 14356 Hatfield South Africa

3.6.2 PCR applied to the current study

The method that was optimised and described in section 3.6.1 was then applied to the 23 TR*EC* of the current study.

3.6.2.1 DNA extraction

DNA was extracted from isolates using the boiling method adapted from that described by Boerlin *et al.* (2005) and Miller *et al.* (1997) during the pilot study.

A loopful of bacteria from a fresh overnight culture on a MacConkey agar plate was resuspended homogeneously in 0.6mL centrifuge tubes (Plastpro)[1] containing 200µL of 0.5% Tween 20 (Sigma Aldrich)[2] in sterile water. The tubes were boiled for 10min at 95℃ in a water bath (Labotec)[3]. They were then immediately transferred into a liquid nitrogen freezer (K series, Cryostorage system, Labotec)[3]. After 5 minutes, the tubes were transported on ice to the water bath where they were boiled again for a further 10 minutes. They were then centrifuged (Eppendorf centrifuge 5417C)[4] for 3 min. at 13000 rpm and the supernatant was transferred into new sterile tubes. The validity of the DNA extraction was done as described in section 3.6.1.1.

3.6.2.2 Amplification of *tet(A)* and *tet(B)*

A 1µL volume of the supernatant was used as a template for each PCR mixture. A multiplex PCR was run for *tet(A)* and *tet(B)* genes.

The PCR mix consisted of:

- 12.5µL of Platinum® Quantitative PCR Super-Mix-UDG (Invitrogen, The Scientific Group)[5] containing: Platinum® *Taq* polymerase, Mg^{++}, uracil DNA

[1] PlastPro, Ltd South Africa
[2] Sigma-Aldrich SA PTY LTD PO box 10434, Aston Manor 1630, South Africa
[3] Labotec group PO Box 6553 Halfway House 1685 South Africa
[4] Eppendorf, supplied by Merck, PO Box 1998 Halfway House, South Africa
[5] The Scientific Group, Box 4261, Randburg 2125,S.A.

glycosylase (UDG), proprietary stabilizers, and deoxyribonucleotide triphosphates with UDP instead of dTTP.

- 0.5µL forward and 0.5µL reverse primer of 32pmol solutions of *tet(A)* and *tet(B).* The primers used are indicated in Table 3.1. Primers were synthesized at Inquaba[1].
- 9.5µL of sterile water.

The DNA was amplified using a thermocycler PE2700/2600 (Applied Biosystems)[2] as published by Boerlin *et al.* (2005):

- 1 cycle of 4 min at 95°C;
- 35 cycles each consisting of 1 min at 94°C, followed by annealing at a 64°C and elongation at 72°C for 1 min
- 1 cycle of 7 min at 72°C.

A list of the primers, as described by Boerlin *et al.* (2005) is given in Table 3.1 below.

Table 3.1: List of *tet(A)* and (*B)* primers and their main characteristics, as published by (Boerlin *et al.,* 2005).

GENE	PRIMERS		ANNEALING Temp. (ºC)	FRAGMENT Size (base pairs)
tet(A)	*tetA*-L *tetA*-R	GGCGGTCTTCTTCATCATGC CGGCAGGCAGACCAAGTAGA	64	502
tet(B)	*tetB*-L *tetB*-R	CATTAATAGGCGCATCGCTG TGAAGGTCATCGATAGCAGG	64	930

3.6.2.3 PCR analysis

After amplification, PCR products were separated by electrophoresis as described in section 3.6.2.1 and then visualized under UV illumination.

The size of the PCR products were determined by comparison of the migration to a standard 100bp molecular marker (GeneRuler™ Fermentas, Inquaba)[1] and it

[1] Inqaba Biotechnical Industries (PTY)Ltd PO Box 14356 Hatfield South Africa
[2] Applied Biosystem, supplied by PE Biosystem SA (Pty) Ltd, PO Box 48453, South Africa

was determined whether isolates had one or more of the two *tet* genes. A gel documentary system (Kodak gel documentation system, Laboratory specialist services)[1] was used to keep a permanent record of the results. The validity of the PCR and products size was ascertained using negative and positive controls. The strains (labelled B 4691/03/poultry for *tet(A)* and B234/2004/equine for *tet(B)*) used for positive controls were obtained from known phenotypically resistant strains of animal origin and had been identified experimentally at an earlier stage (section 3.6.1).

3.7 Data analysis

Data were entered and analysed using Microsoft Excel® (2003)[2] software. Qualitative data was presented as pie charts and histograms. Quantitative data was analysed for significant co-relations. The odds ratio was used to determine the strength of association between TR*EC* in water and impala faeces samples. The significance of the odds ratio was then estimated using the χ^2 test. The accuracy of the replica plating results and the MIC technique were compared by calculating the kappa value. Maps were elaborated using ArcGIS[3] and Mapsource (Garmin)[4].

---

[1] Laboratory Specialist Service cc, South Africa, www.lss.co.za
[2] Microsof Corporation, USA
[3] ArcGIS: www.esri.com
[4] Garmin ®: www.garmin.com

CHAPTER 4. RESULTS

4.1 Introduction

In this chapter details are given about the retrospective data obtained on water quality of the rivers. The analyses of these data were of fundamental importance in dividing the rivers into the two groups: polluted and unpolluted. It was presumed that rivers polluted with human or animal faeces had a higher probability of containing *E. coli* and thus TR*EC*. The decision about the possibility of these rivers may contain TR*EC* was based on their upstream land-use and their physical-chemical characteristics. Thereafter, the division of the rivers into two groups (TR*EC* positive and TR*EC* negative) was confirmed by the cultures of the water samples collected.

Faecal samples were also collected and examined for tetracycline resistance at the same time. The results of bacteriological analysis and of the resistance tests performed on the water and impala faeces are also described in this Chapter. The OR between TR*EC* isolated from water samples and TR*EC* isolated from impala faecal samples was calculated and the level of concordance between the RPL and MIC methods were compared. In addition, the results of the screening of resistant *E. coli* isolates for the most common *tet* genes , *i.e. tet(A)* and *tet(B)*, is reported.

4.2 Determining pollution status of rivers using retrospective data

All the rivers sampled originated outside and to the West of the KNP, where there is a high population density, including towns and industrial areas (see section 3.2). The approximately calculation of the total inhabitants and total human density along the river, made using data in Appendix 1, is reported in Table 4.1.

Table 4.1: Human populations along the different rivers.

Area	Population	Population density (inhabitants/Km^2)
Sabie-Sand	700803	701
Crocodile	506546	549
Letaba	275627	116
Olifants	407762	713

Data on livestock population was only available for comparison at provincial level when the last census was done in 2001 (Table 4.2).

Table 4.2: Number of livestock in the provinces of the study area (StatsOnline, 2001).

Province	Cattle	Sheep	Goats	Pigs
Mpumalanga	1199917	1842378	82097	174527
Limpopo	1339298	853686	911407	191508

The main sewage plants inside the KNP that discharge waste into the studied rivers are shown in Table 4.3 (du Plessis B., personal communication 2007).

Table 4.3: Characteristic of the main STPs along the studied rivers in KNP (du Plessis B., personal communication 2007).

River	Location	Flow m^3/day	Anaerobic ponds			Septic tank
			Total ponds	Ponds Area (m^3)	Retention time (Days)	
Sabie	Kruger Gate	10	0	0	0	1
	Skukuza	338	7	15800	92	0
	Nkuhlu	10	0	0	0	1
	Lower Sabie	84	5	8750	30	0
Sand	Sand	23	4	1500	30	0
Crocodile	Malelane	15	2	1000	42	0
	Berg en Dal	106	5	1790	35	0
	Crocodile Bridge	56	0	0	0	1
Letaba	Shimuweni	8	0	0	0	1
	Letaba	117	5	5400	37	0
Olifants	Olifants	54	4	2400	35	0

Data on the general water quality that are recorded during periodic monitoring of rivers in the KNP, were considered for the classification of rivers as polluted or

unpolluted. These data were obtained from the DWAF through searching their data bases. The rivers are sampled for the RHP (WRC, 2001 a, b) at the sites showed in Figure 4.1 and the resulting water samples are sent to the Institute of Resource Quality Studies (RQS) in Pretoria for analysis.

Figure 4.1: Location of principal water collection site for RHP (blue), camps (brown) and waste water discharge points (red) (ArcGIS)[1].

At the RQS the following parameters were analysed: pH, nitrate (NO_3), ammonia (NH_4), phosphates (PO_4), sodium (Na), chloride (Cl), fluoride (F), conductivity (EC), calcium (Ca), magnesium (Mg), sulphate (SO_4) and total dissolved solids (TDS). Unfortunately data on bacteriological hazards were not available. Among these data particular importance was given to the substances that are general

[1] ArcGIS: www.esri.com

indicators of water quality as TDS, Ph and NO_3 (DWAF, 2001 b), see Table 4.4. below.

Table 4.4: Mean of water indices considered for the classification of the rivers.

River	Monitoring station	Mean value					
		Ph (6.5-7.5)	**TDS** (max 500mg/L)	**NO_3** (0.1-3 mg/L)	**Cl** (max 250 mg/L)	**F** (max 0.5 mg/L)	**SO_4** (max 250 mg/L)
Sabie	Phabene	7.71	78	0.19	6.4	0.13	5.8
	Powerline	7.21	75	0.14	8.2	0.11	4.6
	High water bridge	7.29	76	0.13	8.75	0.11	4.95
	Lower Sabie	7.75	98	0.08	9.3	0.15	6.6
Sand	Exeter	7.53	105	0.02	12.5	0.16	4.6
Crocodile	Malelane	8.06	229	0.54	15.6	0.21	18.1
	Riverside	8.06	252.58	0.54	17.6	0.21	23.4
	Crocodile bridge	8.04	260	0.47	20.8	0.27	17
	Ten Bosch	8.2	318	0.45	25.65	0.28	23.9
	Komatipoort	8.22	322.58	0.38	35.47	0.27	21.01
Olifants	Phalaborwa	7.9	308	0.09	33	0.29	21
	Phalaborwa Sewage work	7.21	534.49	8.39	86.4	0.39	67.13
	Mamba	8.14	472	0.27	51.7	0.61	101.75
	Balule	8.27	522.88	0.04	59.8	0.63	106.75
Letaba	Letaba ranch	7.81	231	0.17	39.7	0.23	12
	Black Heron	8.15	302.5	0.06	44	0.24	13.7

The mean for these parameters, collected more or less periodically from 1977 to 2006, were extrapolated from the datasets and compared. As can be seen from Table 4.4, these substances had high mean values mainly in the Crocodile, Olifants and Letaba rivers, but were very low in the Sabie and Sand rivers. For this reason the Sabie and the Sand Rivers were classified as possible TR*EC*neg rivers compared to the Letaba, Olifants and Crocodile, which were classified as possible TR*EC*pos. Although bacteriological data was not available, it was presumed that polluted rivers would be more likely to contain *E. coli* and TR*EC.* This assisted with the initial design of the case–control study, as impala could be classified on the basis of exposed or non-exposed based on whether the river was polluted or not polluted. The supposition was confirmed by the laboratory analysis for TR*EC* in

the water samples taken when faeces from the impala were collected (See Table 4.7).

4.3 Escherichia coli cultured from water samples

In the six rivers tested, 63.64% (n=21) of the 33 water samples were found to be contaminated by *E. coli*. In particular, 2 of the 3 samples collected from the Sand, 3 of the 9 samples from the Sabie, 7 of the 9 samples from the Crocodile, 5 of the 6 samples from the Olifants and 4 of the 6 samples from the Letaba were positive for *E. coli*. Table 4.5 summarizes these findings. There was no clear distinction between rivers classified as unpolluted and those classified as polluted, although the Sabie River (unpolluted) had the lowest number of positive samples and the Olifants (polluted) the highest. The enumeration of *E. coli* in each sample was not done.

Table 4.5: Prevalence of water samples positive for *E. coli* from each river.

River	Number of samples	Positive cultures
Sand	3	66.66% (n=2)
Sabie	9	33.33% (n=3)
Crocodile	9	77.77% (n=7)
Olifants	6	83.33% (n=5)
Letaba	6	66.66% (n=4)
TOTAL	33	63.64% (n=21)

4.4 Tetracycline resistant E. coli from water samples

Replica Plating of Lederberg (RPL) and MIC methods (as described in Chapter 3) were used to evaluate tetracycline resistance in *E. coli* cultured from the river samples. This method was used to screen for the presence of TR*EC* and the MIC

to quantify the actual susceptibility of the *E. coli* to tetracyclines in mg/L. The RPL screening was only precautionary, in case the bacterial population of the impala, under no selective pressure, consisted of *E. coli* with different tetracycline resistant patterns. Because the RPL is only a qualitative method, the resulting resistant and sensitive colonies were then subjected to the quantitative MIC method. For a double check on each sample, the MIC was conducted on both a resistant and sensitive colony, when present. However, in accordance with the aim of the research, only the result of one colony showing the highest level of resistance was taken into account. The results obtained from these two different methods (RPL and MIC) are shown below in more detail.

4.4.1 Replica plating results from water samples

It was found that 21 of the river water samples contained *E. coli*. These *E. coli* were tested using RPL, where bacteria were considered to be resistant to tetracycline at a breakpoint of ≥4 mg/L.

Table 4.6: Distribution, per river and total, of tetracycline resistant *E. coli* isolated from water samples tested using RPL.

River	Number of samples tested for RPL	Samples resistant to tetracycline (RPL 4 mg/L)
Sand (n=3)	2	50% (n=1)
Sabie (n=9)	3	0% (n=0)
Crocodile (n=9)	7	100% (n=7)
Olifants (n=6)	5	100% (n=5)
Letaba (n=6)	4	75% (n=3)
TOTAL (n=33)	21	76.19% (n=16)

It was found that 76.19% (n=16) of these *E. coli* positive samples (n=21), were resistant to tetracycline. Three samples were collected from each of the sites in each river where impala were drinking. The distribution of resistant samples differed among the rivers (see Table 4.6).

4.4.2 MIC results from water samples

The distribution of the MIC values for *E. coli* obtained from water samples was found to differ between the rivers. The percentage distribution for each dilution is shown in Table 4.7. Details on the dilutions and methodology used were reported in Chapter 3.

Table 4.7: Percentage distribution of tetracycline MICs for *E. coli* from water samples (n=21) for each river.

MIC(mg/L) / River	0.25	0.5	1	2	4	8	16	32
Sand (n=2)				50.00	50.00			
Sabie (n=3)		33.33		66.66				
Crocodile (n=7)		28.58			57.14	14.29		
Olifants (n=5)		20.00	20.00	40.00		20.00		
Letaba (n=4)					50.00	50.00		
TOTAL (n=21)		19.05	4.76	23.81	33.33	19.05		

Considering that the microbiological breakpoint for tetracycline resistance was ≥8mg/L, 19.05% (n=4) of the water samples containing *E. coli* (n=21) were found to be resistant. The distribution of tetracycline resistance among the rivers showed that no resistant *E. coli* were isolated from the Sand and Sabie Rivers, while *E. coli* in 1 sample from the Crocodile, 1 from the Olifants and 2 from the Letaba Rivers, were found to be resistant (Table 4.7).

4.4.3 MIC and river group formation

The MIC results made it possible to finally confirm the division of the rivers into two categories, on the basis of the presence or absence of TR*EC*. The Sand and Sabie Rivers were classified in the group TR*EC*neg, because none of the samples showed tetracycline resistance. The others rivers examined: Crocodile, Olifants

and Letaba were classified as TR*EC*pos, because one or more samples, from each river, were positive (Table 4.8).

Table 4.8: Grouping of rivers into TR*EC*neg and TR*EC*pos.

TR*EC*neg Rivers	TR*EC*pos rivers
Sand Sabie	Crocodile Olifants Letaba

4.4.4 Distribution of MIC values between the river groups

In accordance with the classification of rivers in TR*EC*neg and TR*EC*pos, the percentage of different MIC patterns from the water samples containing *E. coli* (n=21) are shown in Table 4.9.

Table 4.9: Percentage distribution of MIC values for *E. coli* between the groups TR*EC*neg and TR*EC*pos**.**

MIC (mg/L) / Group	0,25	0,5	1	2	4	8	16	32
TR*ECneg* (n=5)		20.00		60.00	20.00			
TR*ECpos*(n=16)		18.75	6.25	12.50	37.50	25.00		

4.5 <u>E. coli</u> cultured from impala faecal samples

The 209 faecal samples collected were distributed among the control and exposed groups as shown in Table 4.10. The control group is regarded as the group of impala drinking from a river belonging to the TR*EC*neg group while the exposed group is regarded as those drinking from a river belonging to TR*EC*pos group.

Table 4.10: Distribution of faecal samples between TR*EC*neg and TR*EC*pos rivers.

Group	River	Faecal samples/river	Faecal samples/TR*EC* group
Control (TR*ECneg*)	Sand	15	104
	Sabie	89	
Exposed (TR*ECpos*)	Crocodile	45	105
	Olifants	30	
	Letaba	30	

E. coli was not detected in all the faecal samples tested, although it was isolated and identified from 91.38% (n=191) of the samples (n=209). The lowest percentage of faecal samples positive for *E. coli* was found in the group of impala drinking from the Sabie River (Table 4.11).

Table 4.11: Distribution of faecal samples positive and negative for *E. coli* according to the river sampled.

River	Samples *E. coli* positive	Samples *E. coli* negative
Sand (n=15)	93.22% (n=14)	6.67% (n=1)
Sabie (n=89)	85.4% (n=76)	14.6% (n=13)
Crocodile (n=45)	93.33% (n=42)	6.67% (n=3)
Olifants (n=30)	96.66% (n=29)	3.34% (n=1)
Letaba (n=30)	100% (n=30)	0% (n=0)
TOTAL (n=209)	91.38% (n=191)	8.62% (n=18)

4.6 Tetracycline resistant E. coli isolated from impala faecal samples

Replica plating of Lederberg (RPL) and MIC (methods described in Chapter 3) were used to evaluate tetracycline resistance in the *E. coli* cultured from impala faecal samples, in the same way that they were used for water samples (see section 4.4).

4.6.1 Replica plating results from faecal samples

Of the 191 samples yielding *E. coli*, only 36.64% (n=70) were tetracycline resistant using the RPL method (breakpoint 4 µg/mL). *Escherichia coli* isolated from impala drinking from the Sand River (n=14) showed resistance in 42.86% (n=6) of the samples; from the Sabie (n=76) 13.26% (n=10); from the Crocodile (n=42) the

59.52% (n=25); from the Olifants (n=29) 55.17% (n=16); and from Letaba (n=30) 43.33% (n=13) (Table 4.12).

Table 4.12: Distribution, per river and total, of TR*EC* in faecal samples using RPL.

Rivers	Number of samples tested for RPL	Samples resistant to tetracycline (RPL 4 mg/L)
Sand (n=15)	14	42.86% (n=6)
Sabie (n=89)	76	13.26% (n=10)
Crocodile (n=45)	42	59.52% (n=25)
Olifants (n=30)	29	55.17% (n=16)
Letaba (n=30)	30	43.33% (n=13)
TOTAL (n=209)	191	36.64 (n=70)

4.6.2 Distribution of MIC values for E. coli in impala faecal samples

The distribution of MIC values among the groups of impala drinking from different rivers is summarized in Table 4.13. Considering that the breakpoint for tetracycline resistance was ≥8mg/L, 9.95% (n=19) of the faecal samples containing *E. coli* (n=191) were found to be resistant. To make the results clearer to understand, they have been shown both in tabular form and as a graphic (pie chart) in Table 4.13 and Figure 4.2, respectively.

Table 4.13: Percentage distribution of tetracycline MICs for *E. coli* isolated from impala faeces (n=191) obtained from different river zones.

River \ MIC (mg/L)	0.25	0.5	1	2	4	8	16	32
Sand				28.57	64.29	7.14		
Sabie		2.63	10.53	72.37	14.47			
Crocodile				23.81	57.14	9.52	7.14	2.38
Olifants	6,9		3.45	51.72	27.59	10.34		
Letaba			6.67	40	30	16.7	3.33	3.33

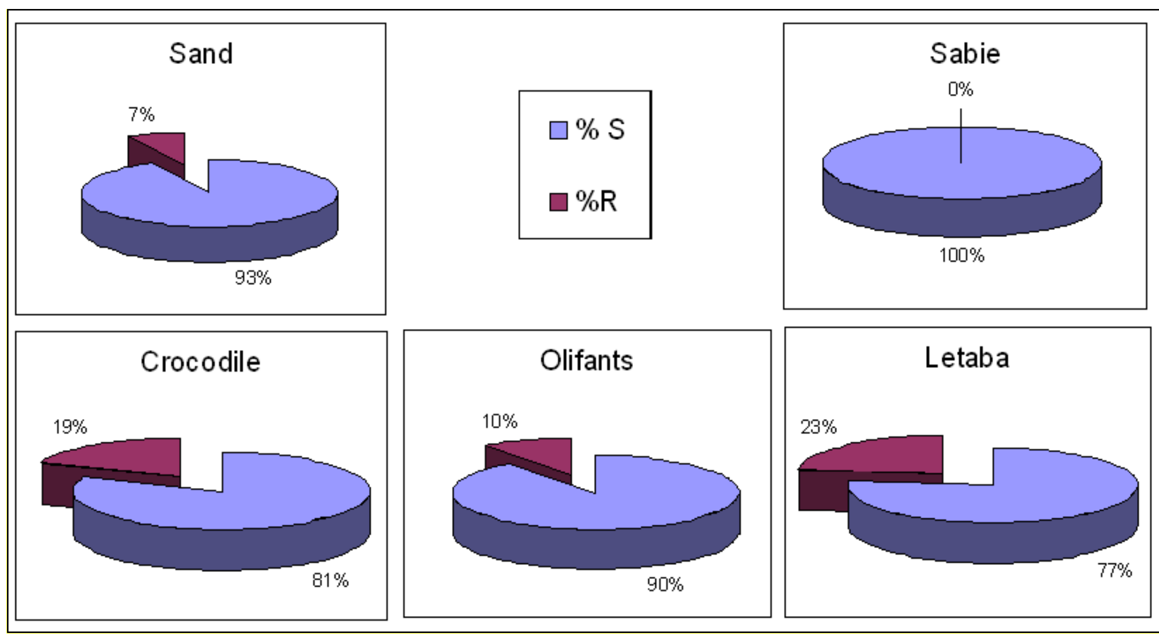

Figure 4.2: Pie chart showing percentage of tetracycline resistant and percentage of tetracycline susceptible *E. coli* in faecal samples per river according to the MIC method.

From the previous Table and graphs, it can easily be seen that the Crocodile River yielded the highest number of samples positive for TR*EC*. In fact, 42,1% (n=8) of the resistant samples (n=19) were isolated from this river. In order of magnitude, the others were Letaba River (36.68%), Olifants (15.72%) and Sand River (5.26%). No resistant *E. coli* were isolated from the Sabie River (Figure 4.3).

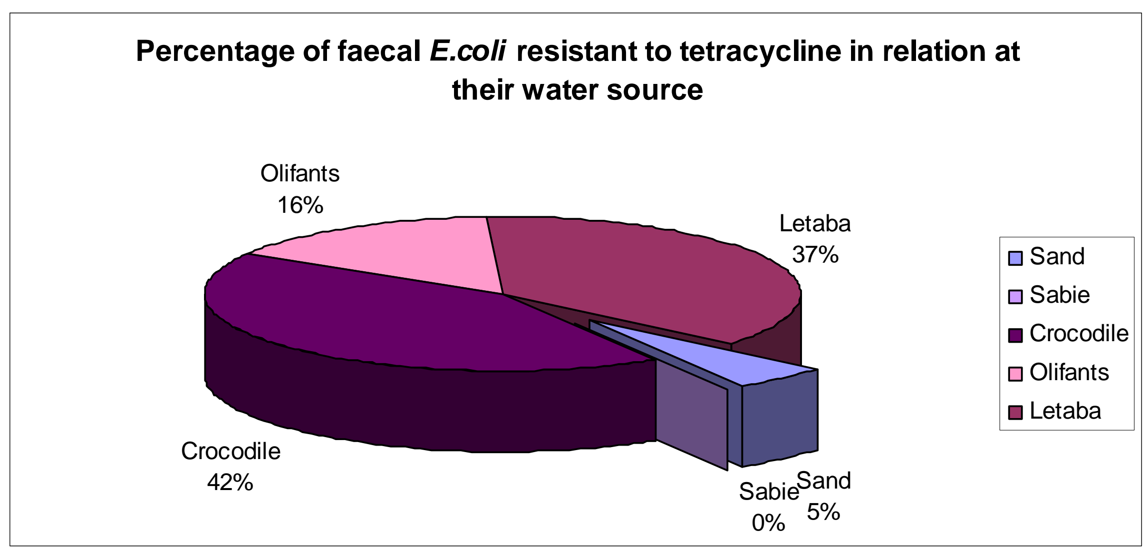

Figure 4.3: Pie chart showing proportion of faecal TREC among the different rivers.

The distribution of the resistance between the rivers showed that no resistant *E. coli* were isolated from the Sabie River, while 1 sample from the Sand, 8 from the Crocodile, 3 from the Olifants and 7 from the Letaba Rivers were found to be resistant (Figure 4.3).

4.6.3 Different distribution of MIC values in faecal samples between the river groups

According to the classification of impala faecal samples (n=191) into non exposed and exposed groups, the percentage of different MIC patterns exhibited from each group are shown in Figure 4.4 below.

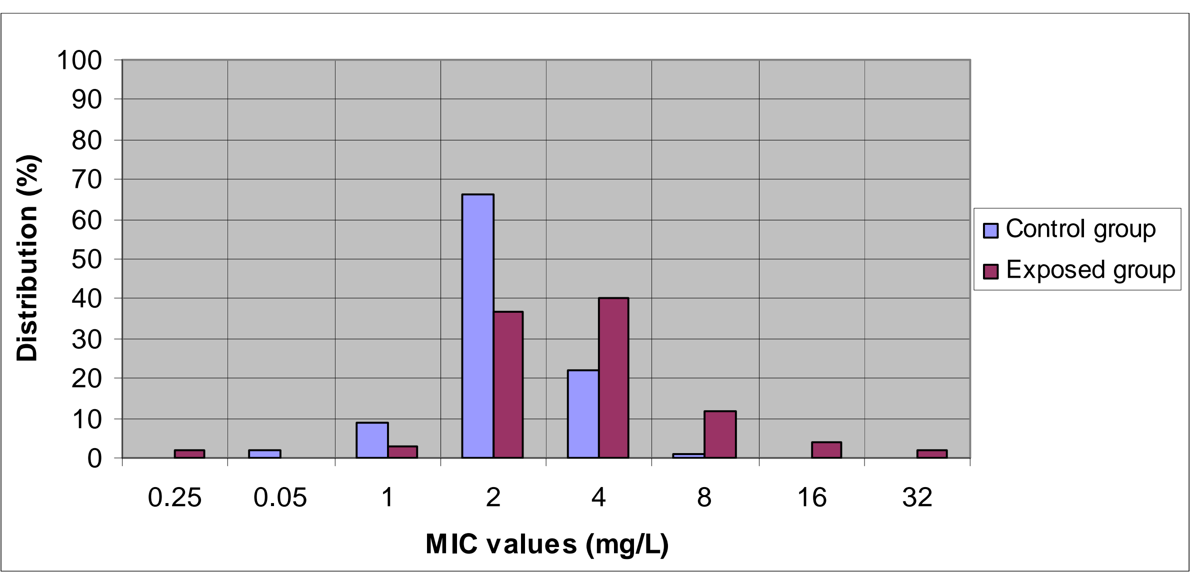

Figure 4.4: Histogram showing the distribution of MIC values in the control and exposed groups.

Considering that the breakpoint for tetracycline resistance is ≥8mg/L, 1.11% (n=1) of the *E. coli* sampled from faeces in the control group (n=90), were resistant to tetracycline in comparison with 17.82% (n=18) of faeces from the exposed group (n=101) (Figure 4.5).

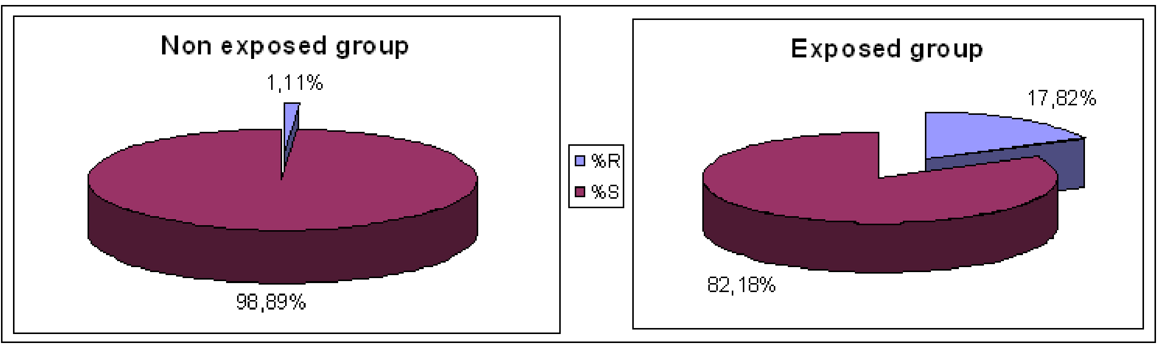

Figure 4.5: Pie chart showing percentage of tetracycline resistant and sensitive *E. coli* in faecal samples per group, using the MIC method.

The above results show clearly that TR*EC* were found mainly in the exposed group. Of the total faecal TR*EC* (n=19) 95% (n=18) were isolated from the group of impala drinking from TR*EC*pos rivers, while only 5% (n=1) of the faecal samples of impala drinking from TR*EC*neg rivers were positive for TR*EC.*

4.7 Resistance pattern related to time scale

All samples were collected at three different times (see Chapter 3). Each time an equal number of samples per point were collected (Table 4.14). Only in one collecting period in March was not possible to collect the desired amount of faeces for the Sabie River as the ambient temperatures were so high that the faeces dried out prior to collection.

Table 4.14: Distribution of total water and total faecal samples time related.

River	Number of water samples			Number of faecal samples		
	March 2007	May 2007	July 2007	March 2007	May 2007	July 2007
Sand	1	1	1	5	5	5
Sabie	3	3	3	29	30	30
Crocodile	3	3	3	15	15	15
Olifants	2	2	2	10	10	10
Letaba	2	2	2	10	10	10
TOTAL	11	11	11	69	70	70

The percentage of isolates with a particular MIC value is shown for each collection period in Table 4.15.

***Table 4.15**: Percentage distribution of MIC values for *E. coli* in water samples during the different collection periods.*

	MIC (mg/L) / Collection	0.25	0.5	1	2	4	8	16	32
Water	**March 2007(n=6)**		16.66		16.66		66.66		
	May 2007 (n=7)		28.52	14.2	14.2	42.8			
	July 2007 (n=8)		12.5		37.5	50			
Faeces	**March 2007 (n=57)**		1.75	5.26	52.63	28.97	8.77	1.75	1.75
	May 2007 (n=66)			10.6	37.88	34.85	10.61	4.55	1.51
	July 2007(n=68)	2.94	1.47	1.47	60.3	32.3	1.47		

Considering the breakpoint to be ≥8mg/L, all the TR*EC* in the water samples was isolated during the collection done in March. Also, the TR*EC*pos faecal samples were isolated mainly from samples collected in March and May (Table 4.16).

Table 4.16: Total distribution of TR*EC* for water and faecal samples in the different collection periods.

Collection date / TR*EC* group	March 2007	May 2007	July 2007
Water (n=4)	100% (n=4)	0% (n=0)	0% (n=0)
Faeces (n=19)	36.84% (n=7)	57.89% (n=11)	5.26% (n=1)

4.8 Measure of association of TREC between impala and their drinking water

The odds ratio (OR) was used to determine whether faeces of impala drinking from TR*EC*pos rivers were more likely to contain TR*EC* than faeces of impala drinking from TR*EC*neg rivers. According to Thrusfield (2005):

$$\textbf{Odds Ratio} = \frac{\text{odds that exposed animal will have the disease}}{\text{odds that nonexposed animal will have the disease}}$$

[equation 5]

Considering the odds (O) formula as:

$$O_{D+/E+} = \frac{\text{probability of disease if exposed}}{\text{probability of no disease if exposed}} = \frac{P(D+/E+)}{P(D-/E+)}$$

[equation 6]

$$O_{D-/E-} = \frac{\text{probability of disease if not exposed}}{\text{probability of no disease if not exposed}} = \frac{P(D+/E-)}{P(D-/E-)}$$

[equation 7]

Where:
D+ = impala with TR*EC*pos faeces
D- = impala with TR*EC*neg faeces
E+ = impala drinking from TR*EC*pos rivers
E- = impala drinking from TR*EC*neg rivers

Then the formula for OR in equation 5 becomes:

$$OR = \frac{P(D+/E+)}{P(D-/E+)} \Big/ \frac{P(D+/E-)}{P(D-/E-)}$$

[equation 8]

Substituting the values in Table 4.17, using a confidence interval (CI) of 95%, it was calculated that the impala drinking from TR*EC*pos rivers were 19.3 (CI: 2,63-141,69) times more likely to have TR*EC* in their faeces than these drinking from TR*EC*neg rivers.

Table 4.17: Values considered for the OR calculation:

	D+	D-	TOTAL
E+	18	83	101
E-	1	89	90
TOTAL	19	173	191

To evaluate whether there was a significant difference between the exposed and control impala, the chi-square (χ^2) statistical test was done. The χ^2 test is the sum of the squared difference between observed (*o*) and the expected (*e*) data (or the deviation), (*d*), divided by the expected data in all possible categories. The formula for calculating χ^2 is:

$$\chi^2 = (o-e)^2/e$$

[equation 9]

The expected value considered for the calculation of the Mantel-Haenszel χ^2, with a degree of freedom of 1 and a p-value of 0,0001 is summarized in Table 4.18.

Table 4.18: Values considered for the χ^2 calculation:

Expected values			
	D+	**D-**	**TOTAL**
E+	10.05	90.95	101
E-	8.95	81.05	90
TOTAL	19	173	191

The χ^2 test value result was 14.76 and so the association between the exposure and the outcome of the resistance in faeces was found to be significant (P<0,0001).

4.9 Correspondence between replica plating and MIC methods

The results of RPL and MICs tests were compared, as shown in Table 4.17 below. As the breakpoint used in the RPL was lower than the breakpoint of the MIC, the correspondence between the two methods performed was calculated at a breakpoint of 4 mg/L.

***Table 4.19**:* Comparison of RPL and MIC test results
Key: R'=resistant ≥ 4 mg/L (for both RPL and MIC)
S'=sensitive ≥ 4 mg/L (for both RPL and MIC)

Samples	Number of samples
R' RLP/S' MIC	17
S' RLP/R' MIC	29
R' RLP/R' MIC	51
S' RLP/S' MIC	93

The agreement between the two tests was calculated using the kappa value (Thrusfield, 2005):

$$\text{kappa} = \frac{\text{OP-EP}}{\text{1-EP}}$$

[equation 10]

Where:

OP (observed proportional agreement) = a+d/N

EP (expected proportional agreement) = (a+b/N x a+c/N) + (c+d/N x b+d/N)

Table 4.20: Data used for calculate Kappa.

	RLP R'	RLP S'	TOTAL
MIC R	(a) 51	(b) 29	80
MIC S	(c) 18	(d) 93	111
TOTAL	69	122	(N) 191

The values used for calculating kappa are in Table 4.20. As result the kappa value calculated by the equation 10 was 0.48. This means that the agreement between the RPL test and the MIC test was 48%.

4.10 Genetic patterns of the isolates

All the positive samples for tetracycline resistance at the MIC method (n=23) were later screened by biotechnology methods for the presence of the most common genetic resistant determinants (see section 2.9.5): *tet(A)* and *tet(B).* The samples were first subject to the extraction of DNA and then the genetic products were processed to amplify the specific genome regions of *tet(A)* and *tet(B).*

4.10.1 DNA extraction and quantification

The efficacy of the DNA extraction was evaluated by the electrophoresis of all the templates obtained. The presence of nucleic acid products was read by the UV transilluminator. The quantification of the amount of DNA present in the samples was evaluated by a randomly reading of the products (Table 4.21).

The reading of the samples at 260 nm revealed the presence of quantity of nucleic acid from 0.147 to 2.565. The reading of the samples at 280 nm revealed the presence of protein into the extract products too. DNA quality was estimated by measuring the 260/280 UV absorbance ratio which varied between 0.978 and 1.794. These results showed that the templates were not completely pure DNA, as

the ratios were less than 1.8. Nevertheless the amount of nucleic acid and purity was sufficient to obtain clear results in a multiplex PCR, as the same amounts of DNA were obtained during a previous trial where good positive results were reached and as the same amount of DNA was detected in the sample used as positive control.

Table 4.21: Results of spectrophotometer reading for five random samples.

Sample	260 nm	280nm	260/280	280/260	Nucleic acid µg/mL
1	2.045	1.874	1.091	0.917	61.143
2	1.261	1.217	1.036	0.965	35.483
3	0.147	0.082	1.794	0.557	6.3139
4	2.565	2.565	1	1	69.004
5	0.418	0.427	0.978	1.022	10.910

4.10.2 PCR results and electrophoresis

All the samples were tested together with a positive and negative control. Not one of the samples tested resulted positive for the presence of *tet(A)* and *tet(B)* genes (Figure 4.6).

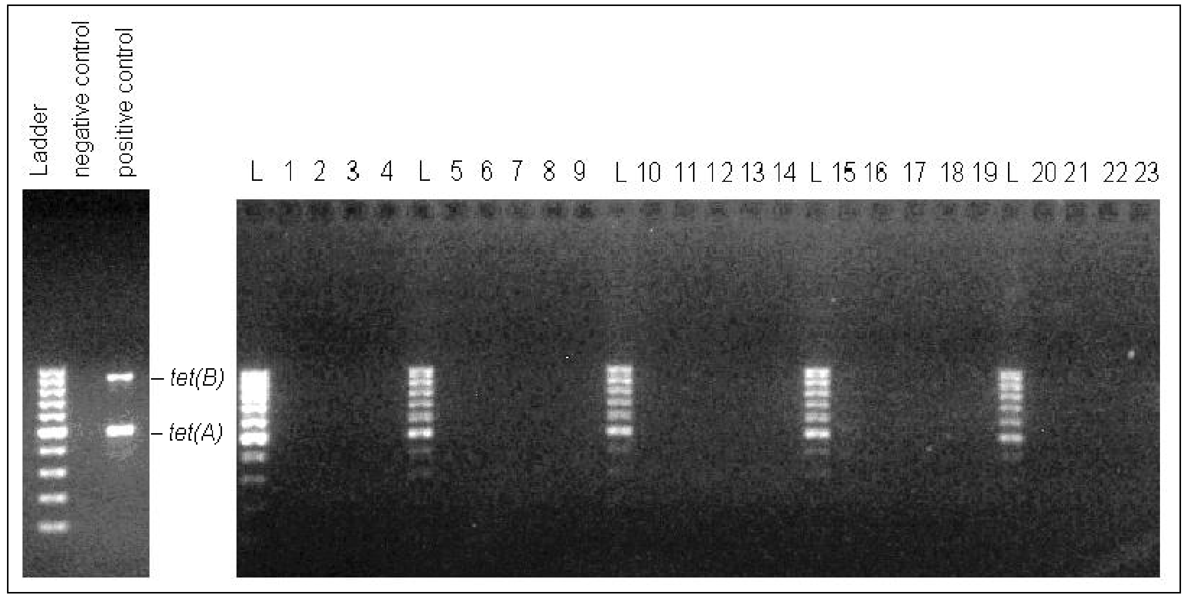

Figure 4.6:PCR results of the 23 TR*EC* samples examined for *tet(A)* and *tet(B)* and their controls.

CHAPTER 5. DISCUSSION

5.1 Introduction

The increased AMR in bacteria, especially pathogens, has received worldwide attention. The increasing failure of therapeutic intervention pushed the WHO to publish the "*Global strategy for containment of antimicrobial resistance*" in 2001 (Okeke *et al.,* 2005). This strategy was intended to minimize the increase of resistant bacterial strains, while maintaining the effectiveness of antibiotics already in use. Although new antibiotics are being developed, *e.g.* glycylglycines (Chopra, 2001), the level of AMR is not decreasing and it seems that development of resistance keeps pace with the development of new antibiotics (Grugel, 2006). Risk management for AMR is heavily biased towards banning the misuse of antibiotics (Grugel, 2006). It is also generally accepted that the high and often inappropriate use of antimicrobials and disinfectants, in both humans and animals, is the main driver of resistance (WHO, 2001). Yet the role the environment plays in the maintenance, dissemination and possible amplification of AMR is not fully understood (Kümmerer, 2004). Increasingly, multi-resistant bacteria are being detected in environmental compartments with a high bacterial density, such as biofilms present in water and soil (Berry *et al.,* 2006; Kümmerer, 2004). The source of these bacteria, as well as the transfer of resistance between different environmental compartments, requires further investigation and should be included in risk analyses. As well as monitoring AMR in environments where there is a high usage of antibiotics, healthy and preferably antimicrobial-naïve populations should also be monitored, to have a better overview of the spread of AMR (Grugel, 2006). Hence, the aim of the current research study was to increase information and to add knowledge on the dissemination of AMR. The results showed that impala drinking from TR*EC* polluted rivers were 19.3 (CI: 2,63-141,69) times more likely to have TR*EC* than unexposed animals. This result increases the awareness of water as important agent for dissemination of AMR.

5.2 Methods used for the detection of tetracycline resistance

The methods used to investigate TR*EC* isolated from water and faecal samples were: RPL, MIC and PCR. The kappa value was calculated to determine the level of agreement between the RPL and MIC methods. The kappa value showed an agreement of only 48% between the two tests when a breakpoint concentration of 4 mg/L was used for both tests (see section 4.10).

However, it has been reported in previous studies that, the RPL may give misleading results because the replicator (velvet-paper) does not produce a uniform pressure over the entire surface of the MacConkey plates, so that not all the colonies are inoculated onto the tetracycline containing plate (Lindstrom, 1977). It is also possible to misjudge sensitive colonies because sometimes the replicator leaves much of the original colony on the new replicated plate, and after incubation, distinguishing them from new-grown colonies becomes difficult. Furthermore it requires a high level of dilutions of the sample, so that single colonies are obtained, lowering the sensitivity of the test. A better screening test would either be to directly plate the sample onto tetracycline containing agar, or for an even higher sensitivity, culture the sample in a tetracycline-containing broth. These improvements would, however, not be able to detect which segment of a bacteria population is resistant to tetracycline, which can be done using the RPL.

In contrast, the standardized MIC method (CLSI, 2002), if done properly, is highly reproducible and is used in all major antimicrobial resistance monitoring programmes throughout the world (Nel *et al.*, 2004; SWARM, 2006). More controls were also in place when performing this test, as each test was performed in duplicate and the purity and concentration of the organisms isolated were tested each time the test was conducted. The accuracy of the test was checked by testing of the control strain *E. coli* ATCC 25922 (reported to have an MIC of 2mg/L) (Andrews, 2001; CLSI, 2002). The test was repeated when the MIC quality control strain MIC disagreed by more than one dilution of its expected results, when the MIC results showed a disagreement of more than one dilution, or when the two results were around the cut-off value.

In this study a cut-off of 8 mg/L was selected. According to the CLSI the MIC breakpoints of *E. coli* for tetracycline are ≤4mg/L for sensitive, 8mg/L for intermediate resistant and ≥16mg/L for highly resistant bacteria (CLSI, 2002). In environmental surveillance programmes, where the microbiological cut-off is used, a value of ≥8mg/L can be considered as resistant. This because the bacteria isolated does not come from an environment with a high selective pressure and the results are not important for the determination of therapeutic success, as they are in the case of clinical environments. Thus the intermediate and highly resistant strains can be considered together as resistant.

Although the RPL is not precise, it was used because little is known about the dynamics in bacterial populations. If, as it was assumed, in an enteric environment a population of bacteria does not come from the same clone and it is not subject of high selective pressure, it could be that the population maintains several different patterns of AMR. A method such as RPL, that could divide the initial population in sensitive and resistant, so as to better analyze the resistant population, was thus necessary. A second method to assure and quantify the resistance, such as MIC broth method, must be conducted thereafter. A lower breakpoint was chosen for the RPL method, considering its probable misleading results. If the same breakpoint as the MIC had been used mutually, it could be possible to lose resistant bacteria, due to the subjective definition of colony growth. Instead, the 4 mg/L breakpoint chosen for the test enabled sensitive bacteria to be discharged, without negating the possibility of using the MIC on 8mg/L border colonies.

The RPL and MIC methods together gave good results, but the amount of time consumed was high. A study recently published seems to have experimented with a new method that can give the same, if not more accurate, results in less time. In this method a certain dilution of water is spread onto a modified Difco MI agar (see section 2.8) impregnated with selected antibiotics, at concentrations that define resistance (breakpoint concentration). Thus, the identification of *E. coli* and its resistance can be recognised in a single step (Watkinson *et al.,* 2007 a). When more specific quantification of the samples is required, another plate with sequential concentration of antibiotics could be spread with the same sample dilution. Until now this method has been used for detecting resistant *E. coli* in

water environments, but with appropriate dilutions, could also be applied to faecal samples. It is suggested that this may be a better phenotypic method to choose for further studies of diffusion of AMR in water and animals.

Biotechnology, with its ability to look at genotypic characteristics, can always be considered the best method to investigate AMR patterns in a population. The use of genotyping method such as the PCR can increase the specificity of association between a particular type of resistance in the environment. This method can detect all the different known antibacterial resistant genes present in a population. Thus by the detection of a known gene or several genes present in a particular system *i.e. E. coli* on a poultry farm, it is possible to trace the movement of these genes in different environments and populations. These methods are also not dependent on the presence of a particular bacterium in a population, but can identify those genes in different bacterial populations or even in free DNA. However, these methods are often expensive, not always easy to interpret and lack of sensitivity. The most sensitive of the technologies *i.e.* the Realtime PCR and microarrays can only detect resistant genes in a minimum of 10^2 bacteria/mL. The simple PCR methods are less sensitive as they will only detect resistance genes in 10^3 to 10^4 bacteria/mL (McPherson & Møller, 2000). In contrast a pre-culturing method is far more sensitive and does not suffer from problems associated with nucleases and other DNA inhibitors being present in the sample. Thus it was decided to rather detect tetracycline resistance in an indicator bacterium such as *E. coli* and then to detect resistance genes in them.

Although, it would have been interesting to screen for all the resistance genes known to code for tetracycline resistance in *E. coli*, financial constraints precluded this. So the two most common genes found in *E. coli*, *i.e. tet(A)* and *tet(B),* were investigated. They were not found, which meant that it was obviously not possible to determine a genetic link between the AMR found in water and impala faeces in the current study, although the phenotypic link was established. However, it opens up the door for future studies where a broader approach to the different resistant genes could be used to establish the genetic epidemiology of AMR in the environment. Thus, future studies could utilise biotechnology not only to identify the genes responsible for TR*EC* but also to determine their source. This could be

done by genotyping, (*e.g.* making use of ribotyping) or by the serotyping of smooth strains. The best method to delineate the faecal pollution source would most probably be by the ribotyping of *E. coli* isolates, as RNA profiles are less susceptible to localized selection pressures than most of the other tests. In particular a marker localized to the glycine decarboxylase gene (*gcvP*) of *E. coli*, has shown some promise for use as a molecular marker for the identification of *E. coli* of human origin (Turner *et al*., 1997). It is, however, expensive and requires sophisticated laboratory facilities and highly trained laboratory personnel and was thus beyond the scope of the current study

5.3 Analysis of retrospective data

The identification of TR*EC* polluted rivers was not easy, because monitoring for AMR in water has not been conducted in South Africa and few studies have been conducted elsewhere (Reinthaler *et al*., 2003; Sayah *et al*., 2005; Watkinson *et al*., 2007 a; Whitlock *et al*., 2002). This was the reason why it was decided to choose rivers within the KNP, in an area highly populated with impala, where the rivers were periodically monitored. No data was available on *E. coli* or the coliform counts in the rivers studied. These would have been very useful to evaluate faecal pollution and thus the presence of AMR in the rivers. Furthermore this would have helped in distinguishing between human and animal sources of pollution. In fact, when the faecal coliforms/enterococci (FC/E) ratio is less than 2, it primarily indicates animal rather than human sources of faecal pollution (Young & Thackston, 1999). Once again, this is a possible direction for further research.

To divide the rivers into the two groups required for calculating the OR (possibly TR*EC* polluted or not) retrospective data on human and livestock density, land use upstream and characteristics of STPs inside the KNP as well as available data on water quality of the rivers, were taken into consideration (Tables 4.1, 4.2, 4.3 and 4.4 in Chapter 4; Table A.1 and Figure A1 in Appendix 1).

The livestock population density data was not useful because there were no specific figures available on the distribution of these populations along the rivers. They were only helpful to understand that livestock-farming activities were present

in the general vicinity of each river. The human population distribution data were also not that helpful as the census districts did not correspond exactly with the communities living and discharging waste along the river banks. In fact, the highly populated census groups (totalling of 701 inhabitants/km^2) along the Sabie and Sand catchment areas could not be correlated to water quality in these rivers and it probably meant that the densely populated areas were not along the river banks. Another reason could be that in the Sabie and Sand catchments, people leave mainly in very small villages and it is probable that larger urbanised areas within a rural setting would be a greater risk for pollution of the rivers (see Figure A.1). Moreover AMR could be related to the misuse of antibiotics by wealthier urban communities as small traditional rural villages in South Africa are known to depend more on herbal remedies (McCrindle *et al.,* 1994). Although should be considered that even in low-income communities, tetracycline resistance is likely because tetracycline is a cheap and broad-spectrum antibiotic (see section 2.9.2). AMR could also be found in river water due to larger communities in rural areas that may be informal settlements and thus not have the necessary sanitation infrastructure to ensure effective disposal of human wastes. If the lack of STPs may increase, in some cases, the possibilities to find AMR bacteria due to inappropriate waste disposal, on the other hand can prevent human faeces from villages that are not located on the river banks to reach and pollute the river water.

Bigger urban areas, towns or cities with a higher number of people using antibiotics, the presence of hospitals and STP's would have a higher likelihood of sewage (and consequently AMR) polluting the rivers. Along the Sabie River, the urban areas are small towns like Skukuza, Hazyview, Graskop, Sabie and Bosbokrand; while along the Sand River only the small town of Thulamanaxi can be found (see Figure 3.4). The area around the Letaba River, with a human density of only 116 inhabitants/km^2, is mainly rural. However, only 40 Km upstream of the KNP, the sewage from the town of Giyani, with a population of 21926 inhabitants (Brinkhoff, 2007), where there is also a hospital, can be considered as a likely source of pollution for the river. The census districts around the Crocodile River catchments record a human density of 549 inhabitants/km^2. However, the large city of Nelspruit, which is located approximately 20Km from the KNP border, has a population of 94714 inhabitants (Brinkhoff, 2007) and could be

considered an important contributor to the pollution of the river. On the Olifants River, there was a more definite link between the human population density (717 inhabitants/km^2) and pollution. This river was later discovered to be one that was most highly contaminated.

In contrast to human density data, the land use upstream of the KNP could possibly be considered a better indicator of the presence of tetracycline resistance in the river. In particular, land use for livestock farming, subtropical fruits, vegetables and summer crops due to the possible use of tetracyclines in these activities (see section 2.9.2). The overall condition of the Sabie and Sand River ecosystems entering the KNP are probably improved by the conservation measures implemented in the neighbouring private game reserves (WRC, 2001 b). In contrast it is possible that the Crocodile, Letaba and Olifants are polluted with TR*EC* due to their upstream land-use, as described below. The land use of the last part of the Crocodile upstream of the KNP is intensive agricultural, ranging from fruit (predominantly citrus) orchards to vegetable and tobacco cultivation, industrial and urban land uses (see section 3.2.2). Apart from intensive agricultural use, urban land-use includes domestic and hospital runoff from Nelspruit, which could have a major impact on the TR*EC* presence in the Crocodile River. On the Letaba, the economy is based on forestry, tea cultivations, subtropical fruits, summer crops, vegetable and livestock farming and this intensive land use for agriculture could also be considered as a possible TR*EC* source for the river (see section 3.2.3). The ecologically insensitive releases of water and sediment from storage dams upstream of the KNP on the Olifants River, can probably be considered the major cause of environmental degradation downstream, which is particularly relevant in the middle and lower parts of the catchments (WRC, 2001 b). The activities in the Phalaborwa area, such as farming, are considered to cause severe damage to the ecosystem of the river and can be included with the above-mentioned as being responsible for the presence of AMR in the river.

The STPs from the main camps inside KNP were considered, to see whether a division among the rivers could be done based on their distribution. As it can be seen from the Table 4.3, the daily flow amount is low (<400 m^3/day) in all of the camps. Moreover all the main camps discharge waste water into the rivers, only

after submitting it to a purification system, either a septic tank or a series of anaerobic ponds. The use of either of these methods as well as the number of ponds, their area and the retention time, is based on the characteristics of the daily flow of the systems. Based on the high level of control and the relatively low volume of treated sewage or waste, it became obvious that the distinction between polluted and unpolluted rivers could not be based on the number of STPs inside the KNP.

The data on the physical-chemical characteristic of the rivers obtained from the RHP were the most useful information to determine the division of the rivers onto two groups for OR. Data about lower water quality of the rivers was presumed to indicate contamination by different pollutants, including human, animal and agricultural waste and thus to have a higher risk of AMR. In particular, pH, TDS, NO_3, Cl, F and SO_4 parameters were considered. It can be noted from Table 4.4 that, although almost all the values were within the normal range for all the rivers, a clear difference can be noted from the mean value of sampling points on the Sabie-Sand catchments and the other three rivers (Olifants, Letaba and Crocodile). On the Sabie-Sand none of the parameters had ever been outside of the normal range but that could not be said for the other rivers. Moreover, the parameters on the Sabie-Sand were consistently below those of the other rivers. In this study, the most important parameters taken into consideration were TDS and NO_3, as they are linked with organic sources and sewage runoff. In particular, NO_3 is considered evidence of pollution from any kind of sewage source. Total dissolved solids, that measures the aggregate of inorganic substances, indicates the presence of organic sources, urban runoff, agriculture runoff (like fertilizer and pesticides) as well as inorganic materials. A wide difference between the mean values of TDS, for the two groups can be seen. In the polluted rivers the mean TDS ranged from 231 to 534 mg/L while in the Sabie and Sand Rivers, the mean ranged from 75 to 105 mg/L. This parameter was therefore considered to be a good indicator of the status of the rivers.

Although the pH, Cl, F were higher for the Crocodile, Letaba and Olifants Rivers, they were still below normal range, thus indicating that inorganic non-metallic substances were not a problem in any of the rivers.

5.4 Study design considerations

It was critical in the design of the study to minimize the source of TR*EC* to that of water only. This was done by selecting a population of animals that would have little if any contact with tetracycline treated animals and their excreta. The other aim was to ensure that there was little contact with humans, another possible source of TR*EC*. For both these reasons the KNP was selected, as the impala population have no contact with domestic stock, where tetracycline treatment is commonplace and the other game, with which they have contact, have never been treated with tetracyclines.

A slight risk is found possible from migratory birds that may have come into contact with livestock or human faecal pollution, using the river water for drinking and bathing. This risk is, however, considered to be negligible. There was always a possibility that wild animals in the KNP could come into direct contact with human excreta. However, the risk is minimal, as the public are never allowed to walk unsupervised, are not even allowed to leave their vehicles outside designated areas and the staff are encouraged to practice good hygiene such as faecal burial.

The other risk factor is the presence of natural resistance of *E. coli* to tetracycline, which will always be present at a low level in a bacterial population, even if the bacterium has never been exposed to antimicrobials. Many microbes produce antibiotics (bacteriocins) which gives them a competitive advantage, that is counteracted by the development of bacterial resistance. However, the level of bacteriocins and consequent AMR is low in natural compartments (Kümmerer, 2004). Even considering a small amount of natural resistance, this would provide a baseline AMR value but not negatively affect the validity of this study.

Animals of the same species were selected for the study, to ensure that natural variation in the different animal species would be minimized. Ingestion of resistant bacteria from soil and plants is another way of contact with resistant bacteria. However, other than natural resistance or animal faeces, the primary way that a AMR bacteria would be acquired from another area would be via water or soil. The presence of bacteria producing tetracycline in soil is also known but the

concentrations have always been recorded as very low, or under detection limits, when synthetic antibiotics are not used (Kümmerer, 2004). Furthermore most *E. coli* originates from faeces or sewage and only survives well under moist conditions (Whitman *et al.,* 2006). The dry conditions of most of the soil in the KNP would not be conducive to the survival of this bacterium.

Davison (1999), postulated the presence of tetracycline resistance in water, both in bacterial flora and as naked DNA. By selecting only *E. coli* for testing, this could have been missed during the current study. However, this would require extensive laboratory testing and it is the belief of Kümmerer (2004), that tetracycline resistance would be more common in commensal bacteria (including *E. coli*) from animals treated with antibiotics. It is thus felt that *E. coli* was a good indicator of the status of tetracycline resistance, both in animals and in water.

5.5 Detection of TREC in water samples

The results show that *E. coli* was only detected in 63.64% of the water samples (Table 4.3). The explanation may lie in the sensitivity and specificity of the test used and the concentration of *E. coli* in the rivers. A previous study reports that after sewage treatment, the presence of *E. coli* in water is around 10^2 CFU /mL (Reinthaler *et al*., 2003). Moreover it should be remembered that the collection sites were located in a conservancy area where the internal sources of pollution are maintained at a minimum. So it is possible that the total amount of coliforms in river water could be lower than that postulated in previous environmental research. It is never possible to detect all the *E. coli* found in water, sampling should, however, aim to collect a representative sample that would for practical purposes detect significant numbers of *E. coli*. The use of a 500mL sample and the pre-enrichment of filter sediment of the water sample, would have met this goal and would have detected *E. coli* in an environment with a level of 10^2 CFU/mL, reported by Reinthaler *et al*. (2003), to be the average level of *E. coli* in surface waters. The belief that 500mL would be sufficient to detect *E. coli* from a level of 10^2CFU/mL arises from the fact that other microbiological methods for enumerating *E. coli,* such as the Colilert^{TM}, are able to detect even 1 CFU/mL from 100mL of sampled water without even pre-filtering the water (EPA, 2001).

As it is known that there can be variable sewage contamination of water due to different seasonal land and human activities (see Chapter 2.5.1), water should always be collected at different times of year to attempt to detect this. For this case study it was done at 3 month intervals: in March, May and July 2007. The fact that less than 100% of the samples contained *E. coli* does not challenge the final results. The assumption that only a single water sample that was TR*EC* positive could indicate the pollution status of the river, was taken into consideration from the start.

5.6 TREC and river pollution

The initial classification of rivers into polluted and unpolluted, based on their upstream land use, was confirmed by the MIC result (Table 4.9). Where 0% of TR*EC* were detected in the *E. coli* from water samples collected in the Sabie and Sand Rivers, while 25% TR*EC* were detected in the *E. coli* isolated from water samples collected from the Crocodile, Olifants and Letaba rivers. These data supported the original hypothesis that bacteria from contaminated water would contain more AMR than those from less contaminated water. The maximum amount of TR*EC* was detected from the Letaba river, where 50% of the *E. coli* isolated were tetracycline resistant, followed by 20% from the Olifants and 14.29% from the Crocodile rivers respectively (Table 4.7). Thus, not only do these rivers pose a threat as a source of potential environmental pollutants, but may also provide a means of transmission of AMR.

5.7 Detection of TREC from faecal samples

It was found that 91.38% of faecal samples cultivated contained *E. coli*. This was not unexpected as *E. coli* is a faecal organism. The percentage distribution of faecal samples in which it was not possible to isolate *E. coli,* differed among the groups, with a peak of 14.6% for the faecal samples collected from impala drinking from the Sabie river (see Table 4.11). The samples where *E. coli* was not detected, were mainly those collected during the heat of the day, as happened

often for the Sabie river, and although attempts were made to ensure that they were fresh, it is possible that the extreme heat (reaching up to 40℃) and UV radiation in that environment could have destroyed the *E. coli* in the faeces.

The different distribution of TR*EC* among the groups is displayed in Figure 4.2. Of the TR*EC*, 42.1%, 36.68% and 15.72% of the total resistant samples, were collected respectively from impala in areas close to the Crocodile, the Letaba and Olifants Rivers (Figure 4.3). Only 5% of the TR*EC* samples originated from the control group of impala, which were drinking from the Sand River. Furthermore, the higher MIC values originated from the exposed group (Figure 4.4). In the control group none of the MIC values of tetracycline resistance exceeded 8mg/L, while in the exposed group 11.88%, 3.96% and 1.98% of the samples had MIC value of 8, 16 and 32mg/L respectively. There were thus clear differences between the two groups. This was further supported by the fact that the OR was calculated as 19.3. This statistical result indicates that there was a significant difference between the case and control groups in the study.

5.8 The effect of season

Although fewer *E. coli* were isolated in the water samples than in the faeces, it was interesting that the isolation of TR*EC* was possible only in late summer, despite the fact that rivers were carrying the greatest amount of water. In fact, in the water samples, all the TR*EC* that were detected were collected in March. As the river was running at its fastest in March, this supported the suggestion that the TR*EC* was not due to multiplication of *E coli* in the river water, but rather from transport from contaminated sources. Previous studies where a regrowth of bacteria were reported were from lake water, in which the physical conditions of the water were more favourable to bacteria growth than rivers. In rivers, a decrease in bacteria count has been recorded by a number of authors (Avery *et al.,* 2008; de Wet *et al.,* 1995; Dutka & Kwan, 1990; Whitman *et al*., 2006).

The presence of TR*EC* in water in March can be explained by the possible higher usage of tetracycline during the summer period. Higher antibiotic usage would increase the possibility of drug residues and AMR bacteria being discharged in

excreta and thus into the water. It is known that most diseases of humans and animals requiring tetracycline treatment such as protozoal and rickettsial infections are vector-borne. These infections tend to be seasonal with a peak in disease incidence in late summer. The KNP is a malaria area and it is recommended that tourists take prophylactic drugs such as doxycycline during the high risk periods from October to April. It is also during this period that domestic ruminants are more commonly treated with tetracyclines for diseases such as heartwater and anaplasmosis. In the summer months, enteric disease tends to increase in intensively farmed livestock, necessitating the use of antimicrobials, one of which would be oral tetracyclines. It must be noted, however, that the largest users of tetracyclines in South Africa, the poultry industry, would use these drugs throughout the year. Unfortunately, due to industrial protection there are no figures on tetracycline use per species, available in South Africa.

In the faecal samples, 36.84% and 57.89% of TR*EC* were detected respectively in March and May, while only 5.26% of TR*EC* were detected in July. The explanation for this trend is not clear and warrants further investigation. The fact that the resistance figures in animal faeces are different from those in water does not exclude the possibility of their correlation with the intake of AMR by drinking water as there is a residual effect. Moreover this is confirmed by the fact that the peak of resistance in faeces is subsequent to the one in water. The most reasonable option that can elucidate the different trend in resistance between water and faecal samples could be explained by a time difference between the transmission of resistance and its amplification within the digestive tract. Very little is known about the maintenance of AMR in a naïve environment so this finding is quite interesting. Theoretically, AMR should not be maintained or amplified where there is no selective pressure, because the acquisition of resistance is associated with a decrease in fitness of the bacterial cell, at least in the short term. However, it has been previously observed that bacteria have the ability to adapt to this deficit and recover in serial passage *in vitro* (Gillespie, 2001). The trend observed in this study led to the belief that resistance could possibly, for some unknown reason, slowly amplify even in an environment with low selective pressure. The long time required for amplification maybe explained by the fact that in the enteric tract of these wild, untreated impala, there was no high selective pressure that could

speed up the selection and amplification of AMR strains. However, it can be also postulated that as water can carry tetracycline resistance, it could also carry some residues of the drug itself. This could present an explanation for the amplification of AMR strains. The presence or absence of such residues, or their level in river water, is unknown and this warrants further research.

5.9 Genetic pattern of the population

The PCR analysis of the resistant samples did not show the presence of either *tet(A)* or *tet(B)* genes, although these two genes for tetracycline resistance were chosen because of their global distribution (see section 2.9.5). Although cost constraints prevented the examination of samples for any of the less common tetracycline resistance genes, it should be noted that resistant bacteria in different geographic locations and different host species can carry different genes (Roberts, 2005). Also, most published studies on tetracycline resistance genetic patterns in *E. coli* are related to clinically resistant strains (Lanz *et al.,* 2003; Guerra *et al.,* 2006; Sengelov *et al.*, 2003 b; Srinivasan *et al.,* 2007). In all of these, genes that codify for efflux proteins have been found (Chopra & Roberts, 2001). Such bacteria come from clinical environments subjected to high selective pressure that are possibly different from the low selective pressure found in natural environments. This result is very interesting and led to the possibility that important differences might be present between the genetic pattern of highly resistant clinical isolates and intermediate resistant isolates. As Bryan *et al.* (2004) stated, only limited studies have examined tetracycline resistance determinants in bacteria isolated in different animal species with different exposures to antibiotics. Even in these cases, only a small number of tetracycline resistant determinants were tested (Sengelov *et al.,* 2003 b) This was one of the reason that led Bryan *et al.* (2004) to test several different animal sources exposed to different levels of antibiotic, for different tetracycline resistance genes. This research led to the discovery of the *tet(M)* in *E. coli* isolates. It is the only gene found in *E. coli* that confers resistance through ribosomal protection (Bryan *et al.*, 2004). An interesting property of ribosomal protection seems to be that this resistance mechanism, when it was cloned into *E. coli,* did not confer high levels of resistance. These bacteria had MICs of 10-25µg/mL whereas those with genes which code for efflux

pumps appears have a 4-10 fold higher level of resistance (Roberts, 1996). Thus it is possible that *tet(M)* was never found previously because it not provide much survival value in the presence of high levels of tetracycline.
In the current study most of the resistant isolates showed an intermediate level of resistance (Table 4.7 and 4.13). This may be the reason why *tet(A)* and *tet(B),* which are the most common genes in clinical isolates codifying for efflux pumps, were not found. It might be interesting to screen the samples for a wider set of *tet* genes. Even more interesting would be to see if these isolates could confirm the presence of *tet(M)* in *E. coli* with intermediate level of resistance, however, such research was beyond the scope of this study, which focussed more on the epidemiological aspects of AMR in impala and their water sources.

5.10 Association between TR<u>EC</u> in water and faecal samples from impala

Although it was not possible to confirm the identity of the genotypic pattern of TR*EC* from water and faecal specimens of the exposed and unexposed groups of impala, the data on phenotypic resistance patterns showed a strong association. Impala from the control or unexposed impala group, had only 1 sample positive for TR*EC*. In contrast, among the case or exposed group of impala, drinking from the TR*EC* positive rivers, 18 samples were found to contain TR*EC.* The OR of 19.3 (CI: 2,63-141,69) clearly shows that faeces of impala drinking from TR*EC*pos rivers were more likely to contain TR*EC* than faeces of impala drinking from TR*EC*neg rivers. The χ^2 test was run to find out whether the value could be significant and the result was 14.76, confirming that the association (OR) between exposed and unexposed groups and the exposure factor (TR*EC*) was highly significant (P<0,0001).

5.11 AMR in water and problem associated with public health

The presence of AMR in surface waters can be considered an area of concern for human public health. This study showed a relationship between AMR in enteric flora of untreated impala drinking from a water source polluted by AMR bacteria

(that is, TR*EC*) and the same could be true for humans. Even groundwater from wells or boreholes, used for drinking, could be a source for the spread and transfer of AMR strains of bacteria. As previous studies report that potable water systems can fail to distribute bacteria-free water (see section 2.7), it could be possible that some of these bacteria can carry AMR even in the household tap water. Some bacteria that are resistant to multiple antibiotics have previously been isolated from chlorinated drinking water (Armstrong *et al.*, 1981; Schwartz *et al.*, 2003). Moreover, even commercially available bottled water can represent a risk. In a recent study it was found that 80% of heterotrophic bacteria isolated from mineral water at different stages of the bottling process were resistant to one or more antibiotics (Messi *et al.*, 2005). Resistant bacteria in water can represent a reservoir of AMR determinants as well as a means for the spread and evolution of AMR genes and their vectors (Young, 1993).

CHAPTER 6. CONCLUSIONS AND RECOMMENDATIONS

The results of this study showed that impala drinking from TR*EC* contaminated rivers were 19.3 times more likely to be infected with TR*EC* than unexposed impala. This is a significant finding, leading to the opinion that surface waters are an important source of tetracycline resistant bacteria for impala. It is thus possible that the same would be true for humans and other animal species that have access to AMR polluted waters. This finding emphasizes the importance of water quality on public health. Furthermore rivers and streams can move over long distances interconnecting different ecosystems and can introduce AMR to populations where antimicrobials are not used. Once the risks associated with the dissemination of antimicrobial resistance in water has been fully studied, it may become necessary for AMR tests to be added to the currently used tests for water quality assessment.

Supplementary studies to identify the most probable contamination source as well as the extent of dissemination and possible amplification of AMR in water, necessary to address risk management, should be considered. Molecular biotechnologies can be used to determine whether resistant *E. coli* come from human or animal sources. A marker localized to the glycine decarboxylase gene (*gcvP*) of *E. coli*, has shown some promise for use as a molecular marker for the identification of *E. coli* of human origin (Turner *et al.*, 1997). Biotechnology can be expensive and it also becomes important to develop low-cost screening tools that can be routinely used to identify the most probable sources of faecal contamination of water (Whitlock *et al.*, 2002). It may be useful to include the classification of pollution sources based on multivariate statistical analysis of AMR patterns (Sayah *et al.,* 2005; Vantarakis *et al.,* 2006). This could also be studied in greater depth and more specifically by the tracing of AMR genes from potential sources to sentinel animals such as impala.

This study also showed a slight increase in the amount of tetracycline resistance in water in correspondence with the summer period when there is a higher usage of tetracycline. This connection could not be proven by this research and thus further research on seasonal variation is suggested.

Several studies have been published on the detection of AMR in the environment, but often different sampling and laboratory methods were used, making it difficult to compare these results. As has been done for the human and veterinary resistance monitoring programmes, where a standardized MIC method is used, this could be done for environmental studies of AMR, possibly making use of methods approved by the EPA and CLSI. Molecular techniques could also be used to improve the knowledge about epidemiology of tetracycline resistance genes. It would be interesting to test samples for a wide range of *tet* genes. Samples with low levels of resistance should also be examined to identify the genetic patterns of isolates not exposed to high environmental resistance, such as that found in clinical situations. In particular further research is advised to confirm if ribosomal mechanisms of tetracycline resistance in *E. coli* can be found in intermediate resistance isolates such as those found in the untreated wild impala.

In the end, research on risk analysis of AMR with the aim to better address an international AMR risk management policy should be supported. To understand the mechanisms of AMR spread, programmes for monitoring of AMR in several environments should be implemented in different countries. In particular research should be directed also in environments where there is low selective pressure for resistance, for instance healthy and wild environments. In this research wild animals, such as impala, were found to be good sentinels for the spread of AMR.

CHAPTER 7. REFERENCES

1. AARESTRUP F.M., AGERSO Y., GERNER-SMIDT P., MADSEN M., JENSEN L.B. 2000. Comparison of antimicrobial resistance phenotypes and resistance in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagnostic Microbiology and Infectious Disease,* 37:127-137.

2. AARESTRUP F.M., SEYFARTH A. M., EMBORG H.D., PEDERSEN K., HENDRIKSEN R.S., 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(7):2054-2059.

3. ADAMS H.R. 1999. *Farmacologia e terapeutica veterinaria.* Seconda ed. Italiana ed E.M.S.I. Roma, Italy.

4. ANDREWS J.M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48:5-16.

5. ARMSTRONG J.L., SHIGEND D.S., CALOMARIS J.J., SEILDER P.J. 1981. Antibiotic resistant bacteria in drinking water. *Applied and Environmental Microbiology,* 42:277-283.

6. AVERY L.M., WILLIAMS A.P., KILLHAM K., JONES D.L. 2008. Survival of *Escherichia coli* 0157:H7 in waters from lakes, rivers, puddles and animal-drinking troughs. *Science of the Total Environment,* 389:378-385.

7. BAIRD D.J., BEVERIDGE M.C.M., KELLY L.A. 1996. Environmental considerations in the use of antibacterial drugs in aquaculture. *Aquaculture and Water Resource Management.* Blackwell Science, Oxford, U.K.

8. BERRY D., CHUANWU X., RASKIN L. 2006. Microbial ecology of drinking water distribution systems. *Current Opinions in Biotechnology,* 17:297-302.

9. BOERLIN P., TRAVIS R., GYGLES C.L., REID-SMITH R., JANECKO N., LIM H., NICHOLSON V., MCEWEN S.A., FRIENDSHIP R., ARCHAMBAULT M. 2005. Antimicrobial resistance and virulence genes of *E. coli* isolates from swine in Ontario. *Applied and Environmental Microbiology,* 71:6753-6761.

10. BONFIGLIO G., SIMPORÈ J., PIGNATELLI S., MUSUMECI S., SOLINAS M.L. 2002. Epidemiology of bacterial resistance in gastro-intestinal pathogens in a tropical area. *International Journal of Antimicrobial Agents* 20:387-389.

11. BOOTH N.H., MCDONALD L.E. 1995. *Veterinary Pharmacology and Therapeutics -7th ed.* Iowa State University Press, USA.

12. BRINKHOFF T. 2007. *Table of urban areas.* http//www.citypopulation.de (accessed March 2008).

13. BRYAN A., SHAPIR N., SADOWSKY M.J. 2004. Frequency and distribution of tetracycline resistance genes in genetically diverse, non-selected, and non-clinical *E. coli* strains isolated from diverse human and animal sources. *Applied and Environmental Microbiology,* 70:2503-2507.

14. BYAPPANAHALLI M.N., FUJIOKA R.S. 1998. Evidence that tropical soil environments can support the growth of *Escherichia coli*. *Water Science and Technology,* 38:171-174.

15. CADIEUX G., TAMBLYN R., DAUPHINE D., LIBMAN M. 2007. Predictors of inappropriate antibiotic prescribing among primary care physicians. *Journal of Canadian Medical Association,* 177:877-883.

16. CAMERON A. 1999. *Survey toolbox for Livestock Disease: a practical manual and software package for active surveillance in developing countries.* ACIAR Monograph 54, vii+330p. Australia.

17. CAPRIOLI A., BUSANI L., MARTEL J.L., HELMUTH R. 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. *International Journal of Antimicrobial Agents*, 14: 295-301.

18. CASTIGLIONI S., FANELLI R., CALAMARI D., BAGNATI R., ZUCCATO E. 2004. Methodological approaches for studying pharmaceuticals in the environment by comparing predicted and measured concentration in the river Po, Italy. *Regulatory Toxicology and Pharmacology* 39:25-32.

19. CHASTRE J. 2008. Evolving problems with resistant pathogens. *Journal of Clinical Microbiology and Infectious Diseases,* 14:3-14.

20. CHOPRA I. 2001. Glycylcyclines: third generation tetracycline antibiotics. *Current Opinion in Pharmacology,* 1:464-469.

21. CHOPRA I., ROBERTS M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews,* 65:232-260.

22. CHUANCHUEN R., BEINLICH K., HOANG T.T., BECHER A., KARKHOFF-SCHWEIZER R.R., SCHEWEIZER H.P. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclorosan selects *nfxB* overexpressing MexCD-OprJ. *Journal of Antimicrobial Agents and Chemotherapy,* 45:428-432.

23. CLSI (formerly NCCLS) 2002. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Approved Standard (second ed),* NCCLS document M31-A2. Clinical Laboratory Standards Institute, Wayne (PA), USA.

24. COBB K. 2004. Basic statistical principles for the clinical research scientist. www.stanford.edu/~kcobb/precept.ppt (visited on July 2006).

25. COL N.F., O'CONNOR R.W. 1987. Estimating world-wide current antibiotic usage: report of task force 1. *Review of Infectious Disease,* 9:S232-234.

26. DANMAP, 2003. *Use of antimicrobial agents and occurrence of antimicrobials resistance in bacteria from food animals*. Food and Humans in Denmark. www.danmap.org (visited on March 2007).

27. DAVIS C.E., CARPENTER J.L., MCALLISTER C.K., MATTHEWS J., BUSH B.A., OGNIBENE A.J. 1985. Tuberculosis. Cause of death in antibiotic area. *Chest,* 88:726-729.

28. DAVISON J. 1999. Genetic exchange between bacteria in the environment. *Plasmid* 42:73-91.

29. DE WET C.M., VENTER S.N., RODDA N., KFIR R., STEYNBERG M.C., HOHLS D., DU PLESSIS G. 1995. A survival study of *Escherichia coli* in a South African river using a membrane diffusion chamber. *Water and Science Technology,* 31:185-188.

30. DIAZ–CRUZ M.S., LOPEZ DE ALDA M.J., BARCELO D. 2003. Environmental behaviour of veterinary and human drugs in soils, sediments and sludge. *Trends in Analytical Chemistry,* 22: 340-351.

31. DOH 2003. Medicines and related substances control act n°101 of 1965. www.doh.gov.za/docs/regulations/2003/medcontrol1.html (visited on April 2008).

32. DURIEZ P., TOPP E. 2007. Temporal dynamics and impact of manure storage on antibiotic resistance patterns and population structure of *Escherichia coli* isolates from a commercial swine farm. *Applied and Environmental Microbiology,* 73:5486-5493.

33. DUTKA B.J., KWAN K.K. 1990. Bacterial die-off and stream transport studies. *Water Research,* 29:1902-1908.

34. DWAF 2001 a. *Quality of domestic water supplies - sampling guide*. Department of Water Affairs and Forestry, Water Research Commission no: TT 117/99, Pretoria, South Africa.

35. DWAF 2001 b. *Quality of domestic water supplies - analysis guide.* Department of water affairs and forestry, Water Research Commission no: TT 129/00, Pretoria, South Africa.

36. EDWARDES M.D. 2001. Sample size requirement for a case-control study design. *BMC Medical Research Methodology* 1:11.

37. EPA 2001. Guidelines establishing test procedures for the analysis of pollutants; analytical methods for biological pollutants in ambiental water: proposed rules. *Federal register,* 66(169):45811-45829. www.epa.gov/EPA-WATER/2001/August/Day-30/w21813.htm (visited on April 2006)

38. EPA 2002. *Method 1064: Total coliforms and Escherichia coli in water by membrane filtration using a simultaneous detection technique (MI medium).* EPA 821-R-02-024. Office of Water, Environmental Protection Agency, Washington, DC, USA. www.epa.gov/microbes/MI_emmc.pdf (visited on April 2006)

39. FAN SOZZI 2006. http:// www.scp.ubc.ca (visited on March 2008).

40. FEUERPFEIL I., STELZER W. 1992. Presence of antibiotic-resistant coliform bacteria in the human intestinal flora. *Bundesgesund-heitsbl* 35:61-65.

41. FRANKLIN A., VAN VUUREN M. 2001. Antimicrobial resistance: harmonisation of national antimicrobial resistance monitoring and surveillance programmes in animals and in animals-derived food. *Revue Scientifique et Technique-Office International des Epizooties,* 20(3):859-870.

42. GAD G.F., EL-DOMANY R.A. ZAKY S., ASHOUR H.M. 2007. Characterization of *Pseudomonas aeruginosa* isolates from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. *Journal of Antimicrobial Chemotherapy,* 60:1010-1017.

43. GANNON J.J., BUSSE M.K. 1989. *E. coli* and enterococci levels in hurban stormwater, river, and chlorinated treatment plant effluent. *Water Research,* 23:1167-1176.

44. GILLESPIE S.H. 2001. Antibiotic resistance in the absence of selective pressure. *International Journal of Antimicrobial Agents,* 17:171-176.

45. GIRAUD E., DOUET D.G., LES BRIS H., BOUJU-ALBERT A., DONNAY-MORENO C., THORIN C., POULIQUEN H. 2006. Survey of antibiotic resistance in an integrated marine aquaculture system under oxolinic acid treatment. *FEMS Microbiological Ecology* 55:439-448.

46. GOLDBERG T.L., GILLESPIE T.R., RWEGO I.B., WHEELER E., ESTOFF E.L., CHAPMAN C.A. 2007. Pattern of gastrointestinal bacterial exchange between chimpanzees and humans involved in research and tourism in western Uganda. *Biological Conservation* 135:511-517.

47. GOMEZ P.A.J., GARCIA DE LO RIOS J.E.,MENDOZA A.R., RAMONET P.P., GARCIA ALBIACH R., RECHE SAINZ M.P. 2004. Molecular basis of quinolone resistance in *Escherichia coli* from wild birds. *The Canadian Journal of Veterinary Research,* 68:229-231.

48. GONZALO M.P., ARRIBAS R.M., LATORRE E., BAQUERO F., MARTINEZ J.L. 2002. Sewage dilution and loss of antibiotic resistance and virulence determinants in *E. coli*. *FEMS Microbiology Letters*, 59:93-96.

49. GRUGEL C. 2006. Meeting report: First national scientific symposium "Risk management for the limitation of antibiotic resistance". *International Journal of Microbiology,* 296:S2,1-3.

50. GUERRA B., JUNKER E., SCHROETER A., HELMUTH R., GUTH B.E.C., BEUTIN L. 2006. Phenotypic and genotypic characterization of antimicrobial resistance in *Escherichia coli* 0111 isolates. *Journal of Antimicrobial Chemotherapy,* 57:1210-1214.

51. GUMMOW B. 2006. *Veterinary Epidemiology*, Course EPI 751, Faculty of Veterinary Science, University of Pretoria, South Africa.

52. HALLING-SORENSEN B., NORS-NIELSEN S., LANZKY P.F., INGERSLEV F., HOLTEN LUTZHOFT H.C., JORGENSEN S.E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment. *Chemosphere,* 36:357-393.

53. HAMILTON W.P., KIM M., THACKSTON E.L. 2005. Comparison of commercially available *Escherichia coli* enumeration tests: implications for attaining water quality standards. *Water Research,* 39:4869-4878.

54. HARTMAN A.B., ESIET I.I, ISENBARGER D.W, LINDLER L.E. 2003. Epidemiology of tetracycline resistance determinants in *Shigella* spp. and enteroinvasive *Escherichia coli*: characterization and dissemination of *tet*(A)-1. *Journal of Clinical Microbiology,* 41(3):1023-1032.

55. HÄTCHLER H., COHEN S., LEVY S. B. 1991. marA, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *E. coli*. *Journal of Bacteriology,* 173:5532-5538.

56. HIRSCH R., TERNES T., HABERER K., KRATZ K.L. 1999. Occurrence of antibiotics in the aquatic environment. *The Science of the Total Environment,* 225:109-118.

57. HOPKINS W.G., HAWLEY J.A., BURKE L.M. 1999. Researching worthwhile enhancements. Sportscience 3, sportsci.org/jour/9901/wghnews.html (visited on January 2007).

58. HSUEH P., CHEN W., LUH K. 2005. Relationship between antimicrobial use and antimicrobial resistance in Gram-negative bacteria causing nosocomial infections from 1991-2003 at a university hospital in Taiwan. *International Journal of antimicrobial Agents,* 26:463-472.

59. HUMBERT P., TREFFEL P., CHAPUIS P., BUCHET J.F., DERANCOURT S., AGACHE P. 1991. The tetracycline in dermatology. *Journal of American Academy of Dermatology,* 25:691-697.

60. HUQ A., SACK R.B., NIZAM A., LONGINI I.M., ALI A., MORRIS J.G., HUNA KHAN M.N., SIDDIQUE K., YUNUS M., ALBERT M.J., SACK D.A., COLWELL R.R. 2005. Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. *Applied and Environmnental Microbiology,* 71(8):4645-4654.

61. IVS (INDEX OF VETERINARY SPECIALITY). 2007. Edited by Carrington C. 45:4. MIMS, Johannesburg.

62. KASZANYITZKY E.J.,TENK M., GHIDAN A., FEHERVARI G.Y., PAPP M. 2006. Antimicrobial susceptibility of enterococci strains isolated from slaughter animals on the data of Hungarian resistance monitoring system from 2001 to 2004. *International Journal of Food Microbiology,* 115(1):119-123.

63. KEMPER N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators*, 8:1-13.

64. KHANNA T., FRIENDSHIP R., DEWEY C., WEESE J.S. 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Veterinary Microbiology,* 128(3-4):298-303.

65. KOIKE S., KRAPAC I.G., OLIVER H.D., YANNARELL A.C., CHEE-SANFORD J.C., AMINOV R.I., MACKIE R.I. 2007. Monitoring and source tracking of tetracycline resistance genes in lagoons and ground water adjacent to swine production facilities over a 3-year period. *Applied and Environmental Microbiology,* 73:4813-4823.

66. KRUSE H., SORUM H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Applied and Environmental Microbiology,* 60:4015-4021.

67. KÜMMERER K. 2004. Resistance in the environment. *Journal of Antimicrobial Chemotherapy,* 54:311-320.

68. LANZ R., KUHNERT P., BOERLIN P. 2003. Antimicrobial resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Veterinary Microbiology,* 91:73-84.

69. LAUTENBACH E., METLAY J.P., BILKER W.B., EDELSTEIN P.H., FISHMAN N.O. 2005. Association between fluoroquinolone resistance and mortality in *Escherichia coli* and *Klebsiella pneumoniae* infections: the role of inadequate empirical antimicrobial therapy. *Journal of Clinical Infectious Disease,* 41:923-929.

70. LECHEVALLIER M.W., WELCH N., SMITH D.B. 1996. Full scale studies of factors related to coliform regrowth in drinking water. *Applied and Environmental Microbiology,* 62:2201-2211.

71. LEDERBERG J., LEDERBERG E.M. 1951. Replica plating and indirect selection of bacterial mutants. *Applied and Environmental Microbiology,* 63:399-406.

72. LEISTEVUO T., TOIVONEN P., ÖSTERBLAD M., KUISTILA M., KAHARA A., LEHTONEN A., HUOVINEN P. 1996. Problems of antimicrobial resistance of faecal aerobic Gram-negative bacilli in the elderly. *Antimicrobial Agents and Chemotherapy,* 40:2399-2403.

73. LEMUNIER M., FRANCOU C., ROSSEAU S., HOUOT S., DANTIGNY P., PIVETEAU P., GUZZO J. 2005. Long-term survival of pathogenic and sanitation bacteria in experimental biowaste composts. *Applied and Environmental Microbiology,* 71:5779-5786.

74. LEVI S., FITZGERALD G., MACONE A. 1976. Changes in intestinal flora of farm personnel after introduction of tetracycline supplemented feed on a farm. *New England Journal of Medicine,* 295:583-8.

75. LEVI S.B. 1992. *The antibiotic paradox: how miracle drugs are destroying the miracle.* Plenum press, New York.

76. LEVI S.B. 2005. Antibiotic resistance: the problem intensifies. *Advanced Drug Delivery Reviews,* 57:1446-1450.

77. LICHT T.R., BJARKE B., CHRISTENSEN B., KROGFELT K.A., MOLIN S. 1999. Plasmid transfer in the animal intestine and other dynamic bacterial populations: the role of community structure and environment. *Environmental Microbiology,* 145: 2615-2622.

78. LILLEHAUG A, BERGSJǾ B, SCHAU J, BRUHEIM T, VIKOREN T, HANDELAND K. 2005. *Campylobacter* spp., *Salmonella* spp., verocytotoxic *E. coli*, and antibiotic resistance in indicator organisms in wild cervids. *Acta Veterinaria Scandinavica,* 46:23-32.

79. LINDSTROM E.B. 1977. Alternative replica plating technique. *Applied and Environmental Microbiology,* 33:224-227.

80. LISS R.H., BATCHELOR F.R. 1987. Economic evaluation of antibiotic use and resistance – a perspective report of Task Force 6. *Reviews of Infectious Disease,* 3:s297-312.

81. MCCRINDLE C.M.E., TICE G., MOGAJANE E.M., SYEWART C.G., MOSUPI H. 1994. An investigation into the potential veterinary needs of semi-rural, low-income community. *Journal of the South African Veterinary Association,* 65:90-96.

82. MCPHERSON M.J., M∅LLER S.G. 2000. *PCR the basics.* BIOS Scientific Publishers, Oxford, UK.

83. MESSI P., GUERRIERI E., BONDI M. 2005. Antibiotic resistance and antibacterial activity in heterotrophic bacteria of mineral origin. *Science of the Total Environment,* 342:213-219.

84. MILLER J., JENNY A., RHYAN J., SAARI D., SUAREZ D. 1997. Detection of *Mycobacterium bovis* in formalin-fixed, paraffin embedded tissue of cattle end elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium*

tuberculosis complex organisms. *Journal of Veterinary Diagnostic Investigation,* 9:244-249.

85. MIRANDA C.D., KEHRENBERG C., ULEP C., SCWARZ S. ROBERTS M.C. 2003. Diversity of tetracycline resistant genes in bacteria isolated from Chilean Salmon farms. *Journal of Antimicrobial Agents and Chemotheraphy,* 47:883-888.

86. NDA, 2007. Fertilizer, farm feeds, agricultural remedies and stock remedies Act, 1947 (Act n°36 of 1947). www.nda.agric.za (visited on April 2008).

87. NEL H., VAN VUUREN M., SWAN G.E. 2004. Towards the establishment and standardization of a veterinary antimicrobial resistance surveillance and monitoring programme in South Africa. *Onderstepoort Journal of Veterinary Research,* 71:239-246.

88. OGDEN I.D., FENLON D.R., VINTEN A.J.A., LEWIS D. 2001. The fate of *Escherichia coli* 0157 in soil and its potentential to contaminate drinking water. *International Journal of Food Microbiology*, 66:111-117.

89. OKEKE I.N., KLUGMAN K.P, BHUTTA Z.A, DUSE A.G., JENKINS P., O'BRIEN T.F., PABLOS-MENDEZ A., LAXMINARAYAN R. 2005. Antimicrobial resistance in developing countries. Part II: strategies for containment. *The Lancet Infectious Diseases*, 5:568-580.

90. ÖSTERBLAD M., LEISTEVUO T., HUOVINEN P. 1995. Screening for antimicrobial resistance in faecal samples by the replica plating method. *Journal of Clinical Microbiology,* 33:3146-3149.

91. ÖSTERBLAD M., NORRDAHL K, KORPIMAKI E., HUOVINEN P. 2001. Antibiotic resistance. How wild are wild mammals? *Nature,* 409:37-38.

92. OTEO J., BAQUERO F., VINDEL A., CAMPOS J. 2004. Antibiotic resistance in 3113 blood isolates of *Staphylococcus aureus* in 40 Spanish hospitals participating in the European antimicrobial resistance surveillance system (2000-2002). *Journal of Antimicrobial Chemotherapy,* 53:1033-1038.

93. PAGANI L., COLINON C., MIGLIAVACCA R., LIBONIA M., DOCQUIER J., NICLEO E., SPALLA M., BERGOLI M., ROSSOLINI G.M. 2005. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-β-lactamase. *Journal of Clinical Microbiology,* 43:3824-3828.

94. PAGANO A., NARDI G., BONNACCORSO C., FALBO V., PASSI C., SANGUINETTI V., MANTOVANI A. 1985. Faecal bacteria of wild ruminants and the alpine marmot. *Veterinary Research Communication,* 9:227-32.

95. PFEIFFER D.U., 2002. *Veterinary Epidemiology - an introduction.* The Royal Veterinary College, London, UK.

96. PHILLIPS I., CASEWELL M., COX T., DE GROOT B., FRIIS C., JONES R., NIGHTINGALE C., PRESTON R., WADDELL J. 2004. Does the use of antibiotics

in food animals pose a risk to human health? A critical review on published data. *Journal of Antimicrobial Chemotherapy,* 53:28-52.

97. PICARD J., SINTHUMULE E., 2002. *VIRBAC Antimicrobial Database Report, 2002.* Department of Veterinary Tropical Diseases, University of Pretoria, South Africa.

98. POETA P., COSTA D., RODRIGUES J.,TOORES C. 2005 a. Study of faecal colonization by vanA-containing *Enterococcus* strains in healthy humans, pets, poultry and wild animals in Portugal. *Journal of Antimicrobial Chemotherapy,* 55:278-80.

99. POETA P., COSTA D., SAÈNZ Y., KLIBI N., RUIZ-LARREA F., RODRIGUES J., TORRES C. 2005 b. Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. *Journal of Veterinary Medicine Series – Infectious Disease and Veterinary Public Health*, 52:396-402.

100. PRIYA S. 2008. Antibiotic resistance: frequently asked questions. http://www.scidev.net/en/health/antibiotic-resistance/features.html (visited on March 2008).

101. PULCINI C., LIEUTIER F., LANDRAUD L., DELLAMONICA P., ROGER P.M. 2007. Antibiotic misuse: a prospective clinical audit in a French university hospital. *European Journal of Clinical Microbiological Infectious disease,* 26:277-280.

102. REINTHALER F.F., POSCH J., FEIERL G., WUST G., HAAS D., RUCKENBAUER G., MASCHER F., MARTH E. 2003. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research* 37:1685-1690.

103. ROBERTS M.C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility and distribution. *FEMS Microbiology Reviews,* 19:1-24.

104. ROBERTS M.C. 2005. Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters,* 254:195-203.

105. ROMANIAN 2002. Distribution and diversity of conjugative plasmids among some multiple antibiotic resistant *E. coli* strains isolated from river waters. *Bacterilogia, Virusologia, Parazitologia, Epidemiologia,* 47:147-153.

106. RUSSEL A.D. 2002. Biocides and pharmacologically active drugs as residues in the environment: is there a correlation with antibiotic resistance? *American Journal of Infectious Disease,* 30:495-498.

107. RYSZ M., ALVAREZ P.J.J. 2004. Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments. *Water Research*, 38:3705-3712.

108. SAYAH R.S., KANEENE J.B., JOHNSON Y., MILLER R. 2005. Patterns of antimicrobial resistance observed in *E. coli* isolates obtained from domestic and

wild-animal faecal samples, human septage, and surface water. *Applied and Environmental Microbiology*, 71:1394-1404.

109. SCHWARTZ T., KOHNEN T., JANSEN B. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology* 43:325-335.

110. SENGELOV G., AGERSO Y., HALLING-SORENSEN B., BALODA S.B., ANDERSEN J.S., JENSEN L.B. 2003 a. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environment International,* 28:587-595.

111. SENGELOV G., HALLING-SORESEN B., AARESTRUP F.M. 2003 b. Susceptibility of *Escherichia coli* and *Enterococcus faecium* isolated from pigs and broiler chickens to tetracycline degradation products and distribution of tetracycline resistance determinants in *E. coli* from food animals. *Veterinary Microbiology,* 95:91-101.

112. SHAH N.S., WRIGHT A., BAI G.H., BARRERA L., BOILAHBAL F., MARTIN-CASABONA N., DROBNIESWKI F., GILPIN C., HAVELKOVE M., LEPE R., LUMB R., METCHOCK B., PATAELS F., RODRIGUES M.F., RUSCH-GERDES S., VAN DEUN A., VINCENT V., LASERSON K., WELLS C., CEGIELSKI J.P. 2007. Worldwide emergence of extensively drug-resistant tuberculosis. *Journal of Emergent Infectious Disease*, 13: 380-387.

113. SKINNER J.D., CHIMIMBA C.T. 2005. *The Mammals of the Southern African Subregion*. 3rd edition, Cambridge University Press, South Africa.

114. SMALLA K., HEUER H., GOTZ A., NIEMEYER D., KROGERRECKLENFORT E., TIETZE E. 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of incQ-like plasmids. *Applied and Environmental Microbiology,* 66:4854-4862.

115. SRINIVASAN V., GILLESPIE B.E., LEWIS M.J., NGUYEN L.T., HEADRICK S.I., SCHUKKEN Y.H., OLIVER S.P. 2007. Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. *Veterinary Microbiology,* 124:319-328.

116. STATSONLINE, 2001. www.statsonline.gov.za (visited on April 2008).

117. STEINFIELD H., GERBER P., WASSENAAR T., CASTE V., ROSALES M., DE HAAN C. 2006. *Livestock's Long Shadow: Environmental Issues and Options.* FAO, Rome, Italy.

118. STUART C., STUART T. 2000. *Field Guide to the Larger Mammals of South Africa.* 2nd edition, Struik Publishers, Cape Town, South Africa.

119. SWANN REPORT. 1969. Joint Committee on the use of antimicrobials in animal Husbandry and Veterinary Medicine. *Report*. HMSO, London.

120. SWARM 2006. *Swedish Veterinary Antimicrobial Resistance Monitoring 2006.* The National Veterinary Institute (SVA), Uppsala, Sweden.

121. SWARTZ M.N. 2002. Pathogens of animal origins. *Clinical Infectious Diseases,* 34(Suppl3):S111-22.

122. TERNES T.A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Research,* 32:3245-3260.

123. TEUBER M. 2001 Veterinary use and antibiotic resistance. *Current Opinion in Microbiology,* 4:493-499.

124. THRUSFIELD M.V. 2005. *Veterinary Epidemiology* 3rd Edition. Blackwell Science Ltd., Oxford, UK.

125. TURNER S.J., LEWIS G.D., BELLAMY A.R. 1997. Detection of sewage-derived Escherichia coli in a rural stream using multiplex PCR and automated DNA detection. *Water Science and technology,* 35:337-342.

126. TURNIDGE J. 2004. Antibiotic use in animals- prejudices, perceptions and realities. *Journal of Antimicrobial Chemotherapy,* 53:2627.

127. VACCHERI A., SILVANI M.C., BERSAGLIA L., MOTOLA D., STRAHINJA P., VARGIU A., POLUZZI E., MONTANARO N. 2008. A 3 year survey on the use of antibacterial . agents in five Italian hospitals. *Journal of Antimicrobial Chemotherapy,* 61:953-958.

128. VAN DE BOGAARD A.E. 1997 a. Antimicrobial resistance - relation to human and animal exposure to antibiotics. *Journal of Antimicrobial Chemotherapy,* 40:453-454.

129. VAN DE BOGAARD A.E., MERTENS P., LONDON N.H. STOBBERINGH E.E. 1997 b. High prevalence of colonization with vancomycin and pristinamycin resistant enterococci in healthy humans and pigs in the Netherlands: is the addition of antibiotics to animal feeds to blame? *Journal of Antimicrobial Chemotherapy,* 40:454-456.

130. VAN DEN BRAAK N., VAN DE BELKUM A., VAN KEULEN M., VLIGENTHART J., VERBRUGH H.A., ENDTZ H.P. 1998. Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in the Nederlands. *Journal of Clinical Microbiology,* 36:1927-32.

131. VAN VUUREN M., PICARD J.A., GREYLING J. 2007. *South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs.* University of Pretoria, South Africa.

132. VANTARAKIS A., VENIERI D., KOMNIOU G., PAPAPETRPOULOU M. 2006. Differentiation of faecal *E. coli* from humans and animals by multiple antibiotic resistance analysis. *Letters in Applied Microbiology,* 42:71-77.

133. VENTER E.H., BOOSMAN A.M. 2003. *Practical PCR course*. Department of Veterinary Tropical Diseases, Faculty of Veterinary Science University of Pretoria, South Africa.

134. VOETS J.P., VERSTRAETE W. 1980. Comparative study of *E. coli* survival in two aquatic ecosystems. *Water Research,* 14:909-915.

135. WARTENBERG D. 2000. *Four types of epidemiological studies.* www.facsnet.org/tool/ref_tutor/epidem/ (visited on July 2006).

136. WATKINSON A.J., MICALIZZI G.R., BATES J.R., COSTANZO S.D. 2007 a. Novel method for rapid assessment of antibiotic resistance in *Escherichia coli* isolates from environmentally waters by use of a modified chromogenic agar. *Applied and Environmental Microbiology,* 73:2224-2229.

137. WATKINSON A.J., MICALIZZI G.R., GRAHAM G.M., BATES J.R., COSTANZO S.D. 2007 b. Antibiotic-resistant *Escherichia coli* in waste waters, surface waters and oysters from an urban riverine system. *Applied and Environmental Microbiology,* 73:5667-5670.

138. WEBSTER L.F., THOMPSON B.C., FULTON M.H., CHESTNUT D.E., VAN DOLAH R.F., LEIGHT A.K., SCOTT G.I. 2004. Identification of sources of *Escherichia coli* estuaries using antibiotic resistance analysis. *Journal of Experimental Marine Biology and Ecology,* 298:179-195.

139. WEIGEL L.M., MUSSER K., THOMPSON J., LIMBERGER R., CLARK N.C., PATEL J.B. 2004. *Genetic analysis of a Vancomycin-resistant Staphylococcus aureus (VRSA) isolated from New York.* 44th Interscience Conference on Anrimicrobial Agent and Chemotheraphy.

140. WHITE D.G., ACAR J., ANTHONY F., FRANKLIN A., GUPTA R., NICHOLLS T., TAMURA Y., THOMPSON S., THRELFALL E.J., VOSE D., VAN VUUREN M., WEGENER H.C., COSTARRICA M.L. 2001. Antimicrobial resistance: standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. *Revue Scientifique et Technique-office International des Epizooties,* 20:849-858.

141. WHITE D.G, ZHAO S., MCDERMOTT P.F., AYERS S., GAINES S., FRIEDMAN S., WAGNER D.D., MENG J., NEEDLE D., DAVIS M., DEBROY C. 2002. Characterization of antimicrobial resistance among *Escherichia coli* O111 isolates of animal and human origin. *Microbial Drug Resistance,* 8:139-146.

142. WHITLOCK J.E., JONES D.T., HARWOOD V.J. 2002. Identification of the sources of faecal coliforms in an urban watershed using antibiotic resistant analysis. *Water research,* 36:4273-4282.

143. WHITMAN R.L., NEVERS M.B., BYAPPANAHALLI N. 2006. Examination of the watershed-wide distribution of *Escherichia coli* along southern Lake Michigan: an integrated approach. *Applied and Environmental Microbiology,* 72:7301-7310.

144. WHITTLE G., WHITEHEAD T.R., HAMBURGER N., SHOEMAKER N.B., COTTA M.A., SALYERS A.A. 2003. Identification of a new ribosomal protection type of tetracycline resistance gene, *tet(36),* from swine manure pits. *Applied and Environmental Microbiology,* 69:4151-4158*.*

145. WHO, 2001. *Global strategy for Containment of Antimicrobial resistance*. World Health Organization, Genevra, Switzerland.

146. WHO, FAO and OIE 2006. *Antimicrobial use in aquaculture and antimicrobial resistance*. World Health Organization Press, Geneva, Switzerland.

147. WRC 2001 a. *State of Rivers Report 2001: Letaba and Luvuvhu river systems.* WRC report n°: TT 165/01. Water Research Commission , Pretoria, South Africa.

148. WRC 2001 b. *State of Rivers Report 2001: Crocodile, Sabie-Sand and Olifants.* Water Research Commission, Pretoria, South Africa. Accessed on line at: http://www.csir.co.za/rhp/state_of_rivers/crocsabieolif (visited on December 2006).

149. YIM G. 2006. Attacks of the superbugs: antibiotic resistance. *The Science Creative Quarterly*, www.scp.ubc.ca (visited on March 2008).

150. YOU J.Y., MOON B., OH I., BAEK B., LI L., KIM B., STEIN B., LEE J. 2006. Antimicrobial resistance of *E. coli* O157 from cattle in Korea. *International Journal of Food Microbiology,* 106:74-78.

151. YOUNG H.K. 1993. Antimicrobial resistant spread in aquatic environments. *Journal of Antimicrobial Chemotherapy,* 31:627-635.

152. YOUNG K. D., THACKSTON E. L. 1999. Housing density and bacterial loading in hurban streams. *Journal of Environmental Engineering,* 125:1177-1180.

153. YOUNG-JU L., JAE-KEUN C., KI-SEUK K., RYUN-BIN T., AE-RAN K., JONG-WAN K., SUK-KYOUNG I., BYOUNG-HAN K. 2005. Fluoroquinolone resistance and *gyrA* and *parC* mutations of *Escherichia coli* isolated from chicken. *The Journal of microbiology*, 43:391-397.

154. ZAGER F.M., MCNERNEY R. 2008. Multidrug-resistant tuberculosis. Bio Med Central *Infectious Disease,* 8:10.

155. ZUCCATO E., CALAMARI D., NATANGELO M., FANELLI R. 2000. Presence of therapeutic drugs in the environment. *The Lancet,* 355:1789-1790.

APPENDIX 1

Table A.1: Population census data for different districts on the catchments of the studied rivers (StatsOnline, 2001).

Census District	Total population	Population density (person/km)
NSIKAZI	348626	474
WITRIVIER	20803	27
NELSPRUIT	60092	27
BARBERTON	76352	21
WITRIVIER	673	0
GIYANI	207725	74
PHALABORWA	30469	3
LULEKANI	37433	39
PHALABORWA	30469	3
LULEKANI	37433	39
NAMAKGALE	48810	485
MHALA	289552	186
PELGRIMSREST (KNP)	1498	0
PELGRIMSREST	39651	14
MHALA	289552	186

Figure A.1: Census districts in the study area (ArcGIS)[1].

[1] ArcGIS: www.esri.com

APPENDIX 2

Bacteriology schedule

Sampling date: …………………..

Locality……………………………………………………

Date start laboratory work…………………………………Date stock…………………………………………

Faecal sample n.	coordinate
	S E
	S E
	S E
	S E
	S E
	S E
	S E

Water sample n.	Related to faecal s. n.	coordinate
		S E

Notes

Faecal sample	F		F		F		F		F		F	
Lactose												
Growth morphology												
	R	S	R	S	R	S	R	S	R	S	R	S
Emolitic												
Gram stain												
M.o. morphology												
Oxidase												
Spot indole												
Catalase												
Citrate												
Motility												
ID:E.coli												

Water sample	W	
Lactose		
Growth morphology		
	R	S
Emolitic		
Gram stain		
M.o. morphology		
Oxidase		
Spot indole		
Catalase		
Citrate		
Motility		
ID:E.coli		

APPENDIX 3

MIC Table

Samples group……………………………….date: ………………………………

	1 name	2 name	3 name	4 name	5 name	6 name	7 name	8 name	9 name	10 name	11 name	12 control
A	32	32	32	32	32	32	32	32	32	32	32	32
B	16	16	16	16	16	16	16	16	16	16	16	16
C	8	8	8	8	8	8	8	8	8	8	8	8
D	4	4	4	4	4	4	4	4	4	4	4	4
E	2	2	2	2	2	2	2	2	2	2	2	2
F	1	1	1	1	1	1	1	1	1	1	1	1
G	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25

APPENDIX 4

1. TITLE

A study to determine whether there is transfer of tetracycline resistant in water-born E. coli to enteric E. coli impala.

2. SENIOR RESEARCHER AND CO-WORKERS:

Supervisor: Prof. CME Mc Crindle

Co-supervisor: Dr. J Picard

Researcher: Dr. V Mariano

3. KEYWORDS

Antimicrobial resistance, water pollution, faeces, *Aepycerus melampus, Escherichia coli*

4. PROJECT STATEMENT

This project is being developed for the researcher to obtain the MSc degree in Veterinary Science.

The purpose is to find a relationship between tetracycline resistance shown by *E. coli* in untreated animals and the presence of tetracycline resistance in their water source.

The change that the project will achieve is a different attitude to the presence of antimicrobials in the water, and thus awareness for antimicrobial dissemination based on the environmental ecology of antimicrobial resistance.

Project time: February 2006-October 2007.

The samples will be collected in KNP over a period of three months, February to April 2007, in four different weeks. An attempt will be made to collect 50 faecal samples from impala and related water samples each week.

The study areas of this project involve two kinds of water sources, one polluted with tetracycline resistant *E. coli* and one not. The exact location will be decided on in consultation with the park ecologist and after a laboratory screening of the water. We tentatively propose the unpolluted rivers to be the Mpondo, Nwantindlopfu and Nwantinwambu, and the polluted ones the Crocodile and Sabie rivers.

The estimated cost of the project is R 19 810. Funding will be provided by the University of Pretoria.

Both SANParks and the researcher will benefit from this project.

This project will fit the biodiversity objective of the KNP mission, in particular the one concerning the composition, structure and pattern in the water and landscape, which try to understand the ecology of unnatural threats leading to compositional changes.

Assistance with the collection of samples and the possibility of using the laboratories facility at Skukuza for the first culture of bacteria is requested from KNP.

5.WORK PROCEDURES

Sampling procedures

The intention is to collect a minimum of 8 water samples from two different kinds of water sources. From each kind of water source 10 pooled faecal samples from 10 different impala herds drinking the water will be collected, for a total of 200 faecal samples, 100 samples for polluted water and 100 samples for unpolluted water.

Fresh faecal samples will be collected from the ground. In this way the animals will not be disturbed in any way.

More or less 10 grams of faeces will be sampled and collected into sterile sample bottles while 500 ml of each water sample will also be collected in a sterilized

bottle. All samples will be stored in a cooler bag at ± 4℃ until their arrival at the laboratory.

Laboratory analysis

All the following procedures will be executed in the bacteriological laboratory of the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria.

If it is not possible to bring the samples out of the area, the isolation of *E. coli* will be carried out in the nearest laboratory facilities. All the materials will be provided by the University of Pretoria.

Isolation of E. coli

Once the faecal samples arrive at the laboratory they will be mixed thoroughly and 1gr will be put into a sterile bottle with 10ml of peptone water (Oxoid). Four serial 10-fold dilutions will be made using physiological saline and 0.1 ml of each will be spread-plated onto MacConkey agar (Oxoid) and incubated overnight at 37℃.

The water samples will be filtered by sterile membranes of 45±2 µm pore diameter (Millipore). The membranes will be incubated overnight in peptone water (Oxoid) at 37℃. From each seven serial 10–fold dilutions w ill be made and 0.1 ml aliquots will be collected to be plated onto MacConkey agar plates.

Once the bacteria have grown, the samples can be discarded and the cultures transported to the bacteriology laboratory in the Department of Veterinary Tropical Diseases.

Replica plating

One MacConkey plate for each sample that shows the growth of a good number of separate colonies with the morphology of *E. coli* will be copied onto other MacConkey agar plates to which 4 µg/ml of tetracycline has been added. The breakpoints of replica plates were selected on the basis of previous experiments described in the literature.

Identification

After incubation, three colonies showing tetracycline resistance and three colonies not showing tetracycline resistance, if there are, will be selected from each MacConkey plate and streaked onto Blood agar (Oxoid), incubated at 37℃ overnight, for identification purposes.

Bacteria from the blood agar will be identified using Gram staining, their morphology and the use of the following biochemical tests: oxidase, catalase, spot indole, citrate and motility.

Once *E. coli* is isolated and identified from each sample, it will be stored in Nunc tubes with brain-heart infusion broth (BHI) and glycerol 10% in the freezer (Forma scientific freezer) at - 86ºC.

Testing susceptibility to tetracycline

Once *E. coli* is stored from all the samples collected, antimicrobial resistance patterns will be determined. Susceptibility to tetracycline will be determined using the minimum inhibitory concentrations (MICs) technique. The guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) will be used as the reference method for preparing the varying dilutions and determining the MICs.

Analysis of data

After an accurate monitoring, the water sources will be divided into two categories: polluted with tetracycline resistance and not polluted. Each faecal sample will be classified as resistant or sensitive. The strength of association will be calculated between the pollution of the water source and the percentage of tetracycline resistance faecal samples by a statistical acceptable method. In this case it is going to be evaluated the odds ratio, relative risk, the risk ratio and the attributable risk.

6.MAJOR MILESTONES

The result of the research will be reported in the MSc dissertation of the researcher and as a peer-reviewed scientific publication in an internationally circulated scientific journal. SANParks will obviously be acknowledged.

7.PROJECT RISK

This project is a low risk activity. The animal use and care committee of the Faculty of Veterinary Science has already established that there are no risks associated with this project for animals. The only risks associated with the project are related to the risks for the researcher and an assistant going into the field collecting the samples.

8.DETAILS

Contact details

Researcher: Dr. V Mariano
Postgraduate student MSc (Veterinary Science)
Section Veterinary Public Health
Dept. Paraclinical Sciences
Faculty of Veterinary Science
University of Pretoria
Tel. 012 529 8477
Fax. 012 529 8311
e-mail: valeria_m3@yahoo.it (valeria#underscore#m3@yahoo.it)

Supervisor: Prof. CME Mc Crindle
Section Veterinary Public Health
Dept. Paraclinical Sciences
Faculty of Veterinary Science
University of Pretoria
Private Bag X04
Onderstepoort 0110
Tel. 012 529 8477
Fax. 012 529 8311
e-mail: cheryl.mccrindle@up.ac.za

Co-supervisor: Dr. J Picard
Section of Bacteriology
Dept. of Veterinary Tropical disease
Faculty of Veterinary Science

University of Pretoria
Tel. 012 529 8017
Fax: 012 529 8312
e-mail: jackie.picard@up.ac.za

Location/s of project

The project will be carried out in the Nikayeni and Marula regions, in particular closed to the following river: Mpondo, Nwantindlopfu, Nwantinwambu, Crocodile and Sabie.

9. BACKGROUND INFORMATION

It is undisputed that in the recent years problems associated with the presence of antimicrobial resistance (AMR) have reached epidemic proportions. A direct link between the use of antibiotics in animals and development of resistant infections in humans has been postulated (Van de Bogaard *et al.,* 1997). Many countries therefore are monitoring the use of antimicrobials in animals (Aarestrup *et al.,* 2001; Van de Bogaard *et al.,* 1997). The estimation of the AMR extent is thus of fundamental importance and many surveillance programmes have been established worldwide (Franklin & van Vuuren, 2001).

In South Africa the veterinary profession runs a national strategy for the use of antibiotics in animals as well as a standard antimicrobial resistance surveillance programme. This programme has already established a standard laboratory method for some zoonotic and indicator bacteria, among them *E. coli* (Nel *et al.,* 2004), the bacterium that will be used for this study.

Tetracyclines are the most commonly sold antibiotic in South Africa. They are essential in the treatment of common tick borne diseases such as anaplasmosis and heartwater in ruminants. We also know that tetracycline resistant *E. coli* is highly prevalent in food animals in South Africa (Virbac antimicrobial resistance database, 2002). Recent studies have proved that the various *tet* genes coding for tetracycline resistance can be exchanged between bacteria, including enteric bacteria, in many ecosystems (Bryan *et al.,* 2004). Thus the potential for the

dissemination of tetracycline resistance in the environment, as well as by animals and their products, is high.

The knowledge of how tetracycline resistance moves within animals and their habitat would assist greatly in determining a risk management policy for this antibiotic in South Africa. Although several studies assessed AMR in *E. coli* populations of animal origin, little work has been done on molecular ecology and epidemiology – linking the type of resistance to the environment and the animal host (Boerlin *et al.,* 2005).

Wildlife populations are useful in the study of the dissemination of tetracycline resistance in nature as they are never treated with antimicrobials, have no contact with farm animals and with humans, and often live in areas with so low population densities that resistant bacteria exchanges are extremely difficult. Thus they can be used to study the effect that pollution, in particular polluted water, has in the dissemination of tetracycline resistance.

Impala (*Aepyceros melampus*), which are selective grazer and browser, were chosen for this project because they are common in many regions in South Africa, have a wide range of habitats, and tend to remain in their territoria (Skinner & Chimimba, 2005).

Literature review

Escherichia coli

Escherichia coli is a common inhabitant of the lower bowel of man and animals and, as is the case for other commensal bacteria, it is considered to constitute a reservoir of resistance genes, which may be transferred to pathogenic bacteria causing disease (Sayah *et al.,* 2005). It could itself be the etiological agent for various types of human and animal infections. In fact it is one of the most common bacteria causing diarrhoea, septicaemia, peritonitis, cystitis, other infections of the urinary tract and pyogenic wound infections. There are also certain highly pathogenic strains, such as *E. coli* 0157, that can produce diarrhoea with

abdominal cramping, sometimes with haemorrhagic colitis or complicated by the often fatal haemolytic uraemic syndrome in humans (White *et al.,* 2002).
Recent studies show that the prevalence of AMR in *E. coli* is increasing and it has been suggested that this is due to the increasing use of antibiotics in livestock (Van de Bogaard *et al.,* 1997). There is also a threat that AMR zoonotic bacteria could be distributed to humans through animal products (You *et al.,* 2006).

E. coli is usually the most prevalent bacterium in the intestinal tract of humans and animals and for this reason it is used as the most common indicator of faecal contamination of water (Vantarakis *et al.,* 2006). Because of its particular ability to survive in the environment it could easily transfer AMR to other organisms, particularly other enterobacteria, in the environment (Kűmmer, 2004). AMR is gained though various mobile elements, such as plasmids, transposons, and integrons, which result in mutation in genes responsible for antimicrobial agent uptake or binding sites or activation of bacterial chromosomes (Sayah *et al.,* 2005).

Tetracycline

Tetracyclines are broad spectrum antibiotics that inhibit the growth of a wide variety of bacteria, protozoa and many intracellular organisms, such as mycoplasma, chlamydia and rickettsia (Booth & McDonald, 1988). Their function is to inhibit bacterial protein synthesis by binding the 30S ribosomal subunit of microrganisms and preventing the attachment of aminoacyl-tRNA to the RNA/ribosome complex (Adams, 1999; Chopra & Roberts, 2001).

Because of their broad spectrum and of low cost, tetracyclines have many therapeutic applications in clinical veterinary medicine and as animal growth promoters. In countries where the use of tetracyclines as food additives is still permitted, it has been estimated that approximately 90% of tetracyclines are administered to cattle and swine at sub-therapeutic concentrations whereas only 15% of usage in poultry reflects sub-therapeutic administration (Chopra & Roberts, 2001).

Their use in human therapy has declined, possibly due to increasing AMR. They are only used as drugs of choice as part of a triple therapy for management of gastritis and peptic ulcer disease associated with *Helicobacter pylori* and for prophylaxis of malaria (for some resistant strains of *Plasmodium falciparum* to mefloquine). They are also effective for zoonotic diseases such as *Ehrlichia conori* (tick fever), psittacosis and in combination with other drugs, for brucellosis *(B. abortus* and *B. melitensis*). Because they are cheap they are particularly attractive for use in developing nations. In fact, the HIV Meeting in 2000 suggested the use of tetracyclines to reduce sexually transmitted bacterial diseases in the developing world (Chopra & Roberts, 2001).

Resistance to tetracycline is conferred by twenty-nine different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes. These genes have been associated with mobile plasmids, transposons, conjugative transposons, and integrons. Their transmission in bacterial populations can take place by clonal spread of particular strains or horizontal transfer of resistance by plasmid- or transposon-mediated conjugation (Hartman *et al.,* 2003).

These genes encode for three different mechanisms of resistance: an efflux pump, a method of ribosomal protection or direct enzymatic inactivation of the drugs. Essentially, *tet* and *otr* genes have the same mode of action (Bryan *et al.,* 2004).

Prevalence of antimicrobial resistance in wildlife

In South Africa, there is little published information about AMR in wildlife and in the environment. In fact we cannot be sure whether we should consider wildlife species as source of resistant bacteria or as sentinels for the spread of resistant organisms or genes. There are a few studies in other countries (Poeta *et al.,* 2005). These studies show that AMR is present in low levels and differ from place to place. An example of this has occurred in the USA, in Minnesota where 4% of *E. coli* obtained from wild animals were resistant to tetracycline (MIC>10 μg/ml)(Bryan *et al.,* 2004), while in the Cedar River area in Michigan only 1.85% were tetracycline resistant (Sayah *et al.,* 2005). This is most probably due to the fact that these animals are never treated with antibiotics. In domestic animals resistance is much higher.

Laboratory analysis of antimicrobial resistance

There are several methods to detect AMR in the laboratory: those based on phenotypic resistance and those on genetic determinants of resistance. Obviously the use of both methods together can optimize the collection of information about AMR. Unfortunately the detection of genetic resistance is still expensive and for that reason is not applicable in this project, even it could be interesting for further studies.

For the determination of the phenotypic resistance patterns of bacteria, mainly two antimicrobial susceptibility methods are commonly used. These are the Kirby-Bauer disc diffusion test and the Minimal Inhibitory Concentration method using broth dilutions (White *et al.,* 2001).

The first one is cheaper and faster than the second and so it is of common use in clinical practice. This is a qualitative method that is able to divide bacteria into resistant or sensitive to the antibiotics specifically tested for. The cut-off values in this test are based on clinical cut-off values. The MIC method on the other hand is a quantitative method that enables determination of the lowest concentration of antibiotic that inhibits the visible growth of a micro-organism after overnight incubation. This method is considered the 'gold standard' for laboratory phenotypic studies on the susceptibility of an organism to antimicrobials. It is able to detect low-level resistance as well as trends in resistance over time (Andrews, 2001).

A method that enables detection of the presence of resistant bacteria and their percentage in a mixed population is the replica plating method (Österblad *et al.,* 1995). It is a technique in which one or more Petri plates containing different solid selective media are inoculated with the same colonies of micro-organisms from a primary plate, reproducing the original spatial order.

There are two main different techniques of replica plating. The first is the traditional method, invented by Lederberg (1951), and consists of pressing a velvet-covered disk onto a master plate and then printing it on secondary plates. The alternative method, invented by Lindstrom (1977), uses more complex equipment than the previous; it picks up each single colony to be transferred onto

a filter paper that will function as a stamp. The Lederberg method may give misleading results on the percentage of resistant and sensitive colonies, because the replicator does not produce uniform pressure over the entire surface of the print. It is chosen in this protocol because in this study it is not important determine the percentage of resistant and sensitive strains and also because it is the less expensive and time-consuming method.

References

AARESTRUP F.M., SEYFARTH A. M., EMBORG H.D., PEDERSEN K., HENDRIKSEN R.S., 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(7):2054-2059.

ADAMS H.R. 1999. *Farmacologia e terapeutica veterinaria seconda ed. italiana* ed E.M.S.I. Roma.

ANDREWS J.M. 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 48:5-16.

BOERLIN P., TRAVIS R., GYGLES C.L., REID-SMITH R., JANECKO N., LIM H., NICHOLSON V., MCEWEN S.A., FRIENDSHIP R., ARCHAMBAULT M. 2005. Antimicrobial resistance and virulence genes of *E. coli* isolates from Swine in Ontario. *Applied and Environmental microbiology,* 71(11):6753-6761.

BOOTH N.H., MCDONALD L.E. 1988. *Veterinary pharmacology and therapeutics -6th ed.* Ed. Science Press, Ephrata.

BRYAN A., SHAPIR N., SADOWSKY M.J. 2004. Frequency and distribution of tetracycline resistance genes in genetically diverse, no selected, and no clinical *E. coli* strains isolated from diverse human and animal sources. *Applied and Environmental Microbiology,* 70(4):2503-2507.

CHOPRA I., ROBERTS M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacteria resistance. *Microbiology and Molecular Biology Reviews,* 65(2):232-260.

EPA 2002. *Method 1064: Total coliforms and Escherichia Coli in water by membrane filtration using a simultaneous detection technique (MI medium).* Office of water Washington DC 20460 for EPA 821-R-02-024.

EPA FEDERAL REGISTER ONLINE VIA GPO ACCESS. 2001. *Guidelines establishing test procedures for the analysis of the pollutants; analytical methods for biological in ambiental water: proposed rules.* Federal register, 66(169):45811-45829.

FRANKLIN A., VAN VUUREN M. 2001. Antimicrobial resistance: harmonisation of national antimicrobial resistance monitoring and surveillance programmes in animals and in animals-derived food. *Revue Scientifique et Technique-office International des Epizooties,* 20(3):859-870.

HARTMAN A.B., ESIET I.I, ISENBARGER D.W, LINDLER L.E.2003. Epidemiology of Tetracycline resistance determinants in *Shigella* spp. and enteroinvasive *Escherichia coli*: characterization and dissemination of tet(A)-1. *Journal of Clinical Microbiology,* 41(3):1023-1032.

KŰMMER K. 2004. Resistance in the environment. *Journal of Antimicrobial Chemotherapy,* 54(2):311-320.

LEDERBERG J., LEDERBERG E.M. 1951. Replica plating and indirect selection of bacterial mutants. *Applied and Environmental Microbiology,* 63:399-406.

LINDSTROM E.B. 1977. Alternative replica plating technique. *Applied and Environmental Microbiology,* 33(2):224-227.

NEL H., VAN VUUREN M., SWAN G.E. 2004. Towards the establishments and standardization of a veterinary antimicrobial resistance surveillance and monitoring programme in South Africa. *Onderstepoort Journal of Veterinary Research,* 71:239-246.

ÖSTERBLAD M., LEISTEVUO T., HUOVINEN P. 1995. Screening for antimicrobial resistance in faecal samples by the replica plating method. *Journal of clinical Microbiology,* 33(12):3146-3149.

POETA P., COSTA D., SAÈNZ Y., KLIBI N., RUIZ-LARREA F., RODRIGUES J., TORRES C. 2005. Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. *Journal of Veterinary Medicine series –infectious disease and Veterinary Public Health*, 52:396-402.

SAYAH R.S., KANEENE J.B., JOHNSON Y., MILLER R. 2005. Patterns of antimicrobial resistance observed in *E. coli* isolates obtained from domestic and wild-animal faecal samples, Human septage, and surface Water. *Applied and Environmental Microbiology*, 71(3):1394-1404.

SKINNER J.D., CHIMIMBA C.T. 2005. *The mammals of the Southern African Subregion*. 3rd edition, Cambridge University Press.

STEGEMAN M., BECKMAN G.T. 1994. *In-vitro susceptibility testing in veterinary practise. Indications, technique, interpretation*. Gustav Fischer Verlang Jena.

THE DEPARTMENT OF WATER AFFAIRS AND FORESTRY, THE DEPARTMENT OF HEALTH, WATER RESEARCH COMMISION. 2001. *Quality of domestic water supplies, sampling guide(2).* Water research Commission n° TT 117/99.

VAN DE BOGAARD A.E., MERTENS P., LONDON N.H. STOBBERINGH E.E. 1997. High prevalence of colonization with vancomycin and pristinamycin resistant enterococci in healthy humans and pigs in the Netherlands: is the addition of antibiotics to animal feeds to blame? *Journal Antimicrobial Chemotherapy,* 40:454-456.

VAN DE BOGAARD A.E. 1997. Antimicrobial resistance-relation to human and animal exposure to antibiotics. *Journal Antimicrobial Chemotherapy,* 40:453-454.

VANTARAKIS A.,VENIERI D., KOMNIOU G., PAPAPETRPOULOU M. 2006. Differentiation of faecal *E. coli* from Humans and animals by multiple antibiotic resistance analysis. *Letters in applied Microbiology,* 42:71-77.

WHITE D.G, ZHAO S., MCDERMOTT P.F., AYERS S., GAINES S., FRIEDMAN S., WAGNER D.D., MENG J., NEEDLE D., DAVIS M., DEBROY C. 2002. Characterization of antimicrobial resistance among *Escherichia coli* 0111 isolates of animal and human origin. *Microbial Drug Resistance,* 8(2):139-146.

WHITE D.G., ACAR J., ANTHONY F., FRANKLIN A., GUPTA R., NICHOLLS T., TAMURA Y., THOMPSON S., THRELFALL E.J., VOSE D., VAN VUUREN M., WEGENER H.C., COSTARRICA M.L. 2001. Antimicrobial resistance: standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. *Revue Scientifique et Technique-office International des Epizooties,* 20(3):849-858.

YOU J.Y., MOON B., OH I., BAEK B., LI L., KIM B., STEIN B., LEE J. 2006. Antimicrobial resistance of *E. coli* 0157 from cattle in Korea. *International Journal of food Microbiology,* 106:74-78.