Prevalence of Antibiotic-Resistant *Escherichia coli* in Faecal Samples from Domestic Animals and Wildlife: a Cross – Sectional Study

BY

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This thesis

is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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March 2009

Declaration

I declare that this thesis has not been previously submitted for a degree at this or any other university, and I further declare that the work incorporated in it is my own

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March, 2009

CONTENTS Page

List of Tables

List of Figures

Chapter 2:

Chapter 3:

ACKNOWLEDGEMENTS

I am grateful that the knowledge gained through completing this study is very relevant and useful to my field of work in Libya, and for the support of the Libyan government, who funded my study.

I would like to express my gratitude to all those who made it possible to complete this thesis.

First and foremost, I wish to express my sincere appreciation to my supervisor Professor Malcolm Bennett (Dean of the Faculty of Veterinary Science), for his encouragement and guidance in this project.

Secondly, I wish to extend my gratitude to Dr. Nicola Williams who provided guidance and expertise on all aspects of the antibiotic resistance work carried out.

I am also indebted to Professor Tony Hart for his constructive comments and suggestions before he left. He will be remembered.

My thanks must go to Professor Martin Woodward (Department of Food and Environment Safety, Veterinary Laboratories Agency) and his colleagues who allowed me a wonderful opportunity to learn about molecular biology and who supported my work in the laboratory.

I also thank the other members of my thesis committee Prof. Craig Winstanley, Professor Janet Bradbury, Dr. Rob Christley, and Dr. Julian Chantry.

I am grateful to my two special friends Dr. Trevor Jones for technical assistance and kind help with the lab work, sample processing, antimicrobial sensitivity tests, PFGE, and to Dr. Laura Hughes for her invaluable help and advice on countless occasions, especially at the time of writing up my thesis.

I am very grateful to Dr. Christian Setzkorn for his willing assistance with computer database programmes on many occasions.

I would like to thank all who work in the Department of Veterinary Pathology for their friendship, invaluable help and advice on countless occasions, especially in the lab. Particular thanks to Thelma Roscoe for her excellent technical support.

I would like to say big thank you to everybody who worked on the VTRI project, Especially Dr. Howard Leatherbarrow and Dr. Angela Lahuerta-Marin who helped with sample collection

My sincere appreciation goes to Sylvia Yang for her skilled secretarial assistance on numerous occasions

Particular thanks to my friend Mohamed Ali Ibrahim for being my best friend and for his continued support and interest in what I do, and further I would like to mention my office mate Lukasz Lukomski.

Lastly, and most importantly, my thanks most go to my family, to whom I would like to dedicate this work: my wife Fawzia, sons Momajed, Moesr, Mubasher and Mubien. I want them to know that I am very grateful for their unreserved love and encouragement throughout my studies. You have all been a source of inspiration and moral support. Without their love and patience, I would not have become who I am now. My thanks also go to my family in Libya, my father, brothers and sisters for their unfailing support and encouragement. I hope I can always be there for you in the way you supported me.

ABSTRACT

Antibiotic resistance is a growing problem worldwide: resistance develops not only in pathogens but also in the normal microflora of the treated host. Relatively little is known about the ecology and evolution of antibiotic resistance in these commensal bacteria.

The aim of this work was to determine the prevalence of antibiotic resistance in several wild animal and domestic animal populations on farms, and to investigate the role of wildlife as a potential source of resistant organisms or resistance genes to domestic animals and man. A cross sectional study was undertaken, with faecal samples collected from cattle and wildlife (wild rodents, birds and other wild mammals such as rabbits, foxes and badgers) on six farms (five dairy and one beef) in Cheshire. Isolation and identification of drug-resistant and drug-sensitive *Escherichia coli* was performed using enrichment and EMBA agar containing different antibiotics (ampicillin, chloramphenicol, nalidixic acid, tetracycline, trimethoprim), followed by antibiotic susceptibility testing by the disc diffusion method to the above antibiotics plus augmentin and ciprofloxacin.

A total of 2084 faecal samples were collected and examined, 447 from cattle, 918 from wild rodents, 105 from other wild mammals, and 614 from wild birds. *E. coli* were detected in 1303 animal faecal samples (62%). Of these, 491 samples (38%) contained *E. coli* that were resistant to at least one antibiotic. Antibiotic resistance was less prevalent in *E .coli* from bird and rodent faeces than from cattle and other wild mammals, but varied from farm to farm. Resistance to tetracyclines and ampicillin had the highest prevalence on all farms and in all species. Resistance to nalidixic acid ciprofloxacin had the lowest prevalence compared with other types of

antibiotics. Furthermore, multi-drug resistance (MDR) was common. The MDR profile was used to identify potential transmission between species. The genes responsible for resistance in MDR isolates with similar profiles but from different species were investigated by DNA microarray. There was good correlation between phenotype and genotype for most isolates and in total 52 (28%) isolates from cattle and wildlife were identified with identical patterns of phenotypic and genotypic resistance. Further characterization of these was carried out by pulsed field gel electrophoresis (PFGE): most isolates had different patterns, but a small number had identical patterns, providing evidence for direct transmission between hosts.

This study shows that the ecology and transmission of antibiotic resistant *E. coli* is complex. Transmission between hosts appears unusual, with each host population largely maintaining its own population of resistant bacteria. However, transmission can occur, and thus each host might be able to act as a reservoir of resistant bacteria and resistance genes for the others. Thus the control of antibiotic resistance on farms is more complex than simply reducing the use of antibiotics

Chapter One

Introduction

1.1 Introduction

Antibiotic resistance has received considerable attention in both human and veterinary medicine as it can prevent successful treatment of bacterial infections, and may be associated with an increase in mortality, especially in hospitalized patients. Bacteria have been isolated that resist most, if not all, currently available classes of natural and synthetic antibiotics, and can sometimes only be treated with experimental and potentially toxic drugs (Todar, 2008; Alekshun & Levy, 2007). Recent high profile examples of resistance include methicillin / oxacillin-resistant *Staphylococcus aureus* (MRSA) (Boucher & Corey, 2008; Van Belkum & Verbrugh, 2001) and vancomycin-resistant enterococci (VRE) (Liu *et al.*, 2008; Malathum & Murray, 1999). Another recent example of resistance evolution is the increasing problem of extended spectrum beta-lactamases (ESBL's) in Enterobacteriaceae which are resistant to cephalosporin and monobactamas (Livermore & Woodford, 2006; Paterson & Bonomo, 2005).

Resistance is widespread, and found in normal enteric bacteria as well as pathogens. The studies described in this thesis developed from the finding of resistance in wildlife in Great Britain, some of which had little contact with human beings or domestic animals (Gilliver *et al.*, 1999). More recently it was reported that antibiotic resistance to one or more commonly prescribed antibiotics were found in eight birds from the arctic tundra of north-eastern Siberia, northern Alaska, and northern Greenland (Sjölund *et al.*, 2008).

Levy (1998) mentioned that the indirect transmission route of resistance through commensal bacteria, and the environment, maybe as significant as the route of direct contact between animals and humans or direct transmission through the food chain.

1.2 History and evolution of antibiotics and antibiotic resistance

It has been suggested that the increased prevalence of resistance seen in clinical human and veterinary medicine is largely due to selection through the use of therapeutic antibiotics, or, in animals, antibiotics as growth promoters. Resistance is selected for not only in those microbes that are the targets of antibiotic treatment, but also in those of the normal microflora. Thus commensal bacteria can be a large potential reservoir of resistance genes for bacterial pathogens. The use of some antibiotics, often at sub-therapeutic doses, as growth promoters has led to particular problems (Alexander *et al.,* 2008; Salyers *et al.*, 2004), and has therefore been banned in many countries (Anadon, 2006). The use of avoparcin, for example, in poultry was linked to the emergence of vancomycin resistance in *Enterobacteriaceae* infecting human beings, and thus has been banned in the European Union since December 2005 (Anadon, 2006; Anderson *et al.*, 2003; Aarestrup *et al.*, 2001).

It is important to remember that resistance is not caused by the use of antibiotics, merely selected for. As most antibiotics are either natural substances, or based on natural substances, it is not surprising that the evolution of antibiotics in the environment over millions of years of inter- and intra-specific competition has, naturally, been accompanied by the evolution of resistance. Several groups (e.g. Davies, 1997; Waters & Davies, 1997; Wiener *et al.*, 1998) have commented that the sources of resistance genes in animal and human bacteria are usually not known, rather like the genes encoding the antibiotics themselves. However, Medeiros (1997) in reviewing the structure and evolution of beta-lactamas, pointed out that clinically-significant beta-lactamase resistance may involve only minor changes to pre-existing genes/operons whose function is often communication between bacteria.

Some studies have suggested that the expression of antibiotic resistance in bacteria imposes a fitness cost for the bacterial cell carrying it, so antibiotic resistance is disadvantageous unless the antibiotic is present in the environment (Andersson, 2006; Nilsson *et al.*, 2006; Austin *et al.*, 1997; Levin *et al.*, 1997). Therefore if the antibiotic is not present in the environment, without selection resistance will gradually be lost from the bacterial population (Andersson & Levin, 1999; Austin *et al.*, 1997; Levin *et al.*, 1997). More recent studies indicate that the maintenance of resistance might not impose a significant fitness cost (Khachatryan *et al.*, 2006; Khachatryan *et al.*, 2004; Sander *et al.*, 2002). Moreover the acquisition of the antibiotic resistance genotype may increase the fitness of some bacteria, leading to an increased prevalence of antibiotic resistance (Yates *et al.*, 2006; Dionisio *et al.*, 2005; Enne *et al.*, 2004). For example, Blot *et al* (1994) suggested that the bleomycin resistance gene contained in transposon Tn*5* enhanced greater fitness through improved survival and growth advantage to *Escherichia coli.* Enne *et al* (2004) reported that the enhanced fitness observed is due to an increase in growth rate conferred by the *sul2*-coding plasmid p9123 carriage.

Groh *et al* (2007) noted that the presence of a multidrug resistance pump coded by the MexF gene can conferred ecological fitness of non-pathogenic *Shewanella oneidensis* Mr-1 in sediment, as this bacterium was isolated from an environment without pharmaceutical impact.

1. 3 **Mechanisms of antibiotic resistance in bacteria**

Drug-resistant strains may have multiple mechanisms of resistance for the same antibiotic as well as different mechanisms of resistance for different antibiotics. Resistance can occur to a single antibiotic or to several antibiotics (multiple

resistance). Multiple-resistant strains can survive in the presence of various antibiotics and depending on the mechanism involved. The mechanisms by which bacteria can resist the action of antibiotic are generally known. Bacteria can gain antimicrobial resistance in two main ways;

1. 3. 1 **Inherent resistance**

Vertical gene transfer occurs after the resistant genes are acquired. Resistance genes can be spread by vertical transmission from parent to offspring (Petersen *et al.*, 2oo6; Clark, 2005).

In other words an organism receives genetic material from its ancestor, e.g. parents or a species from which it evolved. Petersen *et al* (2006) reported that vertical transmission of enrofloxacin-resistant *E. coli* from healthy parents resulting in high first week mortality in the offspring illustrates the potential of the emergence and spreading of fluoroquinolones-resistant bacteria in animal husbandry, even though the use of fluoroquinolones is restricted.

There are several reasons why micro-organisms may have inherent resistance to an antibiotic:

- The organism may lack the structure that an antibiotic inhibits.
- The organism may be impermeable to the antibiotics.
- The organism may be able to change the antibiotic to an inactive form.
- The organism may modify the target of the antibiotic: e.g. alteration of PBP – the binding target site of penicillin – in MRSA and other penicillin-resistant bacteria.

Intrinsic resistance can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic alteration.

1. 3. 2 **Acquired resistance**

The genes responsible can spread widely and rapidly through horizontal gene transfer (HGT) or lateral gene transfer (LGT): Any process in which an organism transfers genetic material to another cell that is not its offspring. Resistance genes can be transferred between bacteria, and the ability of *E. coli* to transfer antimicrobial drug resistance is well known (Bennett, 2004; Blahna *et al.*, 2006).

The mechanisms involved in resistance acquisition are generally well understood on the molecular level. Antibiotic resistance genes can be acquired in any of the three ways: transduction, transformation, and conjugation (Aminov & Makie 2007; Bennett, 2004).

Transduction is gene transfer mechanism facilitated by bacteriophages that inject their DNA into the genome of a host bacterium (spontaneous DNA mutation and recombination). Recombination has an important role in the evolution of antibiotic resistance determinants. A good example of this mechanism is resistance to tetracycline and chloramphenicol in some *Enterobacteriaceae* (Murray, 1991). Bacteriophage-associated transduction of antibiotic resistance determinants has been described, and can act as a vector for DNA between bacterial cells (Giraud *et al.*, 2002).

Another mechanism of gene transfer is transformation, whereby one bacterium takes up naked DNA from the environment, via incorporation of chromosomal or plasmid DNA, from a variety of organisms (Levy & Marshall, 2004).

Conjugation results from the transfer of accessory pieces of DNA that are separate from the chromosome, plasmids, that can move from one bacterium to another. Plasmids are independent, self-replicating genetic elements which may fuse with other plasmid and thus acquire new genes. Plasmids are transferred from one bacterium to another by conjugation and continue to multiply once they have entered a new host. Such exchange may occur between related and unrelated bacteria (Yan *et al.*, 2003; Rubens *et al.*, 1979).

Plasmids are considered as major vectors for the dissemination of both antibiotic resistance and virulence determinants among bacterial populations. Plasmids that carry the genes necessary for conjugation are called conjugative plasmids.

Other genetic elements that play a role in the spread of antimicrobial resistance include transposons and integrons.

Transposons are mobile genetic elements that can exist on plasmids or integrate into other transposons. In other words, sequences of DNA that are capable of transposing or moving from one region to another, either by integration in transferable plasmids or by direct conjugation and further integration into bacterial chromosomes; they can contain one or more genes (Roe & Pillai, 2003; Normark & Normark, 2002).

Integrons were first identified in 1980s (Stokes & Hall, 1989) as mobile elements that consist of conserved sequences of DNA bordering "cassettes" of genes, mobilizing by site-specific recombination (Fluit & Schmitz, 2004). Different classes

of integrons have been described (Nield *et al.*, 2001): class 1 integrons are the most common, and occur on bacterial chromosomes and plasmids. Integrons have the ability to spread among different Gram-negative bacteria species (Singh *et al.*, 2005).

1. 4 **Antibiotics and antibiotic resistance**

Antibiotics are chemical substances produced by microorganisms that kill or inhibit other microorganisms, and their use is therefore important in the treatment of infectious disease. Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic.

Antibiotics were discovered by Scottish physician Alexander Fleming in 1928. He realized that the fungus infecting his bacterial culture was excreting a substance that killed bacteria (Fleming, 1929). Fleming was not able to extract pure penicillin. But his was one of the most important discoveries in last century. In 1939, Flory and Chain were able to extract penicillin and it was used to treat bacterial infections during the Second World War. This substance was first isolated from *Penicillium notatum* and is now known as penicillin. In 1943, Selman Waksman's group working on compounds produced from soil microorganisms (Greenwood, 2000), discovered streptomycin (Schatz, 1944). Other antibiotics such as chlortetracycline and chloramphenicol were discovered shortly thereafter (Garrod & O'Grady, 1971), and many more antibiotics were discovered and developed subsequently. From the 1960s until recently there were few discoveries of new antibiotics, and most of the drugs developed have been chemical modifications, or combinations of existing drugs with different mechanism of action, to increase their effectiveness and to help overcome the problem of antibiotic resistance.

Antibiotics can be classified in several ways according to their antimicrobial spectrum (broad versus narrow), route of administration, mechanism of action, producer strains, manner of biosynthesis, type of activity (bactericidal, bacteriostatic), and their chemical structures.

This thesis describes the prevalence of antibiotic-resistant *E. coli* in faecal samples from domestic animals and wildlife. Resistance to seven kinds of antibiotics (ampicillin, augmentin, chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline and trimethoprim) is studied, and these antibiotics were chosen because all are commonly used in both veterinary and human medicine, and they represent different antibiotic classes, each with different mods of action

1. 4. 1 **Ampicillin and augmentin resistance**

Beta-lactam antibiotics are commonly used in human and veterinary medicine. Gram-negative bacteria are normally less sensitive to penicillin derivatives, but other ß-lactam antibiotics such as ampicillin, carbencillin, and cephalosporin also act against a range of Gram-negative enteric bacteria.

Livermore (1996) reported that ß-lactams are the most widely used antibiotics, and that resistance to ß-lactams is a severe threat because these drugs have low toxicity and are used to treat a broad range of infections. The most important groups of ß-lactam antibiotics are the pencillins and cephalosporins (Franklin & Snow, 2005; Mason & Kietzmann, 1999), although the family also includes methicillin, monobactams, cephamycins and carbapenems.

The main feature of these antibiotics is that have beta-lactam ring, and are specific inhibitors of bacterial cell wall (peptidoglycan) synthesis. When growing bacteria

are exposed to ß-lactam antibiotics, the antibiotic binds to regulatory enzymes, such as transpeptidases, carboxypeptidases and endopeptidases (also called penicillin binding proteins, PBPs) in the growing bacterial cell wall, thereby inhibiting synthesis of peptidoglycan and resulting in bacterial cell death (Franklin & Snow, 2005). Much resistance is caused by selection for ß-lactamase genes among Gram negative bacteria. The genes for these enzymes probably originate amongst bacteria found in the soil (Ghuysen, 1991). Clavulanic acid resembles the ß-lactam molecule but has very week antibacterial action. However, it binds to the ß-lactamase, inhibiting its action, so clavulanate is often used with penicillins (for example amoxicillin) in order to overcome resistance caused by ß-lactamase.

1. 4. 2 **Nalidixic acid and ciprofloxacin resistance**

The first quinolone, nalidixic acid, was synthesised in 1962 (Lescher *et al.*, 1962). This group of antimicrobials, which includes ciprofloxacin, norfloxacin, enrofloxacin and ofloxacins, is widely used in human and some of this products are available in veterinary use (Mitsuhashi, 1993).

The quinolones are active against both Gram-positive and Gram-negative bacteria, and inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV (Hooper, 2001; Baucheron *et al.*, 2004; Drlica & Malik, 2003). The most common mechanisms of quinolone resistance are two types of chromosomal mutations in the DNA gyrase and topoisomerase IV or alterations in drug permeability involving mutations that increase expression of endogenous multidrug efflux pumps, alter outer membrane diffusion channels, or both (Jacoby & Munoz-Price, 2005; Ruiz, 2003; Hooper, 2001).

1. 4. 3 **Tetracycline resistance**

Tetracyclines are a commonly used first line antibiotics for many different species of domestic animals. They are a broad spectrum bacteriostat that inhibits bacterial protein synthesis by binding to the A-sit of the 30S subunit of the bacterial ribosome, thus preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (Peterson, 2008; Chopra & Roberts, 2001). Resistance to tetracycline has been reported in many pathogenic and non-pathogenic bacteria due to the acquisition of *tet* genes. At least 29 different tetracycline resistance genes with different mechanisms of action have been reported. Some work by increasing drug efflux; *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), tet(I), *tet*(J), *tet*(Z), *tet*(30)*b*, *tet*(31)*b*, tet(K), *tet*(L), *otr*(B), *tcr3c*, *tetP*(A), *tet*(V), *tet*(y), some by ribosomal protection: *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *otr*(A), *tetP*(B) (Chee-Sanford *et al.*, 2001). Other *tet* genes are enzymes that affect the drug directly, such as *tet*(X), while the mechanisms of action of other genes, such as *tet*(U), *otr*(C) remains unknown.

Tetracycline efflux appears to be more abundant among Gram-negative bacteria. By exporting tetracycline the antibiotic fails to accumulate in the cell in sufficient concentration to inhibit protein synthesis and block growth of the bacterium. To date, eight different *tet* genes for efflux proteins have been sequenced in Gram negative bacteria (Berens & Hillen, 2003; Butaye *et al.*, 2003; Schwarz *et al.*, 2001). Resistance genes tet (A), tet (B), tet (D) and tet (H) are often found on transposons, while resistance genes tet (C), tet (E) and tet (C) are often found on large conjugative plasmids that may harbour other antibiotic resistance genes (Jones *et al.*, 1992).

1. 4. 4 **Chloramphenicol resistance**

Chloramphenicol is a broad spectrum-antibiotic, derived from bacterium *Streptomyces venezualae.* It was introduced into clinical practice in 1949. Chloramphenicol interferes with bacterial protein synthesis by binding reversibly to the 50S subunit of bacterial ribosome, preventing amino acids from binding to the nascent peptide chain. Resistance is caused by a number of enzymes that alter the chloramphenicol molecule to prevent binding to the bacterial ribosome, or by alteration of outer membrane permeability in Gram-negative bacteria thereby preventing the drug from entering the cell (Bryan, 1984). The *catI* gene is the most reported and widespread gene responsible for resistance (Alton & Vapnek, 1979): chloramphenicol acetyltransferase (CAT) catalyses the transfer of an acetyl group from acetyl-CoA to both hydroxyl groups on the drug, preventing it from binding to the ribosome and rendering the drug inactive. Non-enzymatic resistance to chloramphenicol is through drug efflux, associated with *cmlA* genes unique to Gram-negative bacteria (Schwarz *et al.*, 2004; Williams, 1996). The *floR* gene also encodes an efflux pump that confers resistant to both chloramphenicol and fluorfenicol, and transporters can be plasmid or chromosome encoded (Singer *et al.*, 2004)

1. 4. 5 **Trimethoprim resistance**

Trimethoprim is a bacteriostatic antibiotic, used either on it is own or in combination with a sulphonamide. Trimethoprim is a trimethoxybenzyl pyrimidine, and acts by inhibiting the enzyme dihydrofolate reductase (DHFR) which converts dihydrofolic acid to tetrahydrofolic acid, a step in the process leading to the synthesis of purines and thus DNA. These DHFR inhibitors are encoded by *dfr* genes, of which *dfr* A1 is

the most commonly reported and the first identified among Gram-negative bacteria. It is often found as part of cassette in both class 1 and class 2 integrons (Skold, 2001). At least seventeen types of trimethoprim resistance *dfr* genes and nine *dfr* gene cassettes have been identified in Gram negative bacteria: *dfr* genes include *dfrAI, dfrA5, dfrA7, dfrA12, dfrA14, dfrA17, dfrB1, dfrB2 and dfrB3.*

1. 5 The family *Enterobacteriaceae*

There are many different bacteria within the gut and it is to be expected that there will be competition and variation in relationship between these diverse species. The normal bacterial flora establishes itself in the different parts of newborn animal shortly after birth from the environment, and normally from the bacterial flora of the mother (Koutsos & Arias, 2006; Fuller, 1989).

Savage (1986) reported that the intestinal flora of mammals is responsible for a wide variety of metabolic reactions and assists in the enzymatic breakdown of food and production of useful vitamins. The normal micro flora in the gut is able to work as a protective barrier against infections from other bacteria, such as pathogens, through competition, and may also improve host immunity by adhering to mucosa of intestine and stimulating immune response (Koutsos & Arias, 2006; Salminen *et al.*, 1995). It also stimulates the development of certain tissues, such as lymphatic tissue in gastro intestinal tract (Clavel & Haller, 2007).

Jeurissen (2002) reported that the *Enterobacteriaceae* family represents the largest and most heterogeneous group of aerobic Gram-negative bacilli and is one of the most widely studied families worldwide. A total of 32 genera and more than 130 species have been described, based on biochemical properties.

The digestive tract of animals can be a major bacterial reservoir where resistant strains can be emerge and resistance genes can be acquired (Hershberger *et al.*, 2005).

1. 6 *Escherichia coli*

Escherichia coli was first identified in 1885 (Escherich, 1885), and is a common commensal in the colons of human beings and other vertebrates. Some strains of *E. coli* are pathogenic, usually causing self-limiting gastroenteritis.

Pathogenicity depends on the presence of virulence factors (Brussow *et al.*, 2006; Donnenberg & Whittam, 2001). The *E. coli* that cause enteric disease in human beings are often divided on the basis of virulence properties into enterotoxogenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), verotoxigenic (VTEC), enterohaemorrhagic (EHEC) and enteroaggregative *E.coli* (*EAggEC*): all can cause diarrhoea (Hammerum & Heuer, 2009). In addition to gastrointestinal disease *E.coli* can cause disease when introduced to tissues other than the GI tract, for example sepsis, peritonitis, mastitis, urinary-tract infections and meningitis (Kayser *et al.*, 2005).

Aims and objectives

The work in this thesis was undertaken in order to investigate the ecology of antibiotic resistance on farms. In particular, the aim was to determine whether or not wildlife play a role in the epidemiology of antibiotic resistance in domestic cattle. Although definitely answering such questions once and for all is beyond the scope of a single project, the approach here, as part of a larger project investigating the transmission of various bacterial pathogens, was to study possible transmission of resistance between wildlife and cattle on dairy farms in Cheshire. *Escherichia coli* were chosen as the model bacterial host for resistance, owing to the near ubiquity of this species in the GI tracts of all vertebrate hosts and its ability to survive in the environment. The approach was structured, and each stage in the investigation, and each hypothesis, is described in a separate chapter as follows:

1. Hypothesis: antibiotic resistance in *Escherichia coli* is transmitted freely amongst wildlife and cattle. Therefore similar patterns of resistance will be seen in *E. coli* from most, if not all, hosts. To investigate this, the patterns of resistance in *E. coli* isolated from the faeces of healthy cattle and wildlife will be determined and compared.

2. Hypothesis: the resistance patterns shared between *Escherichia coli* isolated from different hosts are caused by the same genes, as shared patterns represent transmission. Alternatively, if transmission between vertebrate hosts does not occur, similar resistance phenotypes will be associated with different resistance genotypes. Therefore the genotypes of *Escherichia coli* isolates with similar resistance phenotypes will be compared, in this case using microarray approach.

3. Hypothesis: isolates with identical (or very similar) resistance phenotypes and genotypes result from transmission of *E. coli* between different vertebrate hosts. Therefore these isolates, from different hosts, will have identical restriction patterns following pulsed field gel electrophoresis.

Chapter Two

Materials and Methods

General Material and Methods

2. 1 Study area

The study areas were three pairs of neighbouring farms from within the 10x10km study area in Cheshire intensively studied by the Defra Epidemiology Fellowship unit (Richard, 2005; Robinson, 2004). The area was originally chosen to represent typical land use in the region (e.g. dairy, beef, maize, and variety of other livestock and crops are all found in the study area), and a diversity of wildlife habitats (grazed plain and hills, mixed wood land, abandoned rail way line, small ponds, a canal and tributaries of the river Mersey and river Dee).

The six farms were chosen for intensive study within the Liverpool Veterinary Training Research Initiative (VTRI) research programme 2, which focuses on the transmission of potentially zoonotic enteric pathogens between wildlife and domestic livestock. These six farms were chosen based on their representing different husbandry and management regimens, and the distances between the farms. Pairs of neighbouring farms were chosen in order to investigate differences in agents, and thereby potential transmission, over short distances, but under different managements (i.e. to help address the question, what is a greater barrier to transmission-management, or distance?). The pairs of farms were chosen, to represent slightly different habitats within the overall $100km^2$ study area, and distances covered by, perhaps, some birds and wild mammals, but which largely prevented opportunities for direct transmission of enteric agents.

A composite aerial view of the six farms is shown in figure 2. 1.

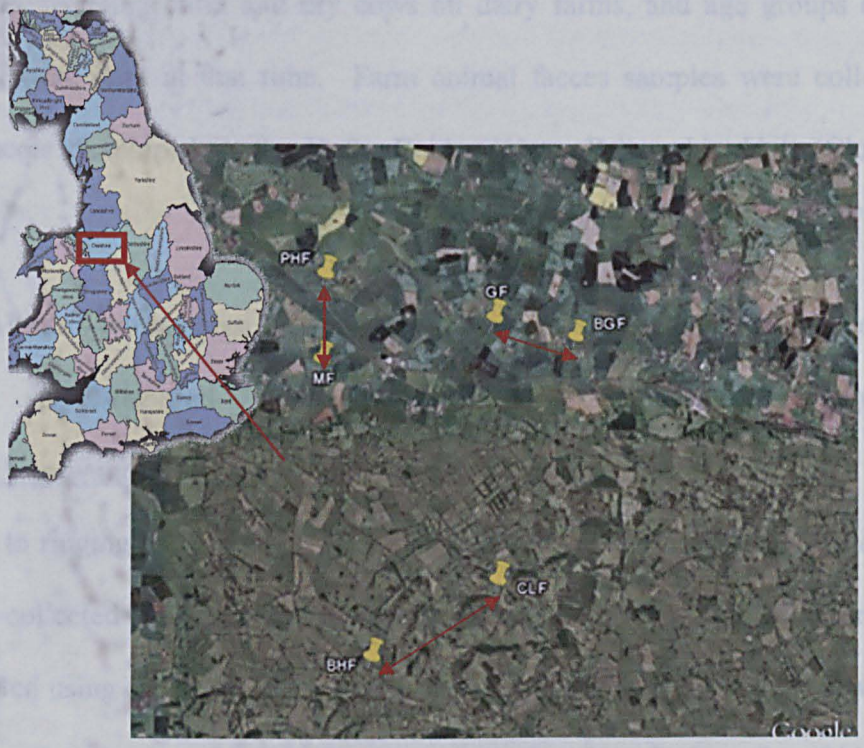


Figure 2. 1: composite aerial photograph of the six study farms, PHF, MF, GF, BGF, BHF and CLF.

2. 2 **Animals and the collection of faecal samples**

Almost all samples were collected in the morning, and returned to the laboratory for processing the sample afternoon.

Each of the farms held cattle – five farms focussed on dairy cows, while one (MF) had beef cattle. Samples of cattle faeces were collected from fresh pats in fields or

animal pens. Several grams of faeces were scooped into sterile plastic containers, and immediately taken back to the laboratory. Sites of collection were chosen according to where different management groups of cattle (calves, weaned animals, heifers, lactating cows and dry cows on dairy farms, and age groups on the beef farm) were kept at that time. Farm animal faeces samples were collected using protocols developed in the Defra Epidemiology Fellowship Unit (Clough *et al.*, 2003).

Wild birds were sampled by catching them in nets or specialist traps, in collaboration with BTO-licensed ringers from the Merseyside Ringing Group (http://www.merseysiderg.org.uk/). Most birds were placed in clean paper bags prior to ringing and recording, and invariably defaecated in the bag. These samples were collected onto sterile swabs and returned to the laboratory. Larger birds were sampled using cloacal swabs – but this method was found to produce lower bacterial yields when used on small birds. Nets were placed on flight paths of birds in areas of the farm chosen to represent possible contact between birds and cattle, and in order to catch a variety of birds. Similarly, traps (e.g. for house sparrows) were baited and left where sparrows were seen to congregate. Faeces from large birds, such as buzzards and pheasants, were collected fresh from the environment where they were seen to have been deposited.

Wild rodents were sampled by catching them in sterile Longworth, or occasionally Ugglan, traps (voles and mice) or larger wire-cage traps (rats and squirrels), baited with sterile grain (e.g. Telfar *et al.*, 2002). Traps were placed, near holes or runways. To increase the chance of capture, some traps were left permanently in place, locked open and containing food. These were replaced with fresh, sterile traps

during trapping sessions. Traps were set over three nights at each site during each trapping session.

Faeces were scraped out of the traps into sterile containers for transport back to the laboratory. The species, approximate age and the site of capture was recorded for each animal. Other data were also collected for use in other studies.

Whenever possible, animals were released back to the wild, the only real exception to this being rats and house mice.

Faecal samples from other wild mammals (mainly rabbits, foxes and badgers) were collected from the field, without catching the animals themselves. Rabbit faeces were collected largely from around burrow systems, and badger faeces from latrines, mainly in hedgerows. Fox faeces were collected as and when found while walking fields. Large mammal faeces samples were collected similarly to cattle faeces samples.

2. 3 **Processing of the faecal samples**

Isolation of *E. coli* and identification of susceptibility and resistance was performed by using enrichment and selective media, followed by susceptibility testing by disc diffusion method.

Most of the samples used were collected by a team within the Liverpool VTRI, the year before I began my research. Therefore I was presented with a collection of faecal samples, and samples incubated overnight in peptone broth, frozen at -70°C. During my period of research, longitudinal studies were carried out on the same farms, and I was involved in the collection and processing of those samples, using identical protocols to those outlined above in this chapter.

2. 3. 1 **Isolation and characterisation of *E. coli***

After overnight incubation in peptone broth, samples were frozen at -70°C until they could be further examined.

To isolate antibiotic susceptible and antibiotic resistant *Escherichia coli*, 0.5 ml of the faecal suspension in peptone broth was added to 3ml brilliant green bile (BGB) broth (LABM LAB51, Bury, UK) and incubated aerobically over-night at 37°C. BGB inhibits the growth of Gram-positive, and some bacteria Gram-negative, bacteria, and so helps select for coliform. The resultant cultures were then streaked onto an eosin-methylene blue agar (EMBA) plates (LabM, LAB61) and incubated overnight at 37°C. EMBA supports the growth of Gram-negative organisms and inhibits the growth of Gram-positive bacteria, and *Escherichia coli* colonies can usually be differentiated from other coliform bacteria by their metallic green sheen when grown on EMBA. Each broth was inoculated onto a routine EMBA plate, but also plates also contain EMBA plus either tetracycline (4μg/ml), chloramphenicol (8μg/ml), Ampicillin (8μg/ml), nalidixic acid (16μg/ml) or trimethoprim (4μg/ml).

2. 3. 2 **Biochemical identification**

Three colonies with typical *Escherichia coli* morphology (i.e. 2-3 mm diameter colonies, greenish-metallic by reflected light and with dark purple centres by transmitted light) were randomly selected and plated on nutrient agar (LABM, LAB8), and again incubated over night at 37°C. The following day, three colonies were picked from each the nutrient agar plate and inoculated onto Simmons Citrate agar plates (SCA) (LabM, LAB69), MacConkey agar plates (Mac) LabM, LAB30), and Trypton Soy Agar (TSA) (LabM, LAB11), and again incubated at 37°C for 24 hrs for MAC and TSA plates, but for 48 hrs on SCA plates.

Escherichia coli isolates were confirmed based on their being lactose positive, indole positive and negative for citrate utilization, as follows.

Lactose fermentation: MacConkey plates were checked for presence of pink colonies indicative of lactose fermentation.

Indole test: Kovac reagent (bioMerieux, Basingstoke, UK) was added to filter paper and placed on each isolate from TSA. If the paper turned purple immediately, the organism was oxidise positive. *Escherichia coli* caused change in colour to pink as a result of indole production.

Citrate utilization test: SCA plates were examined. *E. coli* cannot utilise citrate, therefore *E. coli* isolates grow poorly on Simmon's citrate agar

Chapter Three

A cross sectional study of antibiotic-resistant *Escherichia coli* from the faeces of farm animals and wild life

A cross sectional study of antibiotic resistant *Escherichia coli* from the faeces of farm animals and wild life

3. 1 Introduction

Antibiotics have been widely used in livestock production for the treatment and prevention of disease, and in some cases animal growth promotion (Soulsby, 2008). This extensive use of antibiotics may have contributed to high prevalences of resistance through providing selection pressure both in animals and the wider environment (Akwar *et al.*, 2008).

The increased prevalence of resistance is assumed to be largely due to selection through the use of antibiotics. Therefore it is assumed that if antibiotic use is restricted, that the prevalence of resistance should decrease. In fact the source of resistance is not always known. There is considerable ignorance regarding both the original sources of the resistant strains, and the genetic elements that encode resistance and the dynamics and persistence of resistance under different antibiotic regimens or indeed in the absence of antibiotics.

As antibiotic resistance continues to increase, researchers have extensively studied the different reservoirs of antibiotic resistance. Dantas *et al* (2008) suggested that soil microbes provide an underestimated reservoir of antibiotic resistance genes that can be spread in the environment. It has also been shown that water sources can act as a reservoir or as a medium for antibiotic resistance genes and their transmission to bacteria occupying this environment, facilitating the evolution of bacteria to become multi-drug resistant variants (Biyela & Bezuidenhout, 2004). There are few studies which have investigated the prevalence and transmission of antibiotic resistance in

wild animal populations. However, some studies have shown that wildlife may act as sources of resistance. Work by Gilliver *et al* (1999) demonstrated high prevalences of antimicrobial-resistant Gram-negative enterobacteriacae in wild rodents that had no apparent contact with antimicrobials or farmed animals. The rodents were caught in two private wood land in North-western England. In contrast Osterblad *et al* (2001) found a much less resistance in commensal bacteria among faecal samples from moose, deer, and voles in Finland. Antibiotic-resistant *E. coli* have also been isolated from wild geese in Georgia and North Carolina (USA), and it was suggested that these animals do not naturally come into contact with antibiotics, but may serve as a reservoirs of resistant bacterial and resistance genes in agriculture environments (Cole *et al.*, 2005).

Escherichia coli are one of the most common organisms isolated from the intestinal tract of a wide variety of animals including human beings (Sorum & Sunde, 2001). *E. coli* is largely regarded as a commensal, although pathogenic variants do exist in a number of animal hosts. It has been suggested that the presence of antibiotic resistance determinants in the enteric microflora may act as a reservoir for resistance genes, as transfer of resistance genetic elements may occur as such resistance determinants are often on mobile elements (Turnidge, 2004; Lipsitch *et al.*, 2002; Levy, 1987). Antimicrobial agents act not only against pathogenic bacteria, but the normal enteric microflora or commensal population are also exposed to such agents. These commensal bacteria, are regularly exposured to a variety of antibiotics and therefore will either develop resistance via mutation or become recipients of antimicrobial resistance determinants and in turn, becoming an important reservoir of resistance genes.

The aim of the work presented in this chapter was to determine the prevalence of antibiotic resistance in commensal *E. coli* of domestic farmed animal populations and wild animals occupying the same farmland environment. A further aim was to investigate the role of wildlife as a source of antibiotic resistance determinants or strains, which may be transmitted to farmed animals and man.

3. 2 **Material and Methods**

A cross sectional study was conducted to determine the patterns of antimicrobial resistance in 2084 *E. coli* from faecal samples from domestic farmed animals and wildlife from 6 farms in Cheshire. The characteristics of these farms and the sampling of animals are detailed in Chapter 2. Samples were stored in brain and heart infusion broth containing 5% glycerol (1:1) and kept at -70°C. Prior to isolation, samples were defrosted at room temperature, with isolation and identification of *E. coli* in enrichment and selective media followed by biochemical tests, as detailed in Chapter 2. All isolates were subjected to antimicrobial susceptibility testing by the disc diffusion method for the following antimicrobials, ampicillin, augmentin, chloramphenicol, nalidixic acid, ciprofloxacin, tetracyclines and trimethoprim as detailed below. These antimicrobial agents were chosen on the basis of their importance in human and / or animal medicine for the treatment of infections, and because they also represented different antimicrobial agent classes.

Each unique resistant phenotype from any sample was stored at -80°C in Microbank tubes (Pro-Lab Diagnostics, Neston, UK) and all isolates assigned a unique culture collection number for this project.

3. 3 Antibiotic Susceptibility Testing;

Escherichia coli isolates were grown overnight on nutrient agar and suspended in 3ml of sterile water to give a turbidity equivalent to 0.5 McFarland's standard and 0.5ml of this suspension (after mixing) was added to 4.5 ml of sterile water (1: 10) and this gave the standard inoculums for susceptibility testing.

A sterile cotton tipped swab was dipped into the suspension and used to inoculate the entire surface of a dried Isosensitest (ISO) agar (LabM) plate using a spiral platter to allow an even spread of the organism over the plate. Antibiotic discs were then placed on the surface of ISO plates using sterile needle to place seven of the antibiotics at even distances around the plate, and the following antibiotic discs were used:

- Ampicillin (Amp) 10µg
- Augmentin (Aug) 30µg
- Chloramphenicol (Chl) 30µg
- Nalidixic acid (Nal) 30µg
- Ciprofloxacin (Cip) 1µg
- Tetracycline (Tet) 30µg
- Trimethoprim (Trim) 2.5µg

Plates were incubated for 24 hrs at 37°C in aerobic conditions. Resistance was indicated by growth up to the antibiotic disc or a zone of inhibition within the range (mm) as interpreted according to British Society for Antimicrobial Chemotherapy (BSAC) guide-lines for *Enterobacteriaceae*.

3. 4 Results

Escherichia coli isolates were classified as "sensitive" or "resistant" according to their susceptibility in disc diffusion tests. Prevalence in this chapter is expressed as the proportion (percentage) of samples from which *E. coli* was isolated, not the number of samples that were collected and processed or the total number of isolates (i.e. the number of samples containing *E. coli* resistant to any antibiotic as a proportion of the number of samples from which *E. coli* was isolated).

A total of 2084 faecal samples were examined, 447 from cattle, 918 from wild rodents, 614 from wild birds, and 105 from other wild mammals (foxes, badgers and rabbits), all farms had all different species. The types and numbers of samples are shown in figure 3. 1.

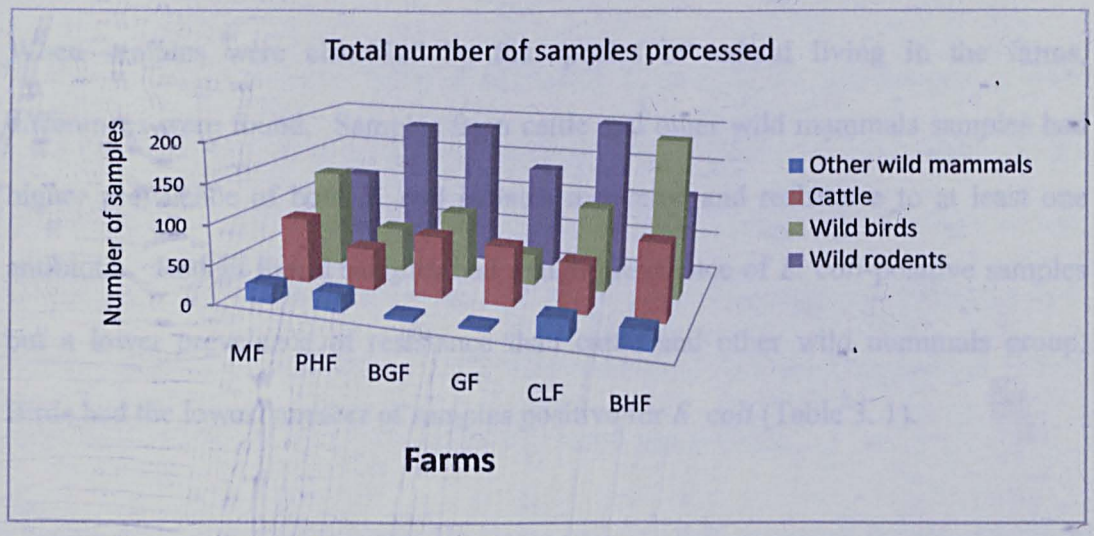


Figure 3. 1. The number of samples collected and tested from each host and farm.

E. coli were detected in 1303 animal faecal samples (63%) (figure 3. 2).

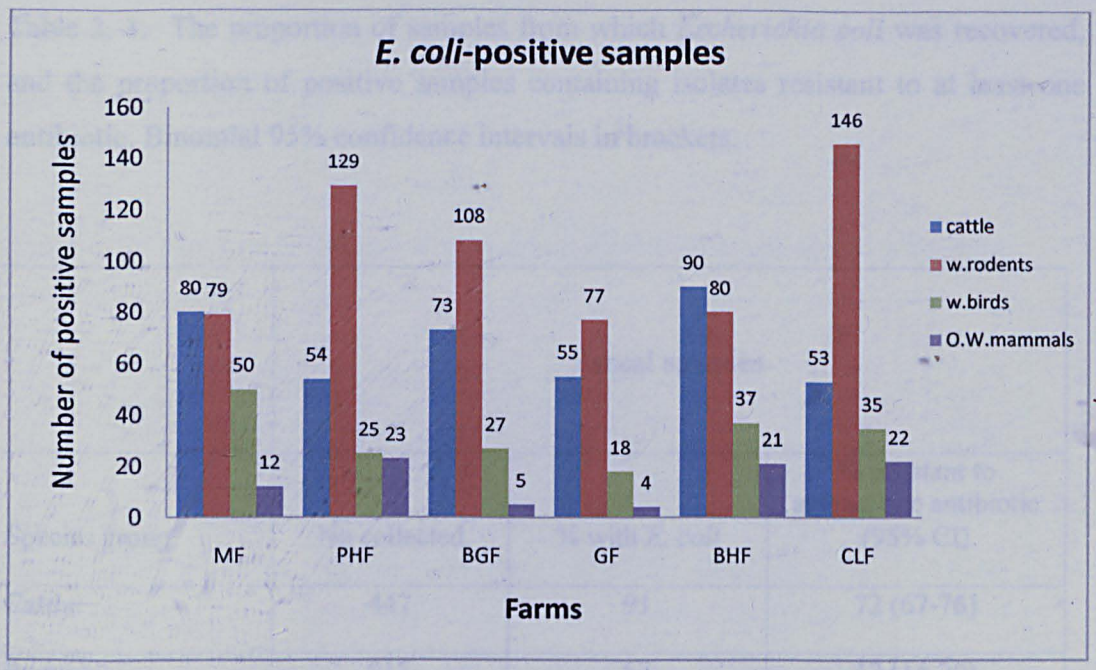


Figure 3. 2. The number of *E. coli*-positive samples by host and farm in the cross sectional survey.

When samples were classified by the species of animal living in the farms, differences were found. Samples from cattle and other wild mammals samples had higher prevalence of both *E. coli* isolates recovered and resistance to at least one antibiotic. Rodent faecal samples had a high prevalence of *E. coli*-positive samples but a lower prevalence of resistance than cattle and other wild mammals group. Birds had the lowest number of samples positive for *E. coli* (Table 3. 1).

Table 3. 1. The proportion of samples from which *Escherichia coli* was recovered, and the proportion of positive samples containing isolates resistant to at least one antibiotic. Binomial 95% confidence intervals in brackets.

	Faecal samples		
Species group	No collected	% with *E. coli*	% resistant to at least one antibiotic (95% CI)
Cattle	447	91	72 (67-76)
Rodents	918	67	17 (14-20)
Birds	614	31	20 (14-26)
Other wild mammals	105	83	66 (55-75)

Antibiotic resistance was detected in faecal *E. coli* of all populations studied, regardless of host species (species group) and farm, but considerable variation in the prevalence was seen between different hosts and farms (Tables 3.1, 3.2, 3.3 and 3.4). Overall, tetracycline resistance was the most commonly detected resistance trait (36%), followed by ampicillin (27%), trimethoprim (17%), chloramphenicol (14%), augmentin (12%), nalidixic acid (6%) and ciprofloxacin (3%).

Overall, the prevalence of samples containing *E. coli* in which the *E. coli* were resistant to one or more antimicrobial was (38%). The proportion of samples containing *E. coli* where the *E. coli* were resistant to one antibiotic was 9%, to two antibiotics 9%, three antibiotics 10%, four antibiotics 7%, five antibiotics 3%, six antibiotics 0.3%, and to seven antibiotics 0.4%. Although the precise figures varied

between farms, a high proportion of multiresistant isolates was consistently seen across farms (Table 3. 2)

Table 3. 2. Percentage of multidrug-resistant *Escherichia coli* samples by farm

SOURCE	No. of samples tested	% resistant to one antimicrobials	% resistant to two antimicrobials	% resistant to three antimicrobials	% resistant to four antimicrobials	% resistant to five antimicrobials	% resistant to six antimicrobials	% resistant to seven antimicrobials
MF	221	20 (9)	22 (10)	21 (10)	17 (8)	7 (3)	2 (1)	1 (0.5)
PHF	231	15 (7)	26 (11)	23 (10)	17 (7)	9 (4)	0 (0)	1 (0.4)
BGF	213	11 (5)	25 (12)	21 (10)	8 (4)	2 (1)	0 (0)	1 (0.5)
GF	154	18 (12)	5 (3)	17 (11)	11 (7)	0 (0)	0 (0)	0 (0)
BHF	228	38 (17)	14 (6)	18 (8)	18 (8)	7 (3)	0 (0)	2 (1)
CLF	256	11 (4)	20 (8)	28 (11)	23 (9)	13 (5)	2 (1)	0 (0)
Total	1303	113 (9)	112 (9)	128 (10)	94 (7)	38 (3)	4 (0.3)	5 (0.4)

Table 3. 3. Prevalence of antibiotic resistance in samples from different hosts

Resistance phenotype	Hosts				
	Cattle (n=405)	Rodents (n=619)	Birds (n= 192)	O W mammals (n=87)	overall (n=1303)
Ampicillin	208 (51)	70 (11)	24 (13)	47 (54)	349 (27%)
Augmentin	86 (21)	35 (6)	9 (5)	26 (30)	156 (12%)
Chloramphenicol	102 (25)	39 (6)	15 (8)	32 (37)	188 (14%)
Nalidixic acid	31 (8)	21 (3)	9 (5)	18 (21)	79 (6%)
Ciprofloxacin	19 (5)	9 (2)	2 (1)	5 (6)	35 (2%)
Tetracycline	278 (69)	99 (16)	33 (17)	54 (62)	464 (36%)
Trimethoprim	129 (32)	44 (7)	13 (7)	40 (46)	226 (17%)

Table 3. 4. Multiple resistance in *E. coli* from different host animals

Source	No. of samples tested	% resistant to one antimi-crobial	% resistant to two antimi-crobials	% resistant to three antimi-crobials	% resistant to four antimi-crobials	% resistant to five antimi-crobials	% resistant to six antimi-crobials	% resistant to seven antimi-crobials
Cattle	405	65 (16)	68 (17)	86 (21)	54 (13)	13 (3)	3 (1)	2 (1)
Rodent	619	27 (4)	25 (4)	21 (3)	15 (2)	17 (3)	1 (0.2)	1 (0.2)
birds	192	12 (6)	9 (5)	8 (4)	8 (4)	1 (0.5)	0 (0)	1 (1)
Other wild mammals	87	9 (10)	10 (12)	13 (15)	17 (19)	7 (8)	0 (0)	1 (1)
Total	1303	113 (9)	112 (9)	128 (10)	94 (7)	38 (3)	4 (0.3)	5 (0.4)

The prevalence of resistance by farm and host is shown in Figures 3.3 – 3.8. Overall, the patterns of prevalence of resistance to individual antimicrobials and multi-resistance by host appeared similar on four of the farms (MF, PHF, BHF and CLF), but less resistance was seen at BGF and particularly at GF.

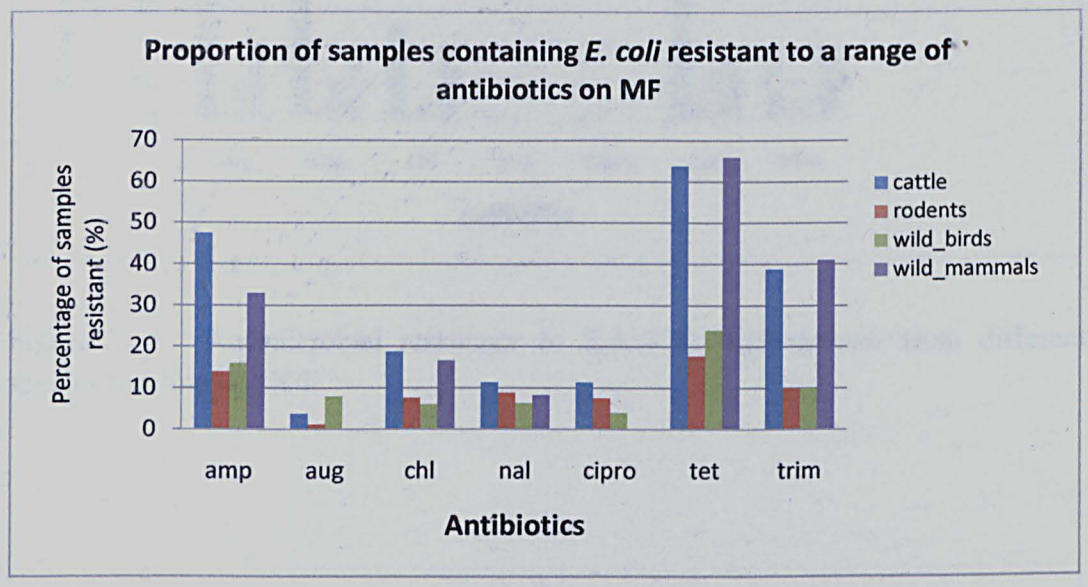


Figure 3. 3. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on MF

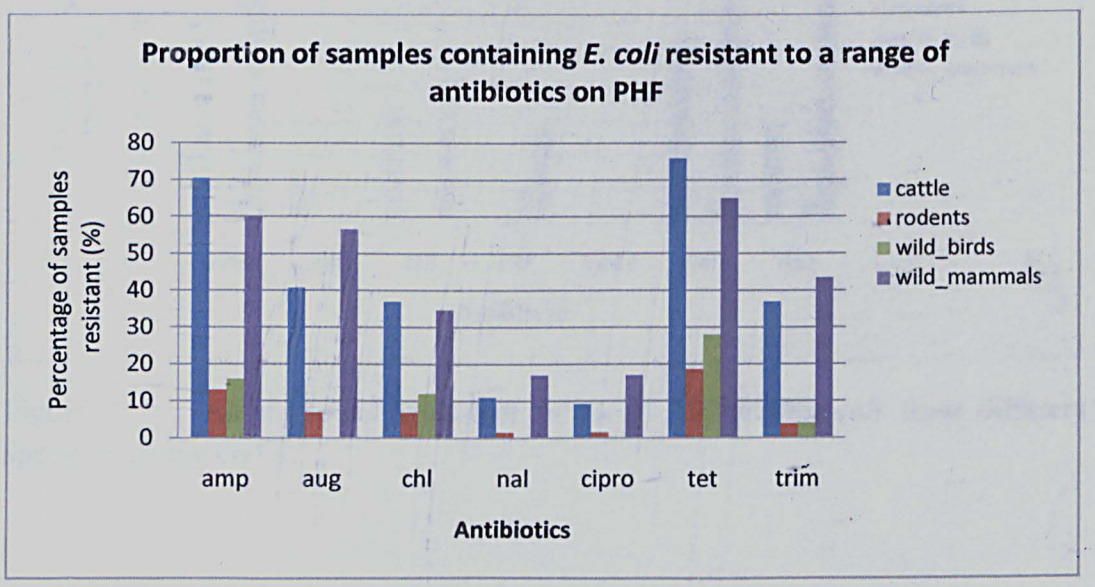


Figure 3. 4. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on PHF

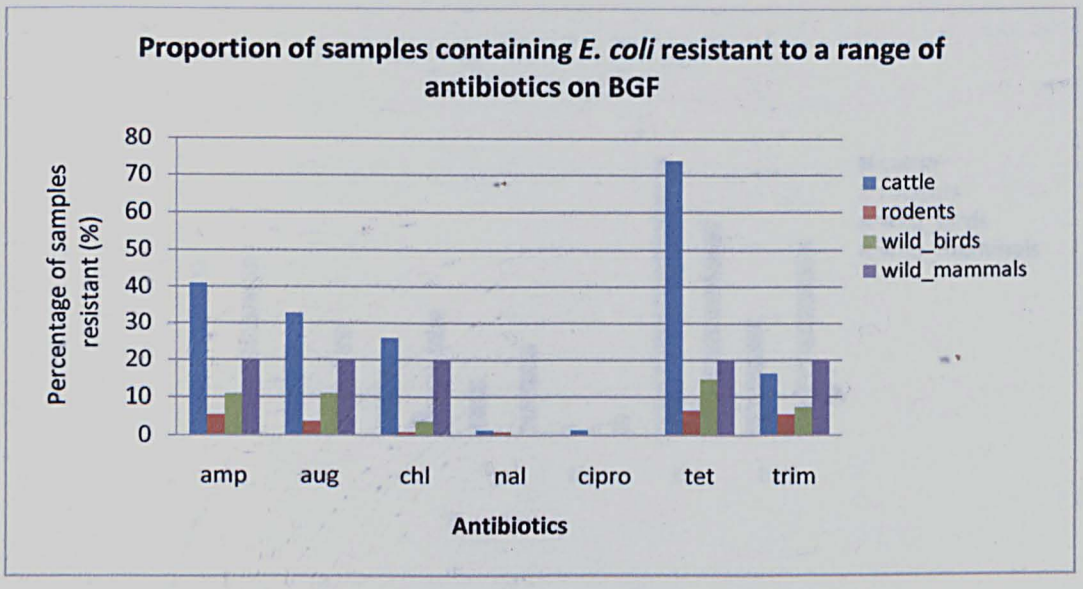


Figure 3. 5. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on BGF

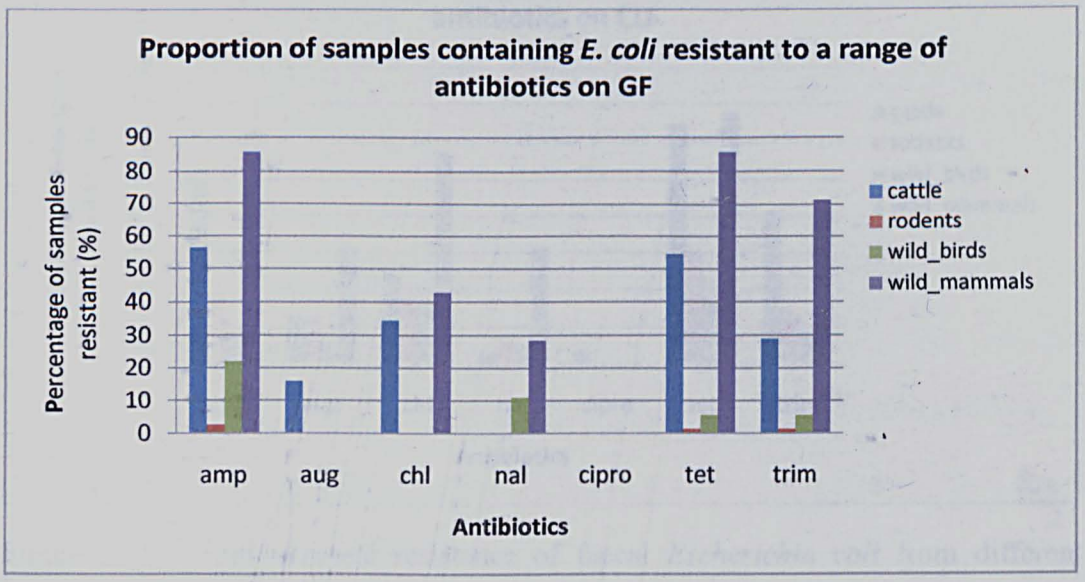


Figure 3. 6. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on GF

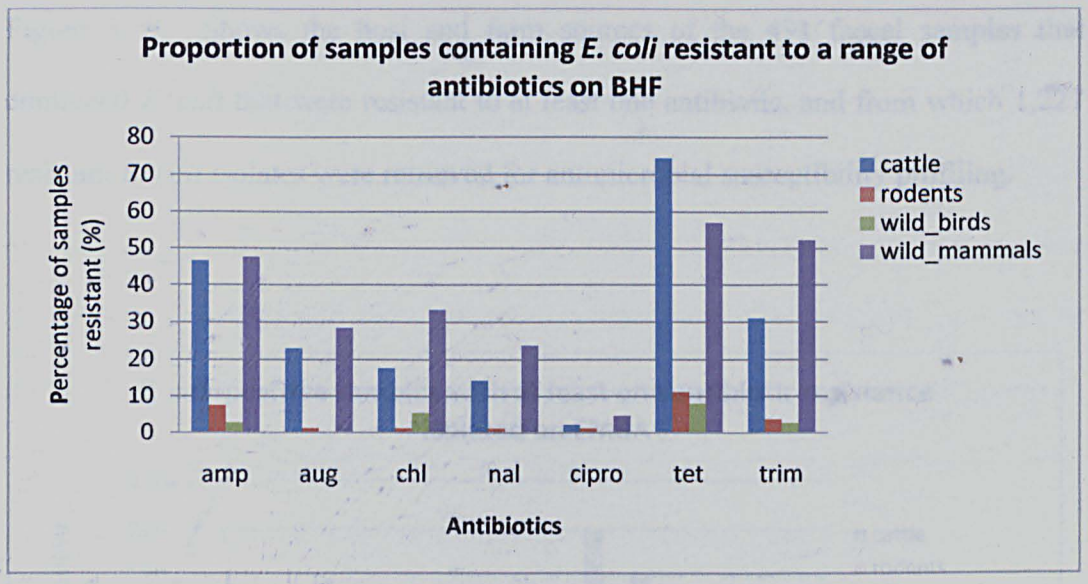


Figure 3. 7. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on BHF

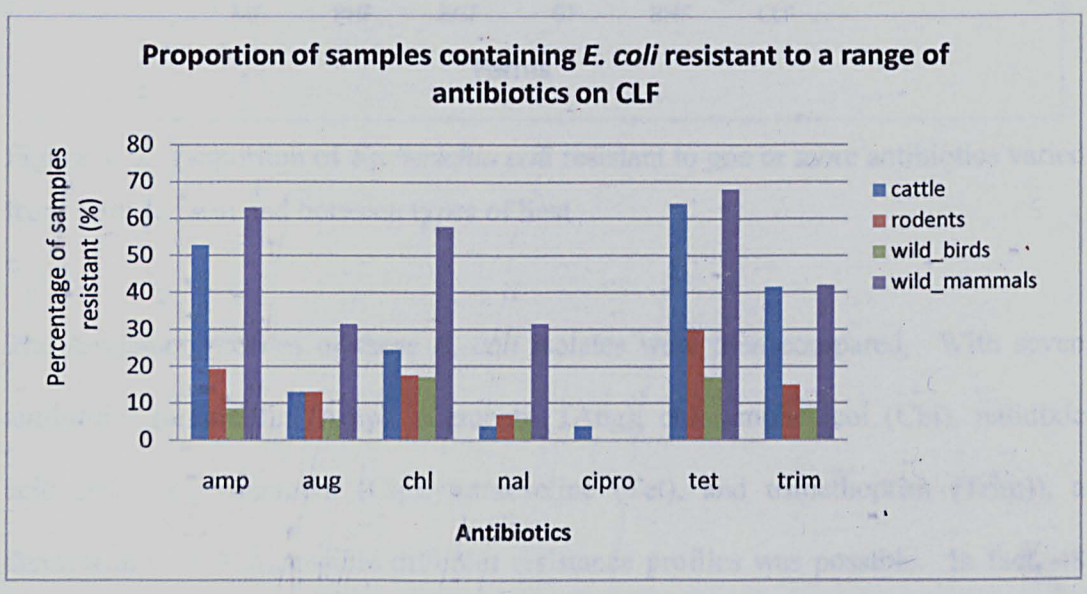


Figure 3. 8. Antimicrobial resistance of faecal *Escherichia coli* from different species residing on CLF

In order to understand better the distribution of multi-drug resistant *E. coli*, the prevalence and distribution of antibiotic resistance profiles among *E. coli* from different sources (species and farms) was investigated.

Figure 3. 9. Shows the host and farm sources of the 491 faecal samples that contained *E. coli* that were resistant to at least one antibiotic, and from which 1,227 resistant *E. coli* isolates were retrieved for antimicrobial susceptibility profiling.

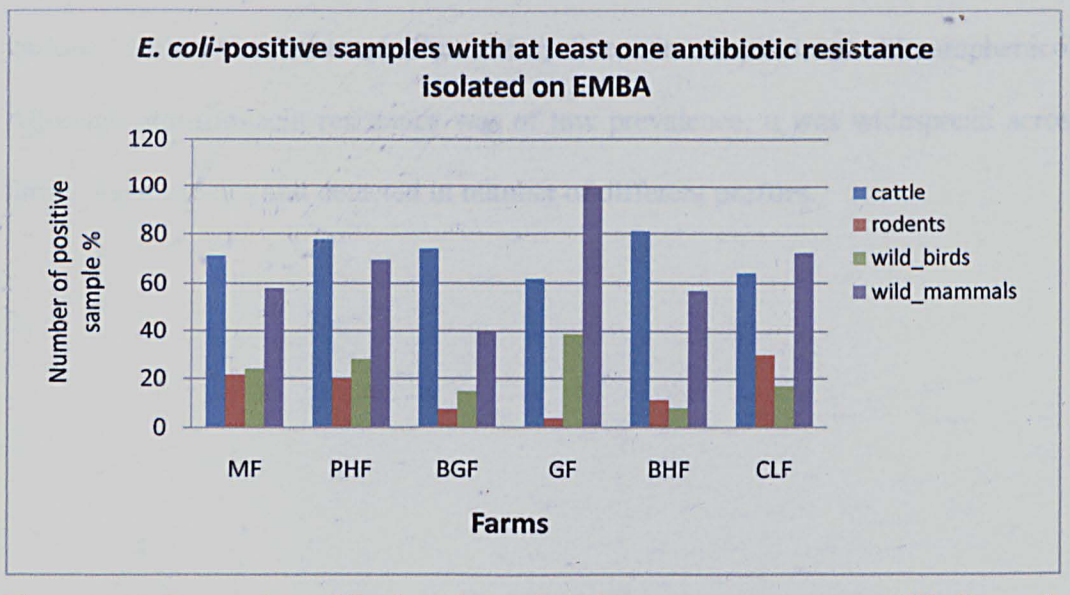


Figure 3. 9. Proportion of *Escherichia coli* resistant to one or more antibiotics varied from farm to farm and between types of host

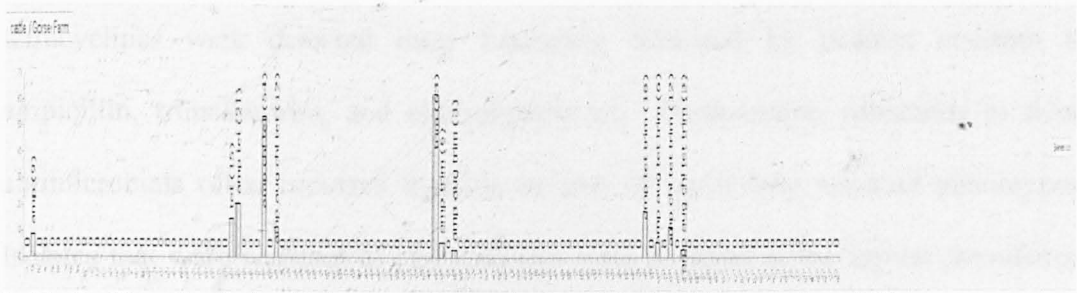
The resistance profiles of these *E. coli* isolates were then compared. With seven antibiotics (ampicillin (Amp), augmentin (Aug), chloramphenicol (Chl), nalidixic acid (Nal), ciprofloxacin (Cip), tetracycline (Tet), and trimethoprim (Trim)), a theoretical $2^7 = 128$ possible different resistance profiles was possible. In fact, 48 resistance profiles were detected, indicating a non-random distribution, i.e. a clustering of resistance phenotypes. Each possible resistance profile was assigned a number (between 1 and 128), and the prevalence of each profile was plotted by species and farm in order to further identify and compare profiles in common and unique across hosts and farms (some examples are shown in Figure 3. 10, all such graphs are shown in appendix1).

Some profiles were detected in *E. coli* from samples distributed widely across cattle and wild animals (Table 3. 5 and Figure 3. 10), but others were limited to particular hosts or farms. *E. coli* was classed as multi-drug resistant (MDR) if isolates were resistant to two or more different antibiotics. The most common profiles comprised resistance to between one and four antimicrobials, and often involved resistance to various combinations of ampicillin, tetracycline, trimethoprim and chloramphenicol. Although ciprofloxacin resistance was of low prevalence, it was widespread across farms / host species and detected in number of different profiles.

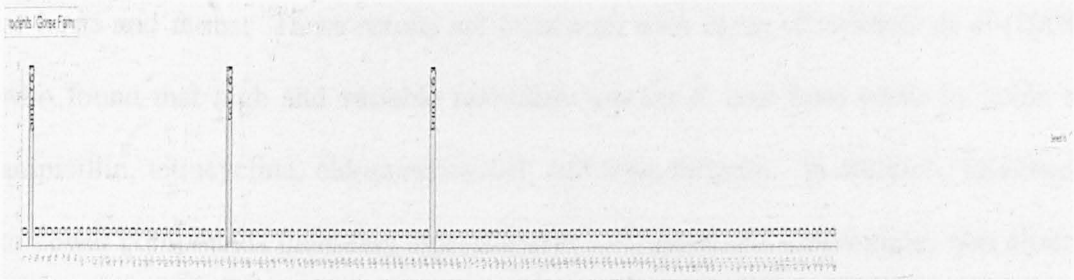
Table 3. 5. The twelve most common antibiotic resistance profiles encountered in *Escherichia coli*, and their distribution by host and farm. (Different colours indicate different level of resistance)

Farm	Host	Antibiotic resistance profile											
		tet	tet, trim	chl, tet	amp	amp, tet	amp, tet, trim	amp, chl, tet	amp, chl, tet, trim	amp, aug, tet	amp, aug, tet, trim	amp, aug, chl, tet	amp, aug, chl, tet, trim
		%	%	%	%	%	%	%	%	%	%	%	%
M F	Cattle	36	6.2	7.5	2.5	26	39	13	10	1.3	10	1.3	
	Rodents	2.5	6.3	1.3		3.8	8.7	3.8	2.5	1.3			
	Birds	6		2		4	2			2			
	O.W. Mammals	33	8.3			17	33	8.3	17				
PHF	Cattle	24	1.8	3.7	3.7	33	7.4	24	1.8	11	26	9.3	13
	Rodents	9.3		1.5		6.2		2.3		5.4	0.8	7.8	6.9
	Birds	12		8		8	4	12				8	
	O.W. Mammals	8.6		8.6		8.6	4.3		4.3	13	60	13	26
GF	Cattle	24			9	20	7.2	45	5.4		3.6	11	1.8
	Rodents	1.2			3.8								
	Birds	11	5.5		22	5.5	11						
	O.W. Mammals	25			25	100	25	50					
BGF	Cattle	27	1.3	26		22	1.3			21	5.5	1.3	
	Rodents		2.7		1.8	1.8	0.9			0.9	4.6	0.9	
	Birds	11	7.4		7					7.4	11	11	
	O.W. Mammals				40		20	20	20		20		
CLF	Cattle	1.8	1.8	17		13	42		21	15	9.4		
	Rodents	10	7.5	9.5		1.3	4.7	1.3	7.5	4.7	6.8	0.7	3.4
	Birds		1.8	5.5			3.6	3.6	3.6				3.6
	O.W. Mammals	9.1	9.1	18		14	14	14	23	9.1	9.1	1	32
BHF	Cattle	38	4.4	5.5	3.3	14	30	5.5	11	16	10	4.4	1.1
	Rodents	7.5				6.3	2.5						
	Birds			8		5.4	2.7						
	O.W. Mammals	38	19	14	4.7	14	14	24	3.3	4.7	14		

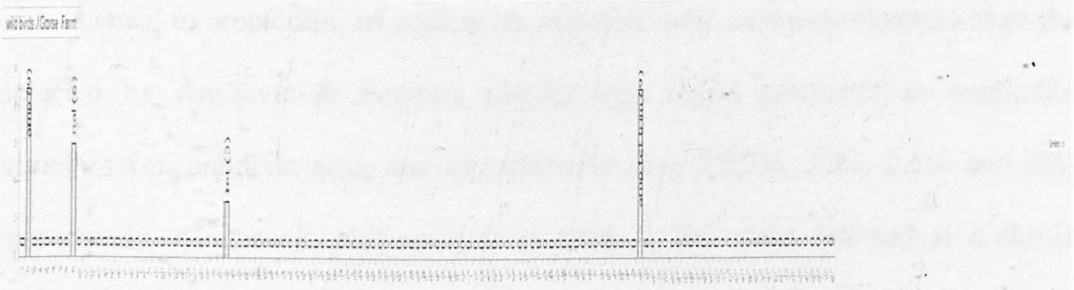
Figure 3. 10. the number of isolates with each of the possible 128 antimicrobial resistance profiles, by farm and host (some examples are shown here, all the profiles are in appendix 1)



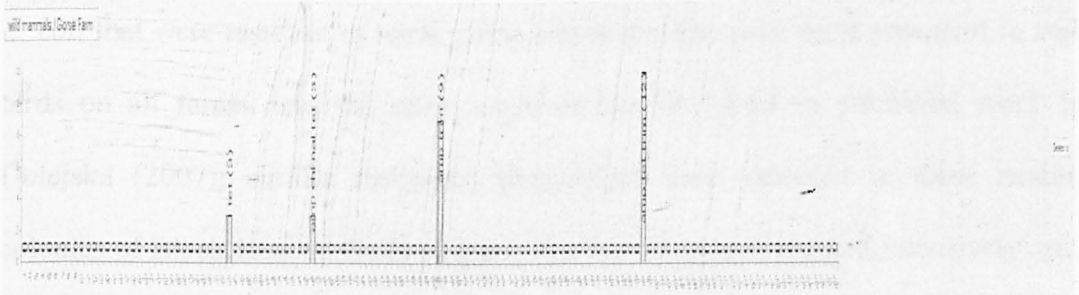
3. 10a Profiles of antimicrobial agent resistance in *E. coli* isolated from cattle on MF.



3. 10b Profiles of antimicrobial agent resistance in *E. coli* isolated from rodents on MF.



3. 10c Profiles of antimicrobial agent resistance in *E. coli* isolated from birds on MF.



3. 10d Profiles of antimicrobial agent resistance in *E. coli* isolated from other wild mammals on MF.

3. 5. **Discussion**

Antibiotic-resistant *E. coli* were detected in faecal samples collected from a range of host species across six farms sampled during. Overall, *E. coli* resistant to tetracyclines were detected most frequently followed by isolates resistant to ampicillin, trimethoprim, and chloramphenicol. Furthermore, resistance to these antimicrobials often occurred together as part of multi-drug resistant phenotypes. Isolates that were resistant to ciprofloxacin were detected at the lowest prevalence. Nevertheless, resistance to this antimicrobial was found in *E. coli* from a wide range of hosts and farms. These results are consistent with those of Bywater *et al* (2004) who found that high and variable resistance among *E. coli* from cattle in Spain to ampicillin, tetracycline, chloramphenicol, and trimethoprim. In addition, resistance to newer compounds including ciprofloxacin, cefetpime and cefotoxime, was absent or low. Although, *E. coli* from cattle in the current study showed a higher prevalence of resistance to ampicillin, trimethoprim, nalidixic acid, and ciprofloxacin than that detected by Knezevic & Petrovic (2008) who found resistance to ampicillin, trimethoprim, nalidixic acid, and ciprofloxacin was (22.5%, 15%, 2.5% and 0%), respectively, whereas *E. coli* resistant to tetracyclines were detected at a similar prevalence in cattle in both studies.

E. coli that were resistant to tetracycline and ampicillin were most prevalent in wild birds on all farms, and the same situation can be found in published work by Dolejska (2007); similar resistance phenotypes were detected in three nesting colonies of Black Headed Gulls situated in a heavily populated and intensively used agricultural area in the Czech Republic. These results were also consistent with various livestock and human populations (Lanz *et al.*, 2003; Bywater *et al.*, 2004).

Antibiotic-resistant *E. coli* appear to be common in a range of wildlife, and not just cattle. In this study, antibiotic-resistant *E. coli* were found in most wildlife species sampled on most farms, but were less prevalent in rodents and wild birds compared to other wild mammals including foxes (*Vulpes vulpes*), badgers (*Meles meles*) and rabbits (*Oryctolagus cuniculus*), and there were variations in the prevalence between farms. Our results are consistent with those of Livermore *et al* (2001) who found that *E. coli* resistant to tetracycline was most prevalent among isolates from rabbits (*Oryctolagus cuniculus*) and magpies (*Pica pica*), but resistance to ampicillin, chloramphenicol, kanamycin, sulphonamides, and trimethoprim was also found. The authors suggest that the differences in the prevalence of antimicrobial resistance observed between bacteria from rabbits and magpies may be caused by differences in diet. Rabbits graze along field edges, whereas magpies are omnivorous and opportunistic feeders.

A study on 270 bank voles (*Clethrionomys glareolus*) and 246 wood mice (*Apodemus sylvaticus*) to determine the prevalence of carriage of Enterobacteriaceae and their antibiotic resistance, within the normal flora of the rodents at different sites Rake Hey and Manor Wood by Gilliver *et al* (1999), found a much higher prevalence of resistance than in this study, with 90% of coliforms resistant to amoxicillin, amoxicillin-clavulanic acid and cefuroxime, resistance to tetracycline at 14 – 76% and to trimethoprim 0 – 67%. It is difficult to compare these results directly since in this study only *E. coli*, not all coliforms, were studied. A recent study by Costa *et al* (2008) that investigated levels of antimicrobial resistance in wild animals from different natural parks in Portugal detected *E. coli* in 78% of the animals sampled. A high proportion of the *E. coli* were resistant to tetracycline, streptomycin and ampicillin (19-35%), and 14% of isolates were resistant to nalidixic acid and 9% to

ciprofloxacin. These results do not agree with the findings of studies carried out by Osterblad *et al* (2000) who found almost no resistance to antibiotics in the Enterobacteriacae that were isolated from wild moose (*Alces alces*), deer spp. and vole spp. in Finland. The factors responsible for these differences are difficult to ascertain – different techniques, different environments (with different degrees of human management) and different host animals doubtless combine to at least contribute to the differences reported – however, that antibiotic resistance is found in wild animals remains a common theme.

In the current study, some of the antibiotic resistance profiles of *E. coli* from cattle faecal samples were found to be similar to those from wild animal faecal samples. This may suggest transmission between the different host species, or alternatively, it may suggest that these species were infected from a common source of resistant bacteria. These numbers suggest that wild animals might function as a reservoir of resistant bacteria for cattle. This idea is further supported by the fact that in some cases higher prevalences of resistant bacteria were detected in other wild mammals isolates than in cattle isolates. The similarities of some resistance patterns seen in the same and different animal species, especially those sampled on neighbouring farms, is suggestive of a common source of resistant bacteria for the different species and cross contamination between close farms. Our results revealed that there were few differences in the prevalence of resistant bacteria between farms in general. For example, the locations with the lowest prevalence of resistance were BGF and GF; this may indicate that these farms were less contaminated with resistant strains of different sources, as those farms further away.

Conversely, some profiles seem limited to one type of host, which suggests that within species transmission also occurs. Overall, our results do not suggest that the

predominant direction of infection of antibiotic resistant bacteria is from livestock to wildlife, or vice versa.

Unidentical patterns of resistance were seen in cattle isolates, and also in wild animal isolates, where the difference in resistance patterns may have been due to exposure to different agents because of differences in husbandry of these species. Alternatively, it could be that other factors, which would help to explain the occurrence of bacteria with unidentical resistance profiles, were not sampled in this study, such as environmental samples, farm workers, or other wild animals.

The small number of antibiotic resistance profiles compared to the total number possible suggests that the genes comprising these profiles are likely to be carried on plasmids. The evolutionary/epidemiological consequence of this is that selection for one gene will result in selection for others so multi-resistance may be selected for over time.

The other important observation found in this study in relation to antibiotic resistance is that resistance to multiple antibiotics was also common. Many animals were carrying bacteria that were resistant to more than one antibiotic; resistance to multiple antibiotics was found in *E. coli* from faecal samples collected from cattle and from all wild animals species that were examined to investigate if antibiotic-resistant *E. coli* was transferable between cattle and wild animals.

These was thought to be due to that selection for one gene will select for others and the genes encoding these profiles are probably carried on conjugative plasmids together which have a sufficient capacity to carry multiple genes, including those carrying antibiotic resistance genes, heavy metals and biocide resistance. It has been shown that there is genetic linkage between antibiotic resistance genes and non

antibiotic agents including mercury and other heavy metals resistance genes (Backer-Austin *et al.*, 2006). The resistance genes are carried on integrons, capturing antibiotic resistance cassette. In the environment, bacteria in metal contaminated area appeared to be more tolerant to metal and antibiotic (Wright *et al.*, 2006). Therefore, the observed multidrug resistance may not be arise due to the selective pressure of antimicrobials but rather reflect a more general resistance mechanism related to the entire plasmid. So as was mentioned before, multiresistance may be selected for over time. In additions, selection might be for other genes not encoding resistance that share the plasmid, and nothing to do with antibiotics being a selection force.

Although mutational change has an impact on drug resistance development, the greatest concern has been the mobile genetic resistance determinants as plasmids, transposons and integrons. Plasmids often carry several different resistances and other life-supporting genes such as transposase or recombinase (Alekshun & Levy, 2007; Canton *et al.*, 2003).

The number of samples collected from some species in this study was limited. For example; the number of large wild mammal samples (fox, badger, and rabbit) was five in BGF, and four from GF. For this reason, *E. coli* possessing resistance profiles detected in cattle faecal samples may be present in large mammal populations but may not have been isolated due a small sample size of large wild mammals. In addition, *E. coli* containing the same antibiotic resistance profiles as cattle may not have been detected in the faecal samples of large wild mammals due to the collection of large wild mammal faecal samples that were of unknown age and therefore may not have been fresh and sometimes only a small quantity of faeces could be collected.

It is likely that there were a number of false negative results with regards to the isolation rate of *E. coli* from faecal samples. For example, samples from wild birds showed an unexpectedly low isolation rate of *E. coli* (31%) The reasons for this remain unclear but might reflect the nature of *E. coli* from bird samples in comparison to samples from other species. As mentioned previously it may also result from the small volume of faecal samples from wild birds. Alternatively it could have been due to the fact that several dilutions were carried out prior to this investigation, which determined only the most abundant bacteria present in the faecal sample.

The delay in samples being processed by being stored in the fridge for periods of time may have had an effect on the isolation of resistant *E. coli*. A study by Kullas *et al* (2002) has highlighted the importance of processing samples as quickly as possible in order to isolate *E. coli*. The study showed that successful recovery of *E. coli* from goose faeces varied with the sampling date. Also, the resistance phenotype can be the result of many genes, so in order to look for more compelling evidence of cross-species transmission, further molecular analysis would be useful (this sets the scene for the microarray chapter).

Chapter Four

DNA microarray for detection of multiple antimicrobial resistance genes in *Escherichia coli* from cattle and wildlife population

DNA microarray for detection of multiple antimicrobial resistance genes in *Escherichia coli* from cattle and wildlife population.

4. 1 Introduction

Escherichia coli is a common part of the commensal bacterial flora of human and other animals, although some strains of *E. coli* can cause enteric or extraintestinal infections. In previous studies (Kozak *et al.*, 2009; Sayah *et al.*, 2005) and chapters in this thesis, it was shown that antibiotic resistance in *E. coli* isolated from domestic animal and wildlife hosts is common, with a range of resistance patterns found. The obvious question is whether this wide host range of species harbouring resistant *E. coli* reflects the transmission of resistant *E. coli*, or at least resistance genes between *E. coli*, or if the resistance derives from separate selection and development of resistance in different mammalian hosts.

One way of approaching this question, is to investigate the genes responsible for the resistance: is the same phenotypic resistance pattern found in *E. coli* from two different animals due to the same genes (consistent with transmission) or not (more consistent with no or rare transmission)?

There are five main mechanisms of resistance to antibacterials:

- Antibiotic modification or in activation;
- Prevention of antibiotic entering the bacterial cell; Changing the target molecules within the bacterium
- Efflux pumps that prevent the antimicrobial achieving an effective intracellular concentration.

- Bypass of the metabolic activity affected by antibacterials

Resistant strains may have multiple mechanisms of resistance for the same antibiotic as well as different mechanisms of resistance for different antibiotics.

There is little information available concerning the prevalence of antimicrobial resistance genes in *E. coli* in domestic animals and wildlife. Until recently it was only possible to investigate a few genes at a time. With the development of DNA microarray techniques, it should be possible to screen many genes at the same time in order to detect the genotype of multiple antibacterial resistance, with a high degree of specificity. This technique may provide an effective, fast, simple, and inexpensive test for gene detection and comparison.

There are several names for this technique: DNA microarrays, DNA arrays, DNA chips, and gene chips. The application of microarrays for expression profiling was first published in 1995 (Hofmann, 2006). Since that time the number of publications regarding to DNA microarrays has increased. DNA microarrays have been used in various studies, including taxonomy, as this approach can be useful for the identification of bacteria as well as determining genetic distance among them (Cho & Tiedje, 2001). Important for this study is that microarrays also have been used previously to detect functional gene diversity and the distribution of important genes in the environment (Bruant *et al.*, 2006; Palaniappan *et al.*, 2006, Dougherty, 2002). Hamelin *et al* (2006) used oligonucleotide microarrays consisting of more than 300 probes representing 189 virulence and virulence-related genes and 30 antimicrobial resistance genes to investigate *E. coli* isolated from Lake Ontario water samples. Microarrays have also been used in a few previous studies on virulence and antibiotic resistance in farm animals. For example, the development of a miniaturised

microarray has been described capable of detecting genes encoding resistance to aminoglycosides, trimethoprim, sulphonamides, tetracyclines and β-lactams, including the detection of extended spectrum β-lactamase genes active in Gram negative bacteria, with high correlation (99%) between PCR and array results (Batchelor *et al.*, 2008). This assay also showed good correlation between phenotypic and genotypic results for a panel of both *E. coli* and salmonella isolates.

The aim of the work described in this Chapter was to investigate the ability of a DNA microarray for the rapid detection of antimicrobial resistance genes in *E. coli* isolated from cattle and wild animals. The antibiotic resistance genes identified, could then be used to investigate the distribution of resistance genes within and between different host reservoirs.

4. 2 Materials and Methods

4. 2. 1 Choosing isolates for testing

The phenotyping results from Chapter 3 were compared, and isolates were selected for study by microarray on the basis of having similar or identical phenotypes within species and farms and across species and farms. Cost precluded examination of all isolates by microarray, so isolates were chosen in an attempt to maximise the chances of identifying identical gene profiles across species and farms.

4. 2. 2 Microarrays

The micro tube based array system used in this study was that of CLONDIAG, Jena, Germany (http://www.clondiag.com/technologies/puplications.php).

The microarrays themselves were developed for the detection of antimicrobial resistance genes in *E. coli* at the Veterinary Laboratories Agency- Weybridge, UK (Defra, report. EU 2136), and contain recent and most common identified antimicrobial resistance genes found in *E. coli*, and all assays were undertaken at the VLA's laboratories. These have been described previously by (Bachelor *et al.*, 2008).

The arrays were 3x3 mm size, with an active surface area of 2.4 x 2.4 mm, mounted onto the bottom of standard 1.5 ml micro reaction tubes.

E. coli isolates were grown overnight on nutrient agar at 37°C and total DNA was obtained by resuspending a colony in 2 ml lysis buffer (0.1 M Tris HCI, PH 8.5, 0.05% Tween 20, 240 mg/ml proteinase K), incubating for 2 hours at 60°C to remove

capsular polysaccharide, followed by a 15 min denaturation step at 95°C. The lysate was spun at 1300 rpm for 5 min, and the supernatant used as the source of DNA.

Sample DNA concentration was measured using a NanoDrop spectrophotometer to quantify DNA, and adjusted to a final concentration of 800ng – 1200ng / ml.

The genes targeted, array probe and primer sequences are shown in Table 1.

Table 4. 1: Genes included in the resistance array, the probes present on the array and the variants of these genes detected by the probe. Also the GenBank accession number for one of the genes detected by the probe and also the control strain used are also included.

Target	Resistance phenotype or-gene description	Additional *Genes* detected by probe	Array probe sequence (5'-3')	Primer sequence (5'-3')
qnr	quinolone	*qnrA*	CAGTGTGACTTCAGC CACTGTCAGC	TCCTCGAAACTGGC ATCC
sulI	sulphonamide		CCTTCCTGTAAAGG ATCTGGGTCCAGC	CGATCGCGTGAAG TTCC
sulII	sulphonamide		TCGATTTGCCGGT GCTTCTGTCTGT	CAGAAAGGATTTGC GCGA
sul3	sulphonamide		GCTCTGCATTTGGT TGAAGATGGAGCA	CGGCTCCCAAATCA ATCAC
tet(A)	tetracycline		CTCATGCTCGGAAT GATTGCCGACG	AGCAGGATGTAGCC TGTG
tet(B)	tetracycline		CGTTTGCTTTCAGG GATCACAGGAGC	GGTATCGGCAATG ACCGA
tet(C)	tetracycline		CTCGCTCAAGCCT TCGTCACTGGT	CTCGCCGAAACGT TTGG
tet(D)	tetracycline		GCACTGTCCAATG TGCTGTGGATGT	AGTGATCCCGGAG ATAATCC
tet(E)	tetracycline		GGTACAGGCACCGT TTATGTTCGCTG	AGGGAGACCAGAA ATGCC
tet(G)	tetracycline		GCTTCACAGCACTC TATTCTGCCACC	CGACAATCCAAACC CAACC
			GCTTCACGGCACTC TATTCTGCCAC*	Primer used same as above.
IntI1	Class 1 integrase		CCATTCCGACGTCT CTACGACGATGA	CTTTCAGCACATGC GTGT
IntI2	Class 2 integrase		GCAAGCCTAGACG GCTACCCTCTG	CGTTGCACTTCATT TGCAG
aadA1-like	aminoglycoside	*aadA aadA1*	AGATTCTCCGCGCT GTAGAAGTCACC	TGATGTCGTCGTG CACA
aadA2-like	aminoglycoside	*aadA2 aadA2a aadA2b aadA2c aadA3 aadA8*	ACGCTCCGCGCTAT AGAAGTCACC	GATGATGTCGTC ATGCACG
aadA4-like	aminoglycoside	*aadA4 aadA5*	CTGGATCACGATCT TGCGATTTTGCTGA	CAAGGCTGTGTTG CCTC
*bla*PSE-1-like	carbenicillinase	bla_{CARB}-1 -2 -3 -8 bla_{PSE}-5	AGCAGATCTTGTGA CCTATTCCCCTGT	CGAGTGTGATTGCC TGC
			AAGCAGATCTTGTT ACGTATTCGCCTGT*	Primer used same as above.
cmlA1-like	chloramphenicol exporter	*cmlA cmlA1 cmlA4 cml A5 cmlA6 cmlA7*	GACCATGTTGCTGG AACGGTCACG	CGATGCTTCCTAG CAGTACA

catA1	chloramphenicol acetyltransferase		CGTCTCAGCCAATC CCTGGGTGAG	GTTGTCCATATT GGCCACG
catIII	chloramphenicol acetyltransferase		TGGGTTCGCCGTGA GCATTTTGAG	GCTAAAACCAC TGGTAAACG
catB3-like	chloramphenicol acetyltransferase	*catB3 catB4*	GCAATGACGTTTGG ATCGGCTCTGAG	CTTGATTCCGGG CATGAC
floR	chloramphenicol/ florfenicol		GCTTTCGTCATT CGTCTCTGGGAG	TGCAGTTGAAGA CCAAGCT
dfrA1	trimethoprim		CAATAGACATCGA GCCGGAAGGTGATG	ACTGGCCTAAA ATTGCTGG
			CAATCGACATTGA GCCAGAAGGTGATGT*	Primer used same as above.
dfrA7	trimethoprim		GGTAATGGCCCTGATC TCCCATGGTC	ATGTGAGCGCTT TAAAGAGT
			GGTAATGGCCCTGATA TCCCATGGTCAG*	Primer used same as above.
dfr12	trimethoprim		CAGTACGCATTTATCT CGTTGCTGCGA	TTGCCAATAACC CGATTGG
dfrA14	trimethoprim		GCCATGGACAGGCTA GCTGAATTCAC	CCACCAGACACT ATAACGTG
dfrA17	trimethoprim		GGTAGTGGTCCTGATA TCCCGTGGTCA	dfrA7 primer used.
dfrA19	trimethoprim		TGTCATAGGCGCTAC TG TCGTTCCTG	AGGTTGATCACA GCACGA
aac(3)-*Ia*	aminoglycoside	also known as *aacC1*	CGTGAGTTCGGAGAC GT AGCCACC	AGTCCGGCTGAT GTTGG
aac(3)-*IVa*	aminoglycoside		CGTTACACCGGACCTT GGAGTTGTCT	GCTGCGCTTTAC ATTTGG
aac(6')-*Ib*	aminoglycoside	also known as *aacA4*aac(6')-1b-cr	CGTCACACTGCGCCTC ATGACTGA	ATAGAGCATCGC AAGGTCA
ant(2")-*Ia*	aminoglycoside		TGGACTATGGATTCTT AGCGGAGATCGG	CAAGCAGGTTC GCAGTC
*bla*DHA-1	plasmidic AmpC	bla_{DHA}-1 -2	TGGTGGACAGCACC ATTAAACCGCT	GGAATATCCTGC TGTGCCA
*bla*ACC	plasmidic AmpC	bla_{ACC-1}	TGTCTGGCAGCAAC TGTCCAAGGT	TCTCATCGATAT TAGCAGCCA
		bla_{ACC-2}	TGCCGAATTTGCTCA CCGGTAACG	CCACGCTTTTCG TCATCG
*bla*MOX	plasmidic AmpC	All bla_{MOX} genes	GCTGCTCAAGGAGCA CAGGATCCC	CCTTGCCATCCTT GAGC
			CGATGCTCAAGGCGT ATCGGATCCC*	Primer used same as above.
*bla*CMY	plasmidic AmpC	All bla_{CMY} genes	ACGAAGAGGCAATGA CCAGACGCG	GCCAGTTTTAA TGGTTGCAG
*bla*FOX	plasmidic AmpC	All bla_{FOX} genes	AGCTTCCAGGCCAATC CGGTTACG	TGTTATAGAGC CGCTGCT

*bla*SHV		All bla_{SHV}	ACAGCTGGAGCGAA AGATCCACTATCG	GAGTAGTCCA CCAGATCCTG
*bla*LEN-1	β-lactam	bla_{LEN-1}	ACAACTGGATCGGC GGATCCACTAC	Shv primer used.
*bla*TEM-1	β-lactam	All bla_{TEM} genes	CGAACTACTTACTC TAGCTTCCCGGCAA	TATCCGCCTCCATC CAGT
*bla*OXA-1	β-lactam	bla_{OXA} –1 –30-31 -33	ACAACGGATTAACA GAAGCATGGCTCG	ACGCAGGAATTGA ATTTGTTC
*bla*OXA-2	β-lactam	bla_{OXA}-2 -3 -15 –20 21 -22 -32 -34 -36	CGATAGTTGTGGCA GACGAACGCC	CCAACATGGCAC GATCC
*bla*OXA-7	β-lactam	bla_{OXA}-7 -10 -11 -13 -14 -16 -17 -19 -28 - 35	CGCAATTATCGGC CTAGAAACTGGTGTC	GGCTTTCCGTCCC ATTTG
*bla*CTX-M1	β-lactam	bla_{CTX-M} -1 -3 -10 -12 -15 -22 -23 –28 Fec-1	CGTCACGCTGTTGTT AGGAAGTGTGC	CCGTTTGCGCAT ACAGC
			GCCACGCTGTCGTTA GGAAGTGTG*	Primer used same as above.
*bla*CTX-M2	β-lactam	bla_{CTX-M} -2 -4 –5 -6 -7-20 Toho-1	GCATTCGCCGCTCA ATGTTAACGGT	TGCTAAATAGC AGGGGTAGC
*bla*CTX-M9	β-lactam	bla_{CTX-M} -9 -14 -16 -17 -19 -21 -24 -27 Toho-2	GCGATGAGACGTTT CGTCTGGATCG	GGAATGGCGGT ATTCAGC
			GGTGATGAGACCTT CCGTCTGGACAG*	Primer used same as above.
*bla*OXA-9	β-lactam	bla_{OXA}-9	GACTCTGTTGTCTG GTTCTCGCAGCA	CCGATCAACTC CCAGACG

DNA labelling was done in 10µl total volumes, comprising 1µl dNTP mix (1mM dACGP; 0.65mM dTTP), 1µl Therminator 10 xamplification buffer, 0.1µl Therminator DNA Polymerase, 0.35µl Biotin-16-dUTP, 1µl primer mix, 1-2µg template, and sample DNA. The PCR conditions were: 5 minutes 96°C, then 40 cycles of 62°C (20s each), 72°C (40s each) and 96°C (60s each). The reaction was then held at 4°C.

The microarray tubes, produced by Clondiag Chips Technology, were placed in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany) and washed with 500µl

of deionised water for 5 min at 55°C and 550 rpm, and with 500µl hybridization buffer 3, DNA for 5 min at 30°C and 550 rpm.

In a separate tube, 10µl labelled DNA (PCR product) was incubated with 90µl hybridization buffer for 5min at 95°C, cooled on ice for 1min (denaturing step) and then added to the array tube. The hybridization was carried out for 60min at 55°C and 550 rpm, and after this step the arrays tubes were washed 3 times with 500µl 2xSSC, 0.01 % Triton, 2XSSC and 0.2xSSC: the first 2 steps were performed at 40°C for 5 min and 55 rpm and the third step at 30°C.

This was followed by 15 min blocking with 100µl freshly prepared milk powder (dissolve 0.02g of milk powder in 1 ml of 6x SSPE/0.005% triton buffer) for 15 min at 30°C and 550rpm.

Finally, 100 µl peroxidase–streptavidine conjugate (poly-HRP Streptavidine) (0.2 µg / ml) was added to each tube for 15 min at 30°C and 550rpm. This was followed by three steps of washing with 2xSSC, 0.01% Triton, 2xSSC, and 0.2xSSC for 5 min at 550 rpm at 30°C for the first step, but for the second and third step at 20°C. Liquid was always drawn off completely with a soft a plastic pipette to avoid scratching of the chip surface at the bottom of the tube.

Visualization of hybridization was achieved by adding 100µl of peroxidise substrate (True Blue/Seramun green) to the array tubes, and signals were detected with an ATROI Array tube reader (Clondiag). Signals were recorded and analysed using Icon Cluster software, according to the manufacturer's instructions.

A photograph was taken to record the staining reaction. After 10 min of staining the resulting picture was analysed by the icon cluster software, which measures the signal intensity and local background for each spot.

Table 4. 2 shows the buffers and solutions used above.

Table 4. 2. Reagent used in microarrays

Reagent	Volume	pH	Storage conditions
3 DNA/SDS Buffer	100ml	Final pH 7.2	Room temp
1M NaPOi	25ml	pH 7.2	"
20% SDS	22.5ml		
0.5M EDTA	200µl	pH 8.0	
20 x SSC	5ml	pH 7.0	
Water	47ml	pH 7.25	
1M NaPOi	1L		Room temp
Sodium phosphate dibasic (anhydrous)	141.96g	Phosphoric acid to adjust to final pH 7.2	
20 x SSC	100ml	Final pH 7.0	Room temp
Sodium chloride	17.53g		
Sodium citrate	8.82g		
Water	100ml		
2 x SSC + 0.01% Triton	100ml		Room temp
20 x SSC	10ml		
Triton x 100	10µl		
Water	90ml		
2 x SSC	100ml		Room temp
20 x SSC	10ml		
Water	90ml		
0.2 x SSC	100ml		Room temp
20 x SSC	1ml		
Water	99ml		

Figure 4. 1. Distribution layout of the oligonucleotide on the microarray.

14x14 0.17 mm

Layout: VLW_ESBL_181104 Absttstand

115	107	108	109	110									115
	90	91	92	93	94	99	100	101	102	103	104	105	106
115	73	74	75	76	77	78	83	84	85	86	87	88	89
115	56	57	58	59	60	61	62	67	68	69	70	71	72
	39	40	41	42	43	44	45	46	51	52	53	54	55
101	102	103	104	105	106	107	108	109	110	35	36	37	38
83	84	85	86	87	88	89	90	91	92	93	94	99	100
61	62	67	68	69	70	71	72	73	74	75	76	77	78
43	44	45	46	51	52	53	54	55	56	57	58	59	60
105	106	107	108	109	110	35	36	37	38	39	40	41	42
87	88	89	90	91	92	93	94	99	100	101	102	103	104
69	70	71	72	73	74	75	76	77	78	83	84	85	86
51	52	53	54	55	56	57	58	59	60	61	62	67	68
115	35	36	37	38	39	40	41	42	43	44	45	46	115

The layout of the spotted probes in the array used in this study is shown in Figure 4. 1. The position of each probe on the array is represented by a number (spot_ID) and the gene sequence is provide with a list of target and corresponding capture probe in Table 2. 1 (Appendix 2). The red numbers (115) indicate the positions of controls, Markin_Mix labelled oliquneucleotides.

4. 3 **Results**

The microarray method was used to identify the resistance genes present in 189 isolates that possessed resistance to at least two antimicrobials. Of *E. coli* isolates tested, 70 came from cattle, 42 from rodents, 46 from other wild mammals (foxes, badgers and rabbits), and 31 from wild birds; the isolates were also chosen from across all six farms (2).

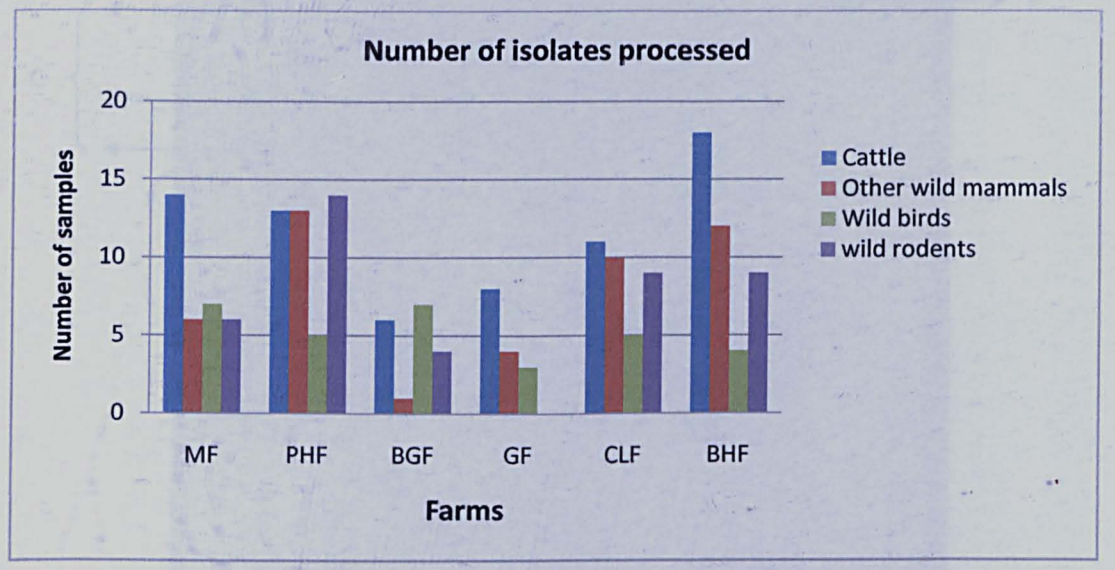


Figure 4. 2. The total number of *Escherichia coli* selected and tested from each host and farm.

Hybridization analyses were performed in triplicate (Figure 4. 3), and the mean signal intensity value of all 3 spots was used for each gene. The hybridization analyses were performed in triplicate using icon cluster software, Clondiag (Figure 4. 4). Mean values below 0.2 were considered negative. Mean values below 0.3 were considered uncertain. Mean values above 0.3 were considered positive.

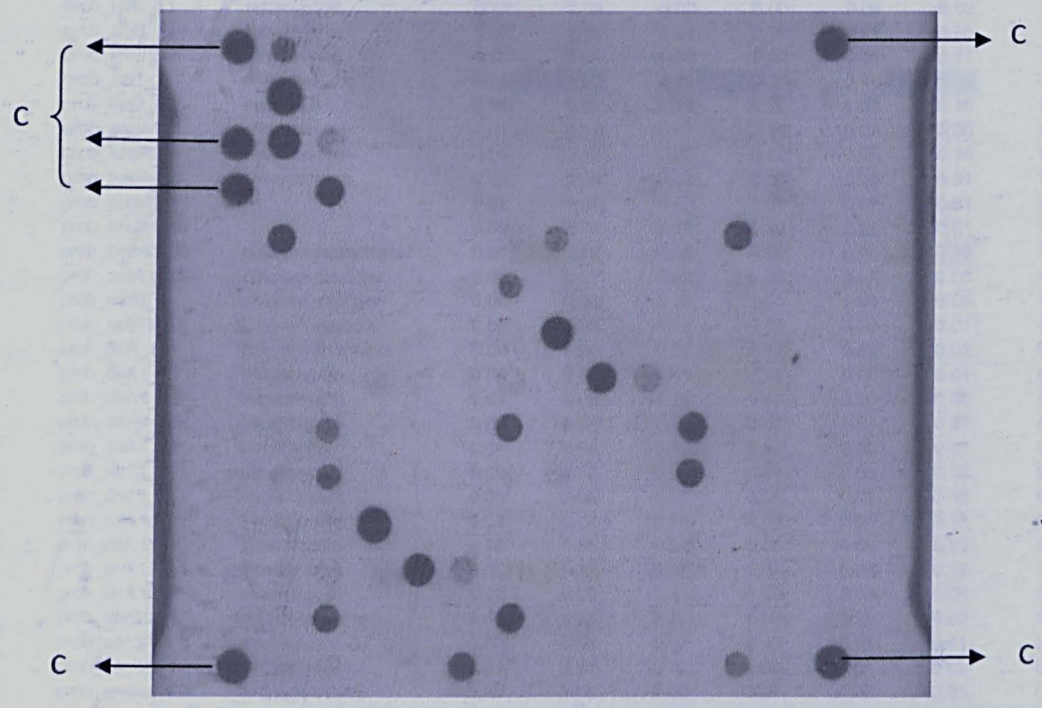


Figure 4. 3: Typical microarray image after hybridisation with sample DNA.

C indicates the positions of controls, Markin Mix labelled oligonucleotides

Figure 4. 4: Screen shot of output of hybridization analyses, using the icon cluster software, Clondiag

Final result sheet		98 GF	104 BHF	98 CLF	104 CLF	37 CLF	37 CLF	98 CLF
		190 M	61 M	100 C	110 C	45 M	58 R	130 R
Gene		10	1048	385	413	433	475	482
		2	2	1	1	2	3	3
prob_qnr_11	quinolone	0.00	0.03	-0.01	0.00	0.00	0.01	0.02
prob_sul2_11	sulphonamide	0.02	0.03	0.04	0.05	0.02	0.02	0.02
prob_sul3_11	sulphonamide	0.00	0.67	-0.02	0.00	0.50	0.61	0.02
prob_tetA_11	tetracycline	0.31	0.03	0.38	0.37	0.21	0.53	0.32
prob_tetB_11	tetracycline	0.00	0.52	-0.02	0.00	0.10	0.01	0.00
prob_tetC_11	tetracycline	0.00	0.05	-0.02	0.00	0.00	0.01	0.04
prob_tetD_1	tetracycline	0.02	0.02	-0.02	0.00	0.00	0.02	0.01
prob_tetE_11	tetracycline	0.01	0.03	-0.02	0.00	0.04	0.02	0.01
prob_tetG_11	tetracycline	0.00	0.02	-0.02	0.00	0.01	0.06	0.01
prob_tetG_12	tetracycline	0.00	0.04	-0.02	0.00	0.01	0.07	0.00
prob_intI1_1	integrase	0.19	0.45	0.29	0.34	0.10	0.38	-0.01
prob_intI2_11	integrase	0.00	0.02	0.13	0.00	0.04	0.19	0.01
prob_aadA1_1	streptomycin	0.01	0.49	-0.01	0.36	0.25	0.50	0.02
prob_aadA2_1	streptomycin	0.00	0.12	-0.02	0.02	0.03	0.10	0.00
prob_aadA4_1	streptomycin	0.00	0.06	-0.02	0.00	0.05	0.01	0.01
prob_ctxM1_11		0.02	0.04	-0.02	0.01	0.01	0.01	0.01
prob_ctxM1_12		0.02	0.04	-0.01	0.02	0.03	0.01	0.04
prob_cmlA1_11	chloram acetyltransf	0.01	0.51	-0.02	0.00	0.15	0.39	0.00
prob_catA1_11	chloram exporter	0.00	0.07	-0.02	0.00	0.02	0.00	0.00
prob_catIII_1	chloram exporter	0.00	0.03	-0.02	0.01	0.00	0.00	0.01
prob_catB3_11	chloram exporter	0.00	0.03	-0.02	0.00	0.01	0.01	0.00
prob_floR_11	chloram florfenicol	0.00	0.04	-0.01	0.45	0.02	0.02	0.01
prob_dfrA1_21	trimethoprim	0.00	0.02	-0.01	0.56	0.02	0.01	0.02
prob_dfrA1_22	trimethoprim	0.01	0.03	-0.02	0.09	0.00	0.03	0.02
prob_dfrA7_11	trimethoprim	0.00	0.05	-0.02	0.02	0.01	0.03	0.02
prob_dfrA7_12	trimethoprim	0.08	0.04	-0.02	0.01	0.00	0.01	0.03
prob_dfr12_11	trimethoprim	0.00	0.10	-0.01	0.01	0.01	0.10	0.03
prob_dfr13_11	trimethoprim	0.00	0.06	-0.02	0.01	0.02	0.12	0.00
prob_dfrA14_21	trimethoprim	0.01	0.21	0.00	0.02	0.03	0.16	0.35
prob_dfrA15_1	trimethoprim	0.06	0.05	-0.01	0.02	0.08	0.02	0.02
prob_dfrA17_11	trimethoprim	0.05	0.32	0.01	0.01	0.04	0.19	0.04
prob_dfrA19_1	trimethoprim	0.01	0.08	-0.01	0.00	0.02	0.01	0.01
prob_aac3Ia_1	aminoglycoside	0.00	0.09	-0.02	0.00	0.00	0.00	0.00
prob_aac3IVa_1	aminoglycoside	0.00	0.04	-0.02	0.10	0.01	0.01	0.01
prob_aac6Ib_1	aminoglycoside	0.01	0.05	-0.02	0.00	0.01	0.02	0.00
prob_ant2Ia_1	aminoglycoside	0.00	0.03	-0.02	0.07	0.03	0.05	0.01
prob_act1_11	plasmidic AmpC	0.06	0.16	0.05	0.06	0.05	0.09	0.02
prob_dha1_1	plasmidic AmpC	0.01	0.03	-0.02	0.01	0.00	0.01	-0.01
prob_acc2_11	plasmidic AmpC	0.01	0.04	-0.02	0.00	0.03	0.01	0.01
prob_mox_1pm	plasmidic AmpC	0.01	0.04	-0.02	0.00	0.04	0.05	0.02
prob_mox_1mm	plasmidic AmpC	0.00	0.05	-0.02	0.00	0.01	0.06	0.01
prob_cmy_11	plasmidic AmpC	0.02	0.09	0.01	0.01	0.02	0.06	0.04
prob_fox_11	plasmidic AmpC	0.01	0.07	-0.01	0.01	0.02	0.10	0.02
prob_tem1_1	β-lactam(penicillinas	0.49	0.67	0.60	0.59	0.19	0.52	0.60
prob_oxa1_21	β- lactam	0.00	0.04	-0.02	0.00	0.00	0.02	0.00
prob_oxa2_11	β- lactam	0.00	0.04	-0.01	0.01	0.04	0.01	0.01
prob_oxa7_11	β- lactam	0.01	0.05	-0.02	0.01	0.01	0.01	0.01
prob_ctxM9_11	sulphonamide	0.08	0.01	-0.03	0.02	0.02	0.00	0.03
prob_ctxM9_12	sulphonamide	0.00	0.03	-0.02	0.01	0.03	0.01	0.03
prob_shv1_11	β- lactam	0.04	0.02	-0.02	0.00	0.03	0.02	0.01
prob_oxa9_11	β- lactam	0.00	0.08	-0.02	0.03	0.07	0.03	0.02
prob_len1_11	plasmidic AmpC	0.00	0.04	-0.03	0.01	0.00	0.03	0.01
prob_acc1_11	plasmidic AmpC	0.01	0.02	-0.02	0.01	0.01	0.01	0.02
prob_ctxM2_11		0.00	0.04	0.00	0.02	0.02	0.04	0.01
prob_per2_1		0.00	0.03	-0.02	0.01	0.00	0.07	0.02
prob_dfrV_21	trimethoprim	0.23	0.21	0.50	0.14	0.10	0.21	-0.01

Of the 47 genes and gene groups represented on the microarray, only 23 genes were detected in *E. coli* isolates, and these were: *sul3_11, tetA_11, tetB_11, tetD_1, aadA1_1, aadA4_1, cmlA1_11, catA1_11, catB3_11, floR_11, dfrA1_21, dfrA1_22, dfrA7_11, dfrA_12, dfrA12_11, dfr13_11, dfrA14_21, dfrA15_1, dfrA17_11, dfrA19_1, dfrV-21, sul1_11, tem1_1* and the class integrase gene *intl1_1* and *intl2_11.*

The average number of genes identified or detected per strain in MF was 2.2, in PHF was 3.2, in BGF was 3.5, in GF was 3.3, in CLF was 3.6 and in BHF was 3.7.

Many resistance genes were found in only a few isolates. To avoid over-estimating the number of resistance genes present in each isolate, and to simplify the analysis, only results for the most common 10 genes were analysed, and the others were excluded from the final analysis. Figure 4. 5 shows the frequency of detection of the ten most commonly detected genes.

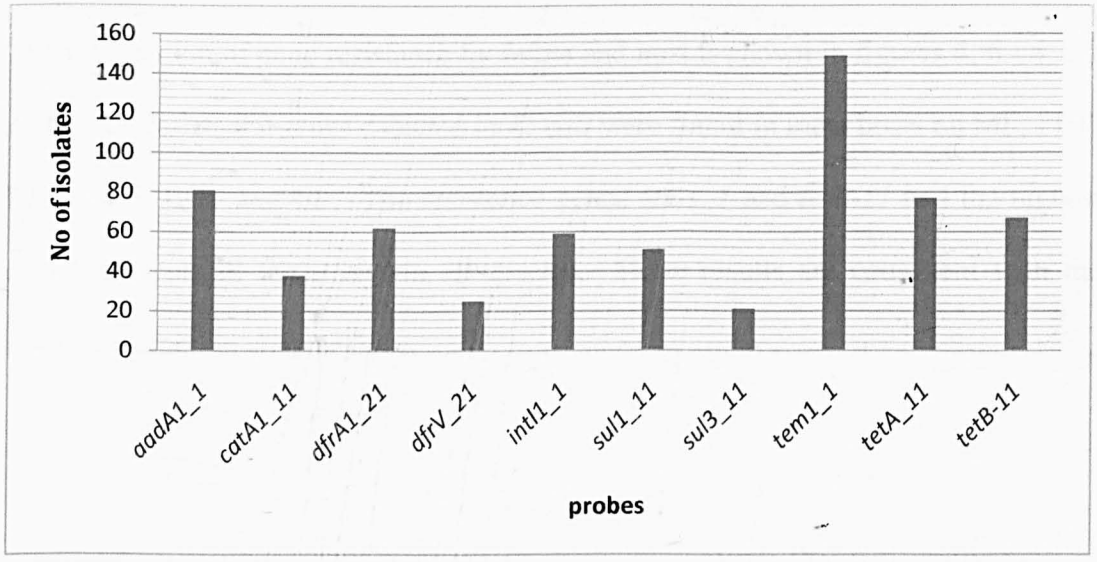


Figure 4. 5. The frequency of detection of resistance genes in 189 *E. coli* isolates (only the ten most frequently detected genes are included).

The genes that were detected most frequently were *teml_1* (74.5%), followed by *aadA1_1* (40.5%), *tetA_11* (38.5%), *tetB_11* (33.5%), *dfrA1_21* (31%), *sul1_11* (25.5%), *catA1_11* (19%), *dfrV_21* (12.5%) and *sul3_11* (10.5%). These genes encode for resistance to the B-lactams (penicillinase), streptomycin, tetracycline, trimethoprim, sulphonamide and chloramphenicol, respectively. In addition, integrase *intl1_1* was also common (29.5%). Most *E. coli* that carried resistance genes had genes typical of class 1 or 2 integrons, which can carry different antimicrobial resistance gene cassettes, and may be involved in the transmission of antimicrobial resistance genes and the dissemination of resistance among resistance bacteria.

4. 3. 1 Distribution of resistance genes on six farms

The number of isolates per individual farms was distributed as follows: 33 isolates from MF; 45 from PHF; 35 from CLF; 43 from BHF; 18 from BGF and 15 from GF.

The distribution of gene resistance by farms and host is shown in figures 4. 6 - 4. 11. Antibiotic resistance genes detected in *E. coli* were found in most hosts on MF, PHF, BHF, CLF, BGF and GF. The resistance genes *teml_1* and *tetB-11* had the highest distribution on all farms and in all species. These results are consistent with the results obtained in chapter three.

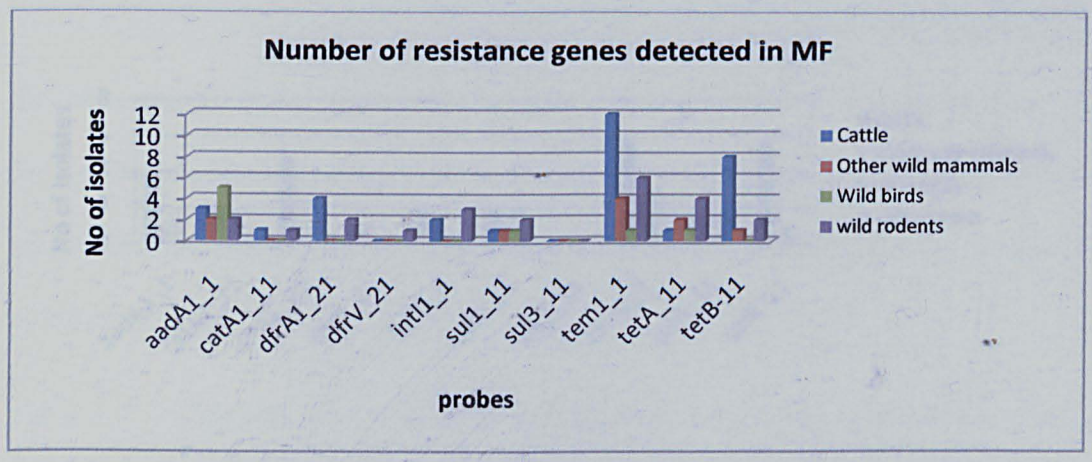


Fig 4. 6. Distribution of antimicrobial-resistance genes in *Escherichia coli* from animals on MF

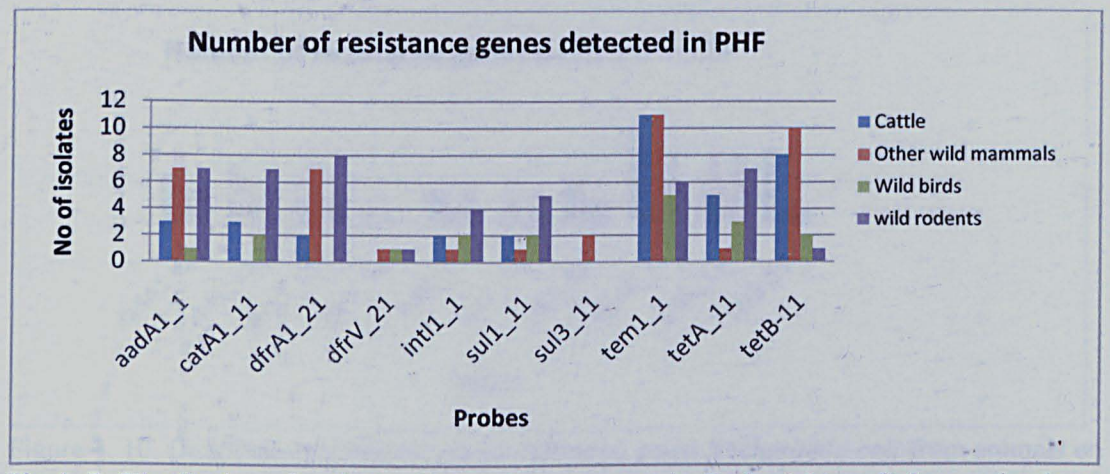


Fig 4. 7. Distribution of antimicrobial-resistance genes in *Escherichia coli* from animals on PHF

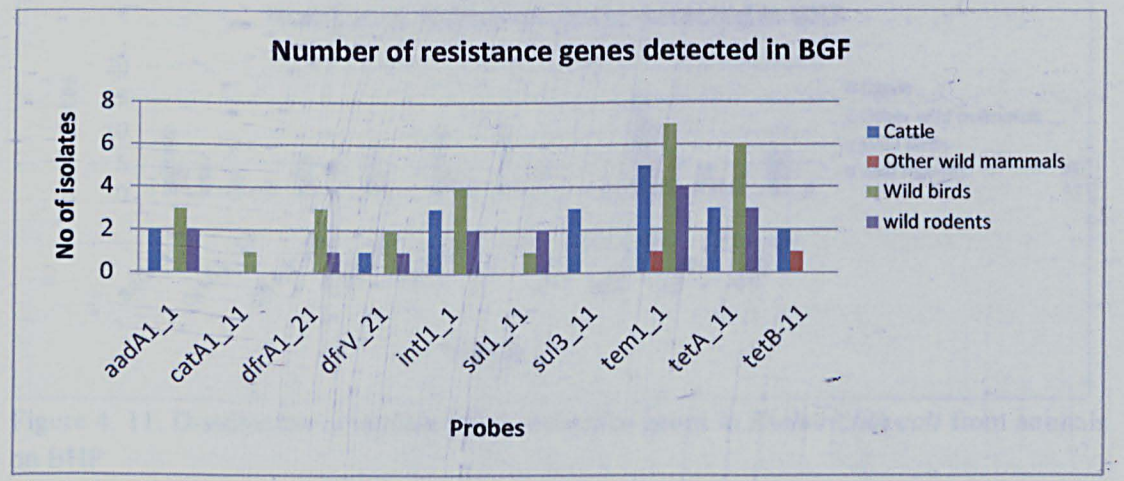


Fig 4. 8. Distribution of antimicrobial-resistance genes in *Escherichia coli* from animals on BGF

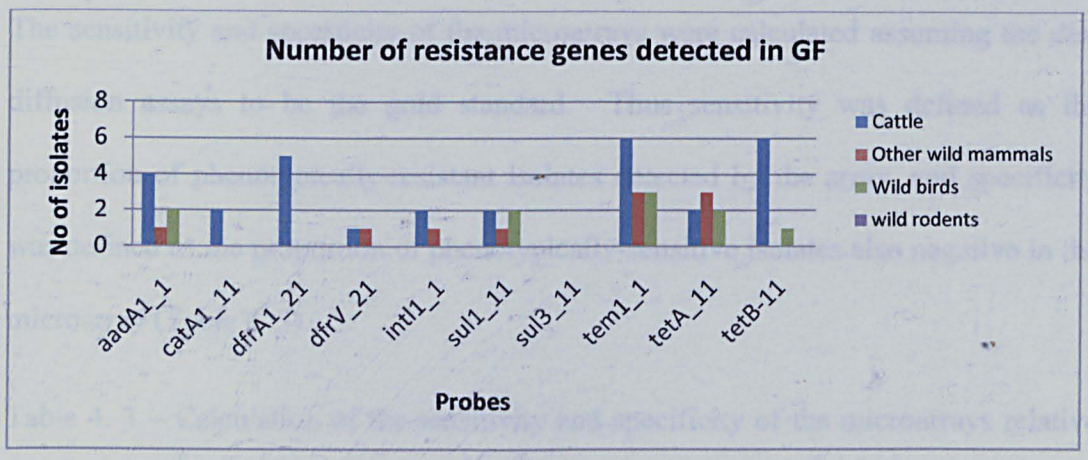


Figure 4. 9. Distribution of antimicrobial-resistance genes in *Escherichia coli* from animals on GF

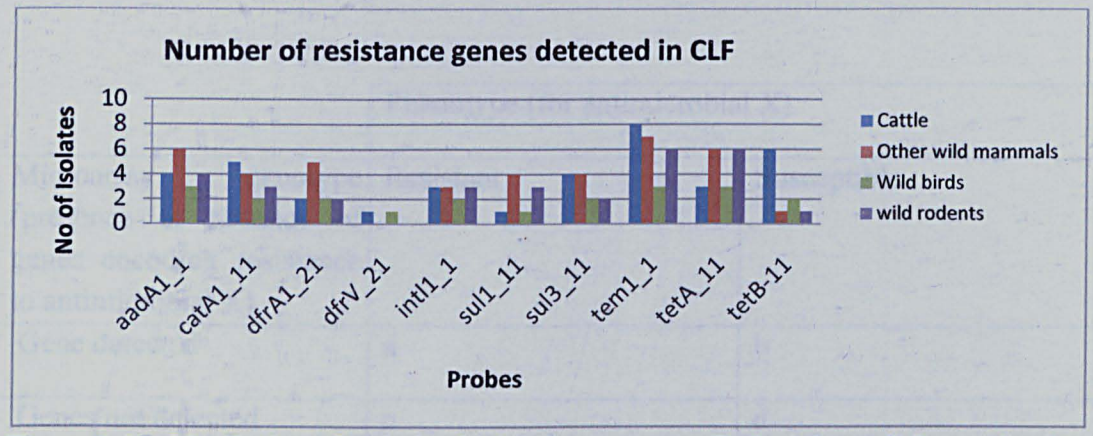


Figure 4. 10. Distribution of antimicrobial-resistance genes *Escherichia coli* from animals on CLF

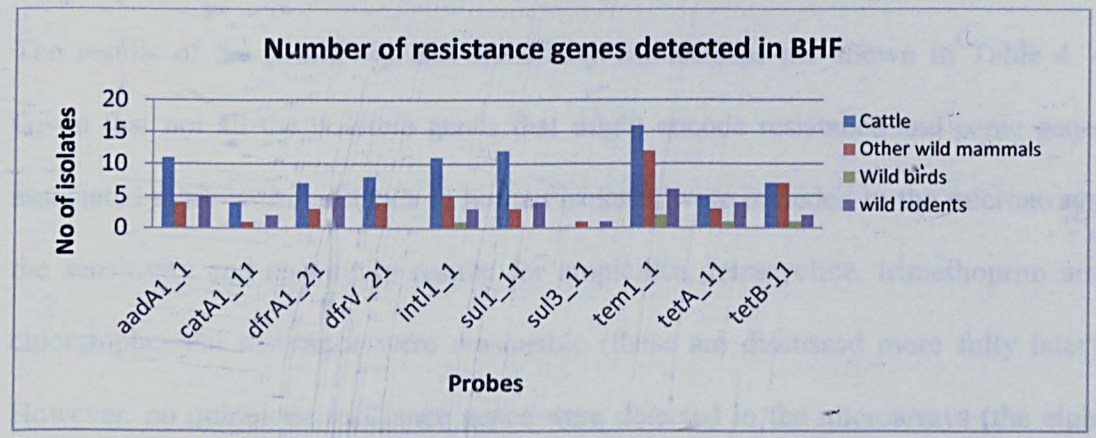


Figure 4. 11. Distribution of antimicrobial-resistance genes in *Escherichia coli* from animals on BHF

The sensitivity and specificity of the microarrays were calculated assuming the disc diffusion assays to be the gold standard. Thus sensitivity was defined as the proportion of phenotypically-resistant isolates detected by the array, and specificity was defined as the proportion of phenotypically-sensitive isolates also negative in the microarray (Table 4. 3).

Table 4. 3 – Calculation of the sensitivity and specificity of the microarrays relative to the disc diffusion test. Sensitivity = a/(a+c) Specificity = d/(b+d)

	Phenotype (for antimicrobial X)	
Microarray genotype (presence or absence of genes encoding resistance to antimicrobial X)	Resistant	Susceptible
Gene detected	a	b
Genes not detected	c	d

The results of the sensitivity and specificity calculations are shown in Table 4. 4 Given that not all the possible genes that might encode resistance and some genes associated more with resistance in human isolates, were included in the microarrays, the sensitivity and specificity results for ampicillin, tetracycline, trimethoprim and chloramphenicol resistance were reasonable (these are discussed more fully later). However, no quinolone resistance genes were detected in the microarrays (the eight isolates that were nalidixic acid and ciprofloxacin resistant in the disc diffusion assays were negative for relevant genes in the microarray). However, this microarray contained only one quinolone resistant gene (*qnr_11*).

Table 4. 4: the sensitivity and specificity of the microarray compared with disc diffusion assays

Gene	Sensitivity %	95% CI	Specificity	95% CI
Ampicillin				
Cattle	0.89 (49/55)	[0.77, 0.95]	0.47 (8/9)	[0.24, 0.71]
Other wild mammals	0.83 (34/41)	[0.67, 0.92]	0.43 (3/4)	[0.12, 0.80]
Wild rodents	0.72 (23/32)	[0.53, 0.86]	0.75 (3/1)	[0.22, 0.99]
Wild birds	1.00 (21/21)	[0.82, 1.00]	0.71 (5/2)	[0.30, 0.95]
Total	0.87 (130/149)	[0.81, 0.92]	0.54 (19/16)	[0.37, 0.71]
Chloramphenicol				
Cattle	0.70 (23/33)	[0.51, 0.84]	0.95 (37/2)	[0.81, 0.99]
Other wild mammals	0.33 (6/18)	[0.14, 0.59]	0.97 (29/1)	[0.81, 1.00]
Wild rodents	0.79 (11/14)	[0.49, 0.94]	0.95 (21/1)	[0.75, 1.00]
Wild birds	0.64 (7/11)	[0.32, 0.88]	0.82 (14/3)	[0.56, 0.95]
Total	0.62 (47/76)	[0.50, 0.73]	0.94 (101/7)	[0.87, 0.97]
Tetracycline				
Cattle	0.83 (52/63)	[0.70, 0.91]	0.67 (6/3)	[0.31, 0.91]
Other wild mammals	0.33 (6/18)	[0.14, 0.59]	0.97 (29/1)	[0.81, 1.00]
Wild rodents	0.71 (25/34)	[0.53, 0.85]	0.00 (0/1)	[0.11, 0.95]
Wild birds	0.81 (21/26)	[0.60, 0.93]	0.00 (0/2)	[0.05, 0.80]
Total	0.79 (132/168)	[0.71, 0.84]	0.56 (9/7)	[0.31, 0.79]
trimethoprim				
Cattle	0.87 (33/38)	[0.71, 0.95]	0.91 (31/3)	[0.75, 0.98]
Other wild mammals	0.83 (25/30)	[0.65, 0.94]	1.00 (18/0)	[0.78, 0.99]
Wild rodents	0.83 (15/18)	[0.58, 0.96]	0.78 (14/4)	[0.52, 0.93]
Wild birds	0.91 (10/11)	[0.57, 1.00]	0.94 (16/1)	[0.69, 1.00]
Total	0.94 (91/97)	[0.86, 0.97]	0.91 (79/8)	[0.82, 0.96]

4. 3. 2 Comparison between resistance gene profile of *E. coli* isolates

The antimicrobial resistance phenotype (as determined by disc diffusion tests) and genotype (as determined by the array) were compared for all isolates (some examples are shown in Table 4. 5 and further tables are shown in appendix 2).

Notable discrepancies between phenotype and genotype results were observed in a number of *E. coli* isolates. Uncorrelated genotypic data included isolates that carried some genes without a corresponding phenotype (i.e. *teml_1, tetA_11*). Furthermore

a number of isolates exhibited a chloramphenicol and tetracycline resistance phenotype, but were negative for all the *catA1_11, catB3_1, floR_11, tetA1_1 and tetB_1* genes included on the array. On the other hand there was complete correlation between susceptibility tests and hybridisation results in many *E. coli* samples.

With 47 resistance genes tested, there were 2^{47} combinations theoretically possible in any isolate. In fact, only 23 genotypes were seen, suggesting that the distribution of these antibiotic resistance genes was not random amongst strains. Most multidrug resistance combinations included tetracycline. The most common patterns detected consisted of *teml_1* and *tetB_11* (found in 29 isolates 15%). The next most common patterns consisted *teml_1* and *tetA_11* (found in 15 isolates 7.5%). Followed by other patterns containing from three to six genes (found in 39 isolates 19.5%) which were detected in between three and six isolates.

In total 52 (28%) isolates from cattle and wildlife hosts (14 from cattle, 13 from other wild mammals, 13 from rodents and 12 from wild birds) had identical patterns of phonotypic and gene resistance with others isolates; 137 (73%) carried the same antibiotic resistance patterns but with different gene resistance profiles.

Table 4. 5. Resistance of phenotypes and genotypes as identified by disc diffusion tests and microarray. Positive hybridization was indicated by (+) squares; Amp, ampicillin; aug, augmentin; chl, chloramphenicol; cip, ciprofloxacin; tet, tetracycline; trim, trimethoprim.

Farms	Species	No of samples	Resistance phenotype	*tetA_11*	*TetB-11*	*intI1_1*	*intL2_11*	*aadA1_1*	*catA1_11*	*dfrA1_21*	*dfrA1-22*	*dfrA14_21*	*dfrV_21*	*tem1_1*	*floR_11*	*sul1_11*
MF	O.W.M	1	amp, tet and trim		+							+		+		
BGF	O.W.M	1	amp, tet and trim		+							+		+		
MF	cattle	1	amp, tet and trim		+									+		
PHF	O.W.M	3	amp, tet and trim		+									+		
PHF	cattle	1	amp and tet		+									+		
PHF	cattle	1	amp and tet		+									+		
PHF	cattle	1	amp and tet		+									+		
PHF	birds	2	amp and tet		+									+		
BGF	cattle	2	amp and tet		+									+		
GF	cattle	2	amp and tet		+									+		
GF	birds	1	amp and tet		+									+		
BHF	cattle	1	amp and tet		+									+		
BHF	rodents	1	amp and tet		+									+		
BHF	birds	1	amp and tet		+									+		
BHF	O.W.M	2	amp and tet		+									+		
CLF	cattle	2	amp and tet		+									+		
MF	cattle	1	amp, tet and trim	+										+		
MF	O.W.M	1	amp, tet and trim	+										+		
GF	O.W.M	1	amp, tet and trim	+										+		
CLF	rodents	1	amp, tet and trim	+										+		
CLF	O.W.M	1	amp, tet and trim	+										+		
MF	O.W.M	1	amp, chl, tet and trim	+										+		
PHF	cattle	1	amp, chl, tet and trim	+										+		
MF	rodents	1	amp and tet	+										+		
PHF	cattle	1	amp and tet	+										+		
PHF	rodents	1	amp and tet	+										+		
BGF	birds	1	amp and tet	+										+		
CLF	rodents	1	amp and tet	+										+		

Farm	Sp. Group	No of samples	Resistance phenotype	tetA_11	tetB-11	intl1_1	intL2_11	aadA1_1	catA1_11	dfrA1_21	dfrA1-22	dfrA14_21	dfrV_21	tem1_!	floR_11	sul1_11
MF	rodents	1	amp, tet and trim	+		+		+		+				+		+
BHF	cattle	1	amp, tet and trim	+		+		+		+				+		+
CLF	rodents	1	amp, tet and trim	+		+		+		+				+		+
CLF	O.W.M	1	amp, tet and trim	+		+		+		+				+		+
MF	birds	1	amp and tet	+				+						+		+
MF	O.W.M	1	amp and tet	+				+						+		+
BGF	rodents	1	amp and tet	+				+						+		+
GF	birds	1	amp, tet and trim	+				+						+		+
GF	bird	1	amp and tet	+				+						+		+
CLF	rodents	1	amp, chl, tet and trim					+	+	+				+		+
CLF	O.W.M	1	amp, chl, tet and trim					+	+	+				+		+
BGF	birds	2	amp, chl, tet and trim	+		+		+		+				+	+	
CLF	birds	1	amp, chl, tet and trim	+		+		+		+				+	+	
BGF	cattle	1	amp, tet and trim	+		+							+	+		
BGF	rodents	1	amp, tet and trim	+		+							+	+		
BGF	birds	1	amp, tet and trim	+		+							+	+		
MF	cattle	1	amp, chl, tet and trim		+	+		+		+				+		
MF	rodents	1	amp, chl, tet and trim		+	+		+		+				+		
MF	birds	1	amp, tet and trim	±									+	+		
BHF	O.W.M	1	amp and trim	+									+	+		
BGF	birds	1	chl and tet	+		+		+	+	+				+		+
BHF	rodents	1	chl and tet	+		+		+	+	+				+		+
CLF	rodents	1	amp, chl, tet and trim	+		+		+	+	+				+		+
CLF	O.W.M	1	amp, chl, tet and trim	+		+		+	+	+				+		+

4. 4 **Discussion**

The numbers and classes of resistance genes were compared between *E. coli* isolates from cattle and wildlife using DNA microarrays. The array was capable of detecting genes encoding resistance to many antimicrobials active in Gram-negative bacteria, and able to identify the cross reaction for a small number of genes, so it ought to be useful for detecting known linkage between genes (Batchelor *et al.*, 2008).

The microtube based array system used in this study was relatively simple to use and had a short assay time due to an amplification step. It may also have the particular advantage of detecting the presence of antibiotic resistance genes that are not phenotypically expressed. It's very important to develop high advanced technique with low cost screening tools that can be used to identify the most antimicrobial resistance genes in different host. Several other *E. coli* resistance arrays for genotyping have been described previously (Grimm *et al.*, 2004; Call *et al.*, 2003). These arrays use mostly other types of technology, such as glass slides printed with short oligonucleotides or polymerase chain reactions products for the target genes as well as fluorescent cyanmine dyes to label the DNA used for hybridisation. Such systems can be more time consuming, require expensive reagents and also highly skilled technicians (Batchelor *et al.*, 2008).

In this study we used the DNA microarray for the detection of antimicrobial resistance genes in *E. coli* isolates. Included on the array were genes encoding resistance to tetracycline, chloramphenicol, trimethoprim, quinolone, β-lactamas, including extended spectrum β–lactamases, sulphonamides and aminoglycoside (streptomycin).

In this study, the most commonly detected genes were *teml_*for ampicillin, *tetA_11*, and *tetB_11* for tetracycline, *catA1_11*, and *floR_11*for chloramphenicol, *dfrA1_11, dfrV_21, and dfrA14_21* for trimethoprim, *sul1_11*, and *sul3_11* for sulphonamide, and *aadA1_1* for streptomycin.

The average number of resistance genes in the observed patterns was 3.3 with a maximum of 13. The highest number of resistance genes detected in an *E. coli* isolate by Batchelor *et al* (2008) was 15, which was isolated from a UK human patient (Batchelor *et al.*, 2008).

There were 171 isolates classified as resistant to tetracycline based on the disc diffusion test, and of these isolates 74 (43%) had *tetA_11*, 68 (39%) had *tetB-11*. 4 (2.3) had *tetA_11* and *tetB_11* together. There were also 151 isolates classified as resistant to ampicillin of this isolates 147 (97%) had *teml_1*. Of the 78 chloramphenicol resistant isolates 39 (50%) had *catA1_11*. 16 (20%) had *floR_11* and 1 (1.2%) had both genes together. And 98 samples were positive to trimethoprim 61 (62%) had *dfrA1_21*. 25 (26%) had *dfrV_21*. 14 (14%) had *dfrA14_21*. 13 (13%) had *dfrA1_21* and *dfrV_21* or *dfrA14_21*, *dfrA1_21* and *dfrV_21*combined together.

Resistance genes were detected in a number of isolates that were not phenotypically tested, including sulphonamide resistance genes *sul3_11* found in 19 (10%) and *sul1_11* found in 51 (27%) of isolates, and streptomycin resistance gene *aadA1_1* found in 79 (42%) isolates.

The class 1 and class 2 integrase genes *intl_11*and *intl2_11* were found in number of isolates from cattle and wildlife (58 (30%) isolates, and 11 (6%) respectively) suggesting that class 1and class 2 integrons may be important in the development of

multiresistance in isolates from both sources. These results are consistent with those of Guerra *et al* (2003) who found about 30% of all resistant *E. coli* isolates from pigs, poultry and cattle carried a class 1 integrons.

In the current study, most of antibiotic resistance genes of *E. coli* from cattle and wildlife faecal samples were found to be similar to those found by other workers. For example, we detected genes *tetA_11* and *tetB_11* in tetracycline-resistant and *aadA1_1in* streptomycin-resistant isolates from different hosts. These results appear to be in agreement with those described by Dolejska *et al* (2007) in Black-headed Gulls in the Czech Republic, Lanz *et al* (2003) in clinical *E. coli* from swine in Switzerland, Guerra *et al* (2003) in *E. coli* isolates from cattle, swine and poultry in German, Dolejska *et al* (2008) who found 70% of tetracycline-resistant isolates with both genes *tetA* and *tetB* in cow on all farms, and Kozak *et al* (2009) in small wild mammals (mice, voles and shrew) who found this resistance associated with farm origin.

Similarly, this study found *sul1_11* and *sul2_11* genes among sulphonamide-resistant *E. coli* isolates from cattle and wildlife were detected, as did Dolejska *et al* (2007) in Black headed Gulls). These genes were also detected in cattle, swine and poultry (Guerra *et al.*, 2003), as well as in commensal microbiota of healthy children living in urban area of Bolivia and Peru (Infante *et al.*, 2005). The majority of chloramphenicol-resistant isolates were positive for the *catA1_11* and *floR_11*, even in birds. These genes have been reported to be frequently responsible for chloramphenicol resistance in *E. coli* from cattle in Western Canada (Gow *et al.*, 2008). Similar resistance genes were also detected in cattle but not in four house sparrow's *E. coli* by Dolejska *et al* (2008).

While there was a good correlation between phenotypic and genotypic results for a large number of samples of *E. coli*, discrepancies were also seen. For example, quinolone resistant *E. coli* showed no *qnr_11* resistance gene. The absence of any correlation with the nalidixic acid and ciprofloxacin might indicate that quinolone resistance is not related simply to the present of *qnr_11*, and other genes may be present. Alternatively, resistance may be due to mutation in gyrase genes. Similarly, a few isolates were phenotypically resistant to ampicillin, chloramphenicol, tetracycline and trimethoprim without, apparently, a corresponding genotype. This may reflect cross resistance, or these isolates may encode resistant genes not present on array.

Conversely, genes were sometimes present without the corresponding phenotype. For example, *E. coli* were identified that were *tetA1_1* and *tetB1_1*, positive, yet tetracycline sensitive. Gene silencing is a possible reason for this. Other examples of potential gene silencing include the detection of *catA1_11*, *floR_11* and *tem1_1* in several *E. coli* isolates that did not demonstrate chloramphenicol or ampicillin resistance. Again *dfrV_21* or *catA1_11* were detected in number of isolates that were not trimethoprim-or chloramphenicol-resistant. Previous studies have demonstrated the possibility of gene silencing for number of genes (Batchelor *et al.*, 2008; Gow *et al.*, 2008; Enne *et al.*, 2006). In conclusion the discrepancies between genotype and phenotype observed in this study may be the result of not testing for all possible resistance genes or of genes not being turned on in number of isolates. Other explanation for the divergence between phenotype and genotype and the presence of resistance genes may be that the misreading for resistance zone (breakpoint) resulting in the misclassification of isolates as susceptible or resistant. Finally, some

resistance phenotype may be caused by mutation rather than gene acquisition. In this case no associated resistance genes would be expected.

A number of samples shared antibiotic resistance patterns and genes profiles yet were isolated from both domestic cattle and wildlife and this might indicate cross species transmission. However more work is needed in to determine whether this suggests cross-species transmission of *E.coli*, or merely some commons assemblages of genes. This question will be addressed by the use of Pulsed Field Gel Electrophoresis (PFGE) in the next chapter.

Chapter Five

Molecular characterization and comparison of *Escherichia coli* using Pulsed Field Gel Electrophoresis (PFGE)

Molecular characterization and comparison of *Escherichia coli* using Pulsed Field Gel Electrophoresis (PFGE)

5. 1 Introduction

Increasingly, the use of antibiotics in human medicine and intensive animal production has been linked to the selection and evolution of antibiotic-resistant pathogenic bacteria (Silbergeld *et al.*, 2008). In addition to the selection of antibiotic resistant strains of pathogenic bacteria, the use of antibiotics has other potentially serious outcomes including the exertion of selective pressures on non-pathogenic commensal bacteria possibly leading to a reservoir of bacteria containing antibiotic resistance genes.

Commensal *E. coli* inhabitant the intestinal tract of many mammals and birds (Pupo, 2000), and may be exposed to antibiotics when used for treatment or prophylactic purposes and subsequently develop the resistance to these antibiotics in order to survive. Consequently, commensal *E. coli* might become an important reservoir of antibiotic resistance genes, which may contribute to increased gene frequency and dissemination of resistance genes through the microbial ecosystem. For this reason, it is very important to investigate the phenotypes, genotypes and the mechanisms of resistance not only in pathogenic bacteria but also in commensal bacteria of the human and animal intestinal tract, which could act as reservoirs for antibiotic resistant bacteria.

Correlation between consumption of antibiotics and the prevalence of resistance in bacteria is complex, and no definitive conclusions have been reached since the prevalence of antibiotic resistance differs between studies. Also, it has been speculated that differences in the patterns of antibiotic resistance may not be directly

related to antimicrobial consumption (Bartoloni *et al.*, 2004). Commensal *E. coli* could serve as a reservoir for tetracycline resistance determinants in dairy cattle environments, even in the absence of tetracycline resistance pressure, suggesting that prevalence is not necessarily related to the recent use of antimicrobial drugs (Khachatryan *et al.*, 2004).

There are other important factors involved in the selection of antibiotic resistance in bacteria such as cross-species transfer of antibiotic resistance genes. It has been previously demonstrated that the transfer of antibiotic resistant faecal bacteria between ranches and resistance can be found in areas with no previous history of antibiotic use (Bartoloni *et al.*, 2004).

Acar and Moulin (2006) reported that factors such as geographical location, the level of hygiene, the size of the farm and the type of integrated farming which takes place can affect the probability of the transmission of antibiotic resistant bacteria between animals and humans. This transmission between animal and human can occur through direct or indirect contact (Price *et al.*, 2007; Armand-Lefevre *et al.*, 2005; Hershberger *et al.*, 2005). Other routes for the dissemination of antibiotic resistant microorganisms could be the food chain (Silbergeld *et al.*, 2008; Johnson *et al.*, 2007), surface water runoff (Davis *et al.*, 2006) and agriculture facilities (Sarmah *et al.*, 2006). In ecological studies, additional routes such as the airborne, the inhalation of air from swine may serve as an additional environmental exposure pathway for the transfer of multidrug resistant bacterial pathogen from swine to human (Chapin *et al.*, 2005). Hospital patients can also disseminate antibiotic resistance (Lipsitch *et al.*, 2002).

Recently, more information has emerged on the occurrence of antibiotic resistance within Enterobacteriaceae isolated from wild mammals and wild birds in a number of different countries (Skurnik *et al.*, 2006; Cole *et al.*, 2005; Sayah *et al.*, 2005; Livermore *et al.*, 2001; Osterblad *et al.*, 2001, Sherley *et al.*, 2000; Gilliver *et al* 1999).

Pulsed-field gel electrophoresis (PFGE) is a technique employed to genotype microorganisms. PFGE is often used to infer genetic relatedness of different bacterial strains. This technique has high reproducibility and discriminatory power in comparing genetic material. It has very high resolution and can discriminate between closely related bacterial strains. For this reason and others, it is considered as one of gold standards in epidemiological studies of organisms (Goerring, 2004). PFGE was used in the current study to characterise *E. coli* isolated from wildlife and cattle faeces. We used PFGE to enable us to determine how similar strains of *E. coli* were that had been derived from the same source and from different sources.

The main aim of this study was to characterise *E. coli* isolates from wildlife and cattle that we identified as having the same resistance phenotype and microarray genotype (Chapter 4). We were interested to determine whether these isolates were identical, which might indicate transmission of *E. coli* between cattle and wildlife populations.

5. 2 **Materials and Methods**

5. 2. 1 Bacterial Isolates

Samples were collected from cattle and wild animals in six farms. A total number of 2084 faecal samples were examined, from which *E. coli* were detected in 1303 (62.5%), 405 from cattle, 192 from other wild mammals, 619 from rodents and 87 from wild birds. Methods used for isolation and identification have been described in previous chapters (materials and methods chapter 2). Resistant bacterial isolates, by phenotyping (disc diffusion method) and genotyping (DNA microarray) were identified as described in previous chapters (chapter 3 and 4). In this chapter further characterization was carried out by pulsed field gel electrophoresis following digestion with *Xbal*, to investigate the relatedness of resistant determinants with similar phenotypes and genotypes.

Pulsed-field gel electrophoresis was carried out as described previously accordance to the Pulse Net Standard Protocol (www.cdc.gov/pulsenet).

The method used is outlined below.

Day one

Isolates were cultured overnight on nutrient agar at 37^0C.

Day two

The bacteria were harvested into 2ml Cell Suspension Buffer (CSB) [100mM Tris, 100mM EDTA, and pH8.0] in sterile plastic bijoux. Suspensions were diluted 1:10. The optical density (OD) of a 1:10 dilution of this suspension was measured using a spectrophotometer to calculate the volumes of cell suspension and CSB required to

make a 200µl suspension with a final OD_{610} of 1.35. Ten microliters of proteinase K solution ($20mgml^{-1}$) was then added to each suspension and mixed gently, followed by 200µl of agarose mixture (1% Bio-Rad PFGE grade agarose, 1% SDS, in 1XTE buffer) made fresh on the day, mixed by pipetting and immediately transferred into duplicate plug molds. These were left to set at 4^0C for 45 minutes.

Duplicate blocks were made in case the enzyme digestion failed and needed to be repeated, or a comparison was needed with a different restriction enzyme.

Three millilitres of cell lysis buffer were added to bijoux tubes and 15µl proteinase K added. The agarose blocks were placed in the solution and incubated with shaking (185 rpm) at 54^0C for 2 hours. The CLB was then removed and 2ml pre-heated sterile water added, which were incubated with shaking for 15 minutes at 54^0C (185 rpm). The plugs were washed twice with sterile distilled water.

Day three

The second water wash was removed and 3ml pre-heated 1XTE buffer was added and this was incubated as above. This was repeated in total of four times. After this, blocks were stored in fresh buffer at 4^oC until digestion with the restriction enzyme.

5. 2. 2 **Restriction enzyme digests**

TE buffer was removed and one agarose block transferred to a sterile eppendorf tube containing 200µl of 1X restriction buffer (specific to the restriction enzyme). Incubation was at 37 ^{0}C for minimum of 15 minutes with shaking as mentioned above to equilibrate blocks. The remaining block was covered in 2 ml 1XTE buffer and stored at 4^0C. After incubation, restriction buffer was removed and 200µl fresh 1X restriction buffer containing 50U *Xbal* were added. This was incubated at 37^0C

for a minimum of 2 hours but could be left longer. The restriction enzyme was stopped by placing blocks in the fridge at 4°C.

5. 2. 3 **Gel electrophoresis**

Digested blocks were run on a 1% PFGE grade agarose gel made with 0.5 X TBE. Blocks were cut in half, one half inserted into the gel, the other half being kept in 1ml of 1 X TBE buffer and returned to the fridge. Bacteriophage λ DNA concatemers embedded in 1% LMP agarose were used as relative molecular weight markers in first, middle and last lane of the gel. The gel was run on a Bio-Rad chef Drill system with 0.5X TBE buffer at 14°C for 20 hours. Initial switch time was 2.2s and final switch time was 54.2s (gradient of 6 V/cm and angle of 120). The gels were stained with ethidium bromide solution (10Mg in 200ml of sterile distilled water for 20-30 minutes, then the bands were visualized under UV light and digitally photographed. Interpretation of these bands by comparison with the marker bands could be carried out to determine whether these isolates were identical or different. This was determined by the number of identical bands within isolates from different host on different farms.

5. 4 **Statistical analysis**

Bands were analysed using Bio Numerics analysis software, version 4.61 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). A percentage similarity between pulse-field banding patterns was calculated according to the Dice similarity coefficient method with a 2% tolerance window, and a dendrogram was constructed using the UPGMA (unweighted pair group method with averages).

5. 3 **Results**

A total of 44 *E. coli* from four different animal sources (16 from cattle, 6 from wild birds, 10 from wild rodents, and 12 from other wild mammals) were analysed by PFGE. However, PFGE patterns could not be generated for four samples so the comparison was limited to 40 isolates.

Although based on a limited number of samples, PFGE revealed that some of the *E. coli* isolates exhibited the same PFGE banding patterns. For example, isolates 228 and 283, which were from a cow and wood mouse respectively from MF, which were both resistant to ampicillin, tetracycline and trimethoprim and carrying resistance genes *teml_1, aadA1_1, tetB_11, dfrA1_21, sul1_11* and *intl1_1* both had the same PFGE banding pattern. Two further isolates that shared an identical PFGE banding pattern were 1016 and 994, which were isolated from cattle and a wood mouse respectively, both from BHF, and were resistant to ampicillin and tetracycline and carried resistance genes *teml_1* and *tetB_11*. Finally, isolates 266 and 48 were isolated from cattle and a dog that were both sampled from CLF, and were resistant to chloramphenicol and tetracycline and carried resistance genes *aadA1_1, tetB_11, catA1_11 and dfrA1_21.*

Table 5. 1. Description of the isolates that were subjected to PFGE.

Isolate number	Genotype	Phenotype	Species	Farm	Date collected
978	*tem1_1 & tetB_11*	amp & tet	badger	BHF	09.09.04
920	*tem1_1 & tetB_11*	amp & tet	unknown bird	BHF	02.02.05
47	*tem1_1 & tetB_11*	amp & tet	bovine	GF	03.05.05
317	*tem1_1 & tetB_11*	amp & tet	bovine	MF	05.07.04
315	*tem1_1 & tetB_11*	amp & tet	badger	MF	05.07.04
92	*tem1_1 & tetB_11*	amp & tet	badger	PHF	08.03.05
380	*tem1_1 & tetB_11*	amp & tet	bovine	CLF	09.08.04
1016	*tem1_1 & tetB_11*	amp & tet	bovine	BHF	31.08.04
994	*tem1_1 & tetB_11*	amp & tet	Wood mouse	BHF	09.09.04
882	*tem1_1 & tetB_11*	amp & tet	unknown bird	GF	07.11.04
757	*tem1_1 & tetB_11*	amp & tet	bovine	PHF	05.07.04
1210	*tem1_1 & tetB_11*	amp & tet	bovine	BGF	18.04.04
702	*tem1_1 & tetB_11*	amp & tet	House sparrow	PHF	05.07.04
509	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	Amp, tet & trim	Bank vole	CLF	26.08.04
1006	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	Amp, tet & trim	House mouse	BHF	09.09.04
437	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	amp, tet & trim	buzzard	CLF	25.08.04
266	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	Amp, tet & trim	Wood mouse	MF	13.07.04
1067	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	Amp, tet & trim	bovine	BHF	06.09.04
1139	*Tem1_1, aadA1_1, tetA_11, Sul1_11, dfrA_21 & intl1_1*	Amp, tet & trim	rabbit	BHF	06.09.04
278	*Tem1_1 & tetA_11*	amp & tet	Wood mouse	MF	13.07.04
792	*Tem1_1 & tetA_11*	amp & tet	bovine	PHF	05.07.04
862	*Tem1_1 & tetA_11*	amp & tet	Wood mouse	PHF	25.08.04
1169	*Tem1_1 & tetA_11*	amp & tet	Wood mouse	CLF	26.01.05
41	*Tem1_1 & tetA_11*	amp & tet	bovine	GF	03.05.05
424	*Tem1_1 & tetA_11*	amp & tet	rabbit	CLF	25.08.04
673	*Tem1_1 & tetA_11*	amp & tet	pheasant	MF	23.02.05
657	*Tem1_1, tetA_11, intl1_1 & dfrV_21*	amp, tet & trim	bovine	BGF	15.11.04
526	*Tem1_1, tetA_11, intl1_1 & dfrV_21*	amp, aug, tet & trim	Pigeon	CLF	25.08.04
32	*Tem1_1, tetA_11, intl1_1 & dfrV_21*	amp, aug, tet & trim	robin	BGF	13.01.05
609	*Tem1_1, tetA_11, intl1_1 & dfrV_21*	amp, aug, tet & trim	Bank vole	BGF	11.11.04
39	*aadA1_1, tetA_11, dfrA1_21 & intl1_1, sul1_11 & catA1_11*	amp, aug, chl, tet & trim	bovine	PHF	04.04.05
46	*aadA1_1, tetA_11, dfrA1_21 & intl1_1, sul1_11 & catA1_11*	amp, aug, chl, tet & trim	Wood mouse	PHF	23.03.05
182	*tetB-11, catA1_11 & sul3_11*	Tet & chl	bovine	CLF	17.01.05

Isolate number	Genotype	Phenotype	Species	Farm	Date collected
531	*TetB_11, catA1_11 & sul3_11*	Tet & chl	dunkock	CLF	26.08.04
178	*aadA1_1, tetB_11, catA1_11 & sul3_11*	Tet & chl	bovine	CLF	17.01.05
460	*aadA1_1, tetB_11, catA1_11 & sul3_11*	Tet & chl	dog	CLF	25.08.04
309	*Tem1_1 & tetA_11*	amp, tet & trim	rabbit	MF	15.08.05
778	*Tem1_1 & tetA_11*	amp, tet & trim	bovine	MF	05.07.04
10	*Tem1_1 & tetA_11*	amp, tet & trim	fox	GF	18.03.05
228	*Tem1_1, aadA1_1, tetB_11, dfrA1_21 & intl1_1*	amp, tet & trim	bovine	MF	05.07.04
283	*Tem1_1, aadA1_1, tetB_11, dfrA1_21 & intl1_1*	amp, tet & trim	Wood mouse	MF	13.07.04
1077	*Tem1_1, intl1_1 & dfrV_21*	chl & tet	bovine	BHF	06.09.04
988	*Tem1_1, intl1_1 & dfrV_21*	amp & trim	badger	BHF	09.09.04
1183	*tem1_1 & tetB_11*	amp, tet & trim	fox	BGF	18.04.05

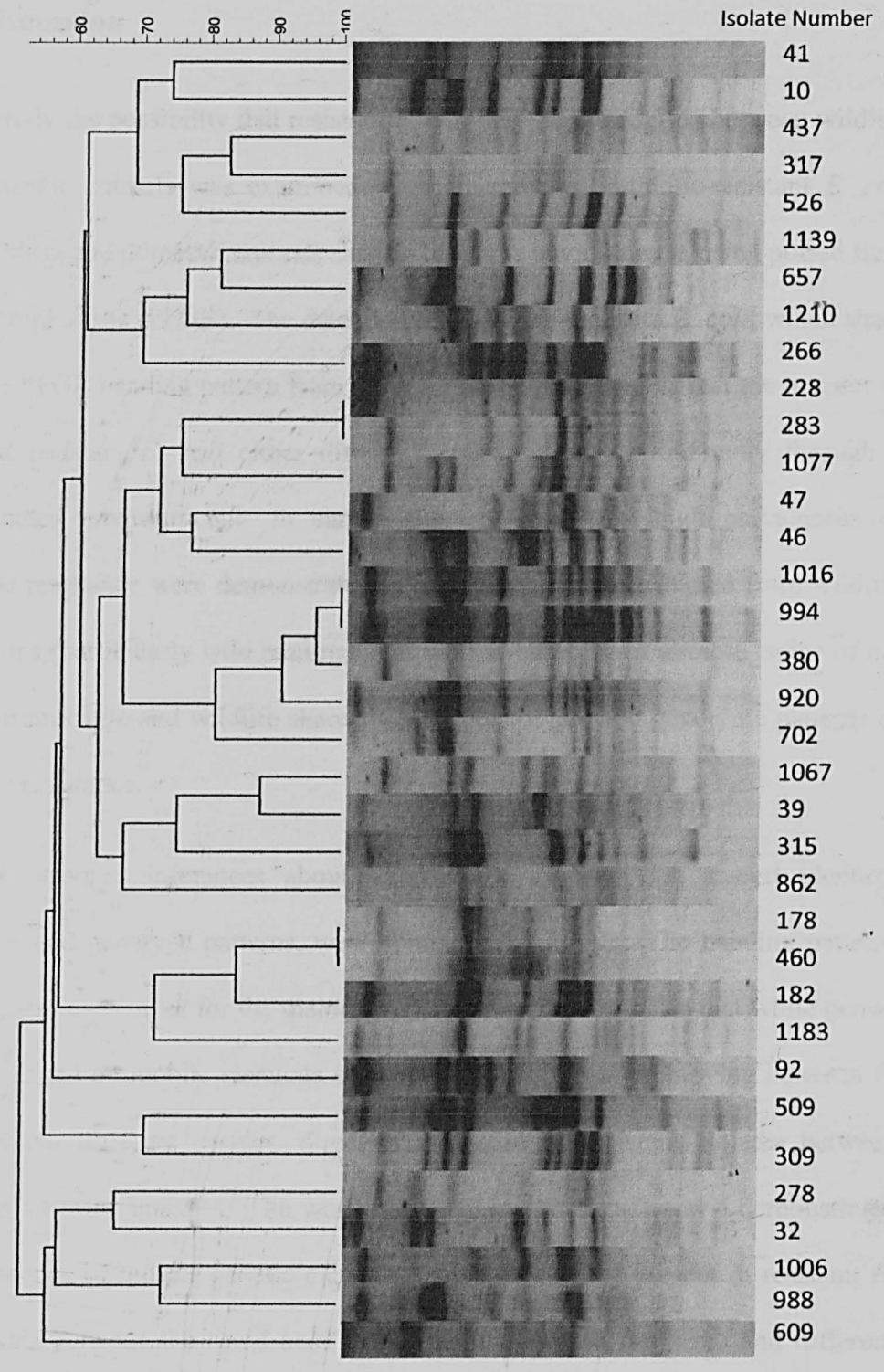


Figure 5. 1. A typical PFGE of *Escherichia coli* from cattle, wild birds, wild rodents, and other wild mammals.

5. 3 Discussion

In this study the possibility that resistant *E.* coli may be transmitted between wildlife and domestic animals was examined by characterising antibiotic-resistant *E. coli* from wildlife and domestic animals sharing the same environments using pulsed field gel electrophoresis (PFGE). The detection of antibiotic-resistant *E. coli*, which share the same PFGE banding pattern from different hosts, may suggest that the transfer of antibiotic-resistant *E. coli* either directly between hosts, or indirectly through a contaminated environment. In earlier chapters, relatively high prevalences of antibiotic resistance were demonstrated in commensal *E. coli* isolated from wildlife populations (particularly wild mammals) as well as cattle. Furthermore, some of the isolates from cattle and wildlife shared similar phenotypic and genotypic patterns of antibiotic resistance.

To draw stronger inferences about transmission isolates that shared identical phenotype and genotype patterns, were compared by PFGE. The banding patterns differed from each other for the majority of isolates. This suggests that while genes, perhaps carried on mobile elements such as plasmids, might be moving between *E. coli* infecting different species, direct transmission of resistant isolates between species is, at least, unusual. The work presented in Chapters 3 and 4 demonstrated some evidence of mobile genetic elements (integrons) being present in resistant *E. coli* isolates between the same host and different hosts on the same and different farms. Further molecular work to determine the presence of plasmids and other transmissible genetic elements would be very beneficial in order to have a better understanding of the antibiotic resistance gene dynamics amongst *E. coli* strains in this population.

Interestingly, six *E. coli* isolates that exhibited similar PFGE banding patterns of *E. coli* were detected in cattle and wildlife faeces. This may suggest that bacterial transfer between cattle and wildlife is possible. If transmission has occurred, there is no indication of the direction of travel: it might be that wild animals can be a source of resistant bacteria for farm animals, or vice versa. Katsunuma *et al.* (2008) found identical clones among *E. coli* and *enterococci* isolates from the faeces of broilers, pigs, cattle and humans, suggesting the possibility of transmission between animals and human on the same farm. However, both wildlife and cattle could have become infected via a common source of resistant bacteria.

Resistant bacteria can be transmitted from animal reservoirs to humans through faecal contamination of food, water and the environment or through direct contact with animals (Wasteson *et al.*, 2005). That transmission did occur, whether indirect or direct, is, however, supported by identical isolates being found on the same, rather than distant farms.

Indeed it is interesting to ask why cross species transmission in not more common, and why so few identical isolates were detected in a range of species on any farm. May be that isolates/strains of *E. coli* are more host limited than often thought. Certainly, the introduction of *E. coli* into a host population does not always lead to its spread. For example, Rice *et al* (1999) found that some *E. coli* subtypes introduced onto a farm were transiently isolated from cattle but failed to be maintained within the herd over long time periods.

PFGE has been demonstrated to be a reliable tool for the differentiation of 'strains' and for use in epidemiological studies of several pathogenic bacteria because of it is high resolution and reproducibility (Shima *et al.*, 2006; Senna *et al.*, 2002). It is

considered to be the gold standard for the differentiation of *E. coli* and *enterococci* strains (Goerring, 2004; Morrison *et al.*, 1999; Swaminathan *et al.*, 2001) and has been used to provide information about the potential clonality of antibiotic-resistant strains (Hoyle *et al.*, 2005).

The use of PFGE has both strengths and weaknesses. The results gained from this study showed that few isolates shared closely related PFGE banding patterns although some were very similar, indicating that some isolates may have been related strains. The major disadvantages of PFGE is that it can be labour intensive and time consuming. Also, there is occasionally difficulty when comparing the results obtained from different gels that have been run under identical conditions. The cost of restriction enzymes is a further disadvantage of the PFGE technique. It should be noted that PFGE banding patterns could not be generated for all isolates analysed; in this study four samples (9%) did not permit effective restriction.

In conclusion, a cross sectional study was carried out to investigate the potential transmission of commensal antibiotic resistant *E. coli* between wild animal and livestock hosts. This demonstrated a high prevalence of antibiotic resistance in the normal enteric bacterial flora of wildlife populations. Isolates from wild and domestic species with similar resistance profiles (as determined by disc diffusion tests and microarray analysis) were compared by PFGE, and some evidence for direct transmission between hosts was found – however, most isolates were different suggesting that inter-species host transmissions at least unusual. Unfortunately PFGE was not carried out to determine relatedness of all isolates, owing to cost. This might have revealed more transmission of strains, gaining and losing resistance in the process.

Chapter Six

General Discussion

6. 1 **Discussion**

As outlined in Chapter 1, it is usually thought that the increased prevalence of antimicrobial resistance seen in bacteria from clinical human and veterinary medicine is largely due to selection through the use of therapeutic antibiotics. Such resistance is selected for not only in those microbes that are the targets of antibiotic treatment, but also in those of the normal, commensal microflora, although this has been less intensively studied. More recently a series of studies have also identified antimicrobial resistance in pathogenic and non-pathogenic bacteria from various species of wild animals including wild birds (Sjolund *et al.*, 2008; Gibbs *et al.*, 2007; Dolejska *et al.*, 2007; Literak *et al.*, 2007), wild mammals (Gilliver *et al.*, 1999; Kozak *et al.*, 2006; Sherley *et al.*, 2000), amphibians (Mitchell, 2003) and reptiles (Ahmed *et al.*, 2007; Gopee *et al.*, 2000). Although it was assumed in all of these studies that the wild animals investigated had never been treated with antibiotics, it is difficult to compare these studies as they differed in the environment and host animals investigated, the methodology used, bacterial species investigated, and classes of antibiotics tested. Moreover, variation in possible contact of wild animals with human beings, domestic animals, and the presence of different possible sources of contamination such as rivers, wastewater from farms, food processing plants or slaughter houses, makes comparing the findings still more difficult.

The work described in this thesis was different from previous studies in that, based on previous studies suggesting that antimicrobial resistant *E. coli* were found in wildlife on Cheshire farms, it aimed more to study possible transmission between wildlife and cattle. Might wildlife be a source of resistance for cattle, or would resistant *E. coli* excreted by cattle so contaminate the environment that these criteria

would be found in all wildlife? Or might the ecology of resistance and transmission be more complicated than this?

Thus the first aim was to investigate the possibility of the transmission of antibiotic resistant bacteria between domestic cattle and wild animals. We assumed that if antibiotic-resistant *E. coli* isolates shared common phenotypic and genotypic characteristics this might suggest transmission of these bacteria does occur between wildlife and domestic farm animals either through direct contact or indirectly through common source of resistance genes or strains.

Thus a cross sectional survey of domestic animals and wildlife was undertaken. Faecal samples from domestic animals and wildlife present on six farms (three pairs of neighbouring farms) in Cheshire in north-west England were collected between 2004 and 2005. Faecal *E. coli* were studied as the model organism as this species is known to have a wide host range, and antimicrobial resistance in *E. coli* has been studied intensively in the past, so much is known about the possible resistance genes involved. *E. coli* isolates were identified using classical biochemical methods. Susceptibility to seven antibiotics (ampicillin, augmentin, chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline and trimethoprim) was tested by the disc diffusion method.

Several resistant isolates with different resistance phenotypes were obtained from single faecal samples and some samples yielded *E. coli* isolates with more than four different resistance profiles. Of the 1303 samples tested, 38% were found to be resistant to at least one antimicrobial. Resistance to tetracycline, ampicillin, and trimethoprim was most common, followed by resistance to chloramphenicol and augmentin. Few isolates were resistant to ciprofloxacin and nalidixic acid. Eighty

four different patterns of multiple antimicrobial resistance were detected. The most common patterns consisted of resistance to ampicillin and tetracycline. The next most common multiple antimicrobial resistance pattern was resistance to ampicillin, tetracyclines and trimethoprim.

Escherichia coli was isolated from the majority of faecal samples from cattle and other wild mammals (foxes, badgers and rabbits). Isolates from these sources were found to have the highest prevalence of resistance; 72 % and 66 % respectively. A lower incidence of antibiotic resistant *E. coli* was detected in wild birds and rodents; 20 % and 17% respectively. Therefore, the general observation was that cattle had greatest proportion of *E. coli* faecal samples that were resistant to at least one antibiotic, and this is consistent with Sayah *et al* (2005) who reported that *E. coli* isolates from domestic animals showed resistance to the largest number of antimicrobial agents compared to isolates from human sewage, wildlife, and surface water. It is interesting that there was nearly as a high a prevalence of antibiotic resistant *E. coli* in other wild mammals as in cattle, as it was initially hypothesised that the prevalence of resistance would be significantly higher in cattle as they are more likely to have been exposed to antibiotics. Indeed, on some farms there was a higher prevalence of resistant bacteria detected in other wild mammals than in cattle. This would suggest that other factors may be playing a role in selecting for resistance to antibiotics than simply antibiotic use. These factors might include possible transmission of antibiotic resistant bacteria between different host species, or multiple host species being infected from one common source of resistant bacteria. It would be interesting to examine this hypothesis in further studies, which could examine environmental samples including soil, slurry, manure and water samples to determine the importance of the environment as a transmission route.

In this study, a high prevalence of resistance to tetracycline, ampicillin, trimethoprim and chloramphenicol was observed in *E. coli* isolates from a range of host species. These findings are also in line with other workers who have reported a high level of acquired resistance in *E. coli* to several classes of antibiotics. Bywater *et al* (2004) found high resistance among *E. coli* isolates from cattle in Spain to ampicillin, tetracycline, trimethoprim and chloramphenicol. Other investigators have also shown similar results in black headed gulls in the Czech Republic (Dolejska *et al.*, 2007). The results were also in agreement with the findings of similar studies carried out on livestock and human populations (Bywater *et al.*, 2004; Lanz *et al.*, 2003). However, higher levels of antibiotic resistant *E. coli* were found in this study than those reported by Mubita *et al* (2008) who found that 3.6% and 8.4% of *E. coli* isolates from pastoral cattle in Zambia were resistant to tetracycline and ampicillin respectively. Aarestrup *et al* (2001) found *Enterococci* isolates were completely susceptible to tetracycline (100%), despite the differences in animal species. Bryan *et al* (2004) reported that the prevalence of bacteria resistant to tetracycline and ampicillin, which were isolated from various animals and the environment, remains high in Europe, despite the fact that these antibiotics have not been used in Europe since 1975 as growth promoters.

The weak response in resistance to augmentin in comparison with ampicillin might be expected; augmentin is a combined antibiotic (amoxicillin plus clavulanic acid) and is available for use when there is high level of resistance to amoxicillin or ampicillin as the clavulanic acid is a strong β–lactamase inhibitor. *E. coli* isolates from cattle and wild animals sampled showed in general a lower percentage of resistance to quinolone antibiotics (nalidixic acid and ciprofloxacin). It is also interesting that ciprofloxacin resistance was detected in around 3% of isolates (but

mostly in cattle and other wild mammals samples) tested in this study. It was rarely found in *E. coli* isolated from samples from wild birds and rodents. The lack of notable resistance to nalidixic acid and ciprofloxacin in either cattle or wildlife suggests a lack of resistant strains to nalidixic acid or ciprofloxacin in this area or that resistance might be due to chromosomal mutations, which cannot be transferred between bacteria horizontally. Our findings differ from those reported by Costa *et al* (2008) who found resistance to nalidixic acid and ciprofloxacin was 14% and 9%, respectively, in wild animals in Portugal. They also report that this resistance was associated with mutations in *gyrA* and *parC* genes. However, *E. coli* isolates from cattle in this study showed higher resistance to nalidixic acid and ciprofloxacin than that detected by Gow *et al* (2008) who found a complete absence of resistant strains.

When we compared the results from different farms, there was similarity in some of the resistance patterns for *E. coli* isolates from different hosts sampled on the same farm. This was also true for some neighbouring farms, for example, the resistance patterns of the *E. coli* isolated from hosts on MF and PHF were similar on both farms. This may be explained by *E. coli* being acquired by hosts on the two farms from a common source of resistant bacteria or cross transmission between the farms. A little more variation was seen when comparing farms that were more distant from each other. For example, samples collected from BGF and GF showed the lowest prevalence of resistance, but these two farms are further apart than the other farms. This may indicate that these farms as neighbouring farms were less contaminated with resistant strains from different sources.

The results also showed that some antibiotic resistance profiles seem limited to one type of host. This suggests that resistance found in *E. coli* faecal samples from wild

animals, or cattle at least, may be subject to different selection pressures, or a wide range of different sources of infection. It could be related to the diet of animals as different animals exhibit different behaviours for example, foxes can move long distances looking for food, and tend to have a broad diet. The diet of foxes can include rodents, rabbits, other small mammals, reptiles (such as snakes) amphibians, grasses, fruits, fish and birds. Whereas the diet of badgers consists largely of earth worms, insects and grubs. Occasionally, badgers will eat small mammals, amphibians, reptiles and birds as well as cereal and fruits. Rabbits are active throughout the year; rabbits are herbivores who feed by grazing or grass and leafy weeds.

It is clear that multi-drug resistant *E. coli* are very common across large range of wildlife and cattle. Resistance to six antibiotics was found in *E. coli* isolated from samples collected on two farms: samples collected from cattle and rodents on MF, and twice in cattle on CLF. In addition, resistance to seven antibiotics was found in samples collected on four farms, *E. coli* from cattle and rodents on BHF, once in birds on MF, once in other wild mammals on PHF, and once in cattle on BGF.

The highest prevalence of multi-drug resistance was detected in isolates from cattle and other wild mammals compared to rodents and wild birds. Most multidrug resistant isolates showed resistance to antibiotics that included tetracycline, which may suggest that *E. coli* resistant to tetracycline are at increased risk for becoming resistant to another antibiotic. A study by Acar and Moulin (2006) showed that multidrug resistant strains can survive in the presence of various antibiotics and use of just one antibiotic may remain the whole set of resistance characters. More recent studies have reported that plasmids may play a role in the development of antimicrobial resistance because they often contain resistance genes (Bennett, 2008;

Boerlin & Reid-Smith, 2008). It is possible that the observed resistance to multiple drugs observed in this study is a reflection of a general resistance mechanism related to plasmid transfer rather than selective pressures. This does not mean that selective pressure is not involved in the dissemination of antibiotic resistance but shows that it may not be sufficient on its own to cause the resistance patterns seen, and that other factors are likely to be involved too.

In this study, the data obtained from a cross sectional study were broken down by host species, farm and farm pair. Using a database for the analysis of resistance patterns provided clear information on the prevalence and potential spread of resistant bacteria between hosts on the farms, and helped to select *E. coli* isolates in sympatric wild animals and domestic animal population that had the same resistance profile for further investigation by using DNA microarray technique. Multidrug resistance was very common, and the multidrug resistance profiles are being used to identify potential transmission between hosts / species.

The microarrays technique used in this study has been shown to be a rapid and simple technique for the screening of resistance genes in Gram-negative bacteria. With this technique, it is possible to detect hundreds of genes encoding resistance to many antimicrobials active in Gram-negative bacteria (Batchelor *et al.*, 2008; Frye *et al.*, 2006). In the near future, these assays will be key in furthering the understanding of the acquisition, transmission and dissemination of antimicrobial resistance in pathogenic and commensal bacteria. In our *E. coli* isolates, the greatest number of resistance genes detected in one isolate was 13, which was isolated from cattle in BHF. The array has highlighted some commonality between the resistance genes carried by *E. coli* from cattle and wildlife. For example, *teml_1, tetA_11* and

tetB_11 were most frequently associated with ampicillin and tetracycline resistance in both cattle and wildlife, and class 1 integrons were also more common in both. These results are consistent with those of (Dolejska *et al.*, 2008) who found that some of the resistance genes carried by *E. coli* isolates from calves, cows, young bulls and house sparrows were *tetA, tetB* (isolates resistant to tetracyclines), bla_{tem} (beta-lactams) positive, and antimicrobial-resistant calf isolates from farms possessed class 1 integrons associated with the acquisition of specific genes of resistance.

In this study the following genes were most commonly identified in *E. coli* isolates: *tetA_11*, *tetB_11* (isolates resistant to tetracycline); *teml_1* (beta-lactams); *aadA1_1* (streptomycin); *sul3_11*, *sul1_11* (sulphonamides); *catA1_11*, *floR_11* (chloramphenicol); and *dfrA14_21, dfrA17_11* and *dfrV_21* (trimethoprim); and class 1 integrons.

In most of the isolates tested, the resistance genes detected by the microarray were consistent with the phenotypes determined by the disc diffusion test with the corresponding antibiotic used. Similar phenotype and genotype patterns were found in wildlife and cattle indicating that there may be a relationship between the resistant isolates that were detected in wildlife and farm animals. Overall, the results generated in the present study suggest that farm animals and wildlife, which interact on the same farm, and neighbouring farms, may be able to share resistance genes through the exchange of genetic elements. The presence of antibiotic resistant *E. coli* isolates in wildlife provides some evidence for the transmission of resistant bacteria, or the genetic elements that encode resistance, from cattle, the environment or vice versa.

There were some exceptions whereby resistance genes were detected by the microarray but were not detected phenotypically. These results were expected, and it may be that these genes are not functioning or are not being expressed during the phenotypic test. On some occasions, the opposite was true, for example, it was found that resistance genes were not detected in many isolates that were phenotypically resistant to ampicillin, chloramphenicol, tetracycline and trimethoprim. This is possibly because enzymes conferring these antibiotics resistance are encoded by a different group of genes, which have not all been included in the array. This is a limitation of the use of this microarray; it does not provide information about other possible genes that could be carried by resistant bacteria.

The use of PFGE was found to be a way of assessing the similar strains isolated from different animals. Two isolates that shared similar PFGE banding patterns were from samples collected from a cow and a wood mouse on MF. Two more *E. coli* isolates sharing closely related PFGE banding patterns were from cattle and a wood mouse from BHF, and isolates derived from samples collected from cattle and a dog from CLF also shared the same banding pattern. But even strains of *E. coli* that did not share PFGE banding patterns that were isolated from domestic cattle and wildlife on farms shared resistance genes that could have been transmitted by plasmid or other genetic elements via direct contact or through the environment. Further work with high numbers of *E. coli* isolates are required to verify this observation and to understand the source and mechanism of transmission involved between wildlife and cattle.

6. 2 Conclusions

The work described in this thesis was undertaken in order to investigate whether or not wildlife play a role in the epidemiology of antibiotic resistance in domestic cattle. Although, as predicted, answering such a large question was beyond the scope of this project, the study has thrown up interesting results and conclusions.

1. Hypothesis: antibiotic resistance in *E.coli* is transmitted freely amongst wildlife and cattle, therefore similar patterns of resistance will be seen in *E. coli* from most, if not all, hosts.

This hypothesis, while not totally disproved, was not upheld. Fewer resistance patterns than might be expected at random were found, suggesting that multiresistance involves the co-transmission of resistance – probably on plasmids. However, *E. coli* with a wide variety of resistance patterns were found, each with different host ranges. There was no indication that a small number of resistant strains were being transmitted widely amongst different hosts. Furthermore, different farms and pairs of farms were associate with particular patterns. This suggests that the ecology of resistance is complex, and not driven simply by selection, for example, cattle. Some isolates with identical resistance patterns were, however, isolated from different hosts on the same farm, suggesting that transmission between hosts might occur.

2. Hypothesis: the resistance patterns shared between *E. coli* isolated from different hosts are caused by the same genes, as shared patterns represent transmission. Alternatively, if transmission between vertebrate hosts does

not occur, similar resistance phenotypes will be associated with different resistance genotypes.

This hypothesis, while not totally disproved, was not upheld. . The genotypes of *E.coli* isolates with similar resistance phenotypes were compared, using a microarray. A wide range of genotypes were found, suggesting that in the majority of cases, similar resistance phenotypes of *E. coli* isolated from different hosts probably did not reflect cross-species transmission. Some isolates with the same phenotypes and genotypes, but from different hosts, were identified, however, and these might suggest that cross species transmission, while unusual does occur.

3. Hypothesis: isolates with identical (or very similar) resistance phenotypes and genotypes result from transmission of *E. coli* between different vertebrate hosts.

This hypothesis, while not totally disproved, was not upheld. . These isolates, from different hosts, were compared by restriction enzyme digestion and pulse field gel electrophoresis. Again, most isolates had different PFGE patterns, but a small number had identical patterns, despite coming from different hosts on the same farm. This strongly suggests that cross species transmission of resistant *E. coli* can occur.

Overall, the finding of this study suggests that wildlife may be involved in the complex ecology of antimicrobial resistance, and may play a role in the transfer of resistant bacteria and / or genetic elements that encode antibiotic resistance. However, this ecology is complex – not simply driven by antibiotic use – and transmission between species appears to be relatively unusual. On the whole,

different hosts sharing the same environment, have their own strains of *E. coli* with their own resistance patterns. That wildlife can be reservoirs of resistance, however, potentially has a far reaching impact on the health of people and livestock (Cole *et al.*, 2005; Gopee *et al.*, 2000; Gilliver *et al.*, 1999).

6. 3 **Further work**

This study raises several questions regarding the evolution and ecology of antimicrobial resistance. The wildlife studied here did not have any obvious contact with antimicrobials that might select for resistance. However, the occurrence of antibiotic resistance in wildlife is likely due to acquired resistance, through horizontal transfer of resistance genes. It may be that wildlife are exposed to antimicrobials in the soil, feed, water, slurry, and / or manure of livestock, which may select for antibiotics resistance.

It would be interesting to undertake a longitudinal study of commensal enteric bacteria involving more samples from wildlife, cattle, environment and possibly human; there are a number of possible exposure routes that have not been investigated by this study.

Mechanisms of transfer of resistance were not investigated by this study. It would be interesting to investigate the presence of mobile genetic elements and the genetic mechanisms that may be underlying multi-drug resistance.

By using advanced DNA microarrays and genotyping techniques, we can determine the genes which are responsible for resistance in wild and domestic animal populations, determine genetic associations, and possibly link the origin of the genes to an external source, such as humans or other animal species.

References

References

AARESTRUP, F.M., SEYFARTH, A.M., EMBORG, H.D., PEDERSEN, K., HENDRIKSEN, R.S. and BAGER, F., 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal *enterococci* from food animals in Denmark. *Antimicrobial Agents and Chemotherapy,* **45**(7), pp. 2054-2059.

ACAR, J.F. and MOULIN, G., 2006. Antimicrobial resistance at farm level. *Scientific and Technical Review,* **25**(2), pp. 775-792.

AHMED, A.M., MOTOI, Y., SATO, M., MARUYAMA, A., WATANABE, H., FUKUMOTO, Y. and SHIMAMOTO, T., 2007. Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. *Applied and Environmental Microbiology,* **73**(20), pp. 6686-6690.

AKWAR, H.T., POPPE, C., WILSON, J., REID-SMITH, R.J., DYCK, M., WADDINGTON, J., SHANG, D. and MCEWEN, S.A., 2008. Prevalence and patterns of antimicrobial resistance of fecal *Escherichia coli* among pigs on 47 farrow-to-finish farms with different in-feed medication policies in Ontario and British Columbia. *Canadian Journal of Veterinary research,* **72**(2), pp. 195-201.

ALEKSHUN, M.N. and LEVY, S.B., 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* **128**(6), pp. 1037-1050.

ALEXANDER, T.W., YANKE, L.J., TOPP, E., OLSON, M.E., READ, R.R., MORCK, D.W. and MCALLISTER, T.A., 2008. Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. *Applied and Environmental Microbiology,* **74**(14), pp. 4405-4416.

ALTON, N.K. and VAPNEK, D., 1979. Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. *Nature,* **282**(5741), pp. 864-869.

AMINOV, R.I. and MACKIE, R.I., 2007. Evolution and ecology of antibiotic resistance genes. *FEMS Microbiology Letters,* **271**(2), pp. 147-161.

ANADON, A., 2006. The EU ban of antibiotics as feed additives: alternatives and consumer safety. *Journal of Veterinary Pharmacology and Therapeutics,* **29**: pp. 41-44.

ANDERSON, A.D., NELSON, J.M., ROSSITER, J. and ANGULO, F.J., 2003. Public health consequences of use of antimicrobial agents in food animals. *Microbial drug resistance*, 9(4), pp. 373-379.

ANDERSSON, D.I. and LEVIN, B.R., 1999. The biological cost of antibiotic resistance. *Current Opinions in Microbiology,* **2**(5), pp. 489-493.

ANDERSSON, D.I., 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? *Current Opinions in Microbiology,* **9**(5), pp. 461-465.

ARMAND-LEFEVRE, L., RUIMY, R. and ANDREMONT, A., 2005. Clonal comparison of Staphylococcus aureus isolates from healthy pig farmers, human controls, and pigs. *Emerging Infectious Diseases,* **11**(5), pp. 711-714.

AUSTIN, D.J., KAKEHASHI, M. and ANDERSON, R.M., 1997. The transmission dynamics of antibiotic-resistant bacteria: the relationship between resistance in commensal organisms and antibiotic consumption. *Proceedings.Biological Sciences,* **264**(1388), pp. 1629-1638.

BAKER-AUSTIN, C., WRIGHT, M.S., STEPANAUSKAS, R. and MCARTHUR, J.V., 2006. Co-selection of antibiotic and metal resistance. *Trends in Microbiology,* **14**(4), pp. 176-182.

BARTOLONI, A., BARTALESI, F., MANTELLA, A., DELL'AMICO, E., ROSELLI, M., STROHMEYER, M., BARAHONA, H.G., BARRON, V.P., PARADISI, F. and ROSSOLINI, G.M., 2004. High prevalence of acquired antimicrobial resistance unrelated to heavy antimicrobial consumption. *Journal of Infectious Diseases,* **189**(7), pp. 1291-1294.

BATCHELOR, M., HOPKINS, K.L., LIEBANA, E., SLICKERS, P., EHRICHT, R., MAFURA, M., AARESTRUP, F., MEVIUS, D., CLIFTON-HADLEY, F.A., WOODWARD, M.J., DAVIES, R.H., THRELFALL, E.J. and ANJUM, M.F., 2008. Development of a miniaturised microarray-based assay for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria. *International Journal of Antimicrobial Agents,* **31**(5), pp. 440-451.

BAUCHERON, S., CHASLUS-DANCLA, E. and CLOECKAERT, A., 2004. Role of TolC and parC mutation in high-level fluoroquinolone resistance in *Salmonella enterica* serotype Typhimurium DT204. *Journal of Antimicrobial Chemotherapy,* **53**(4), pp. 657-659.

BENNETT, P.M., 2004. Genome plasticity: insertion sequence elements, transposons and integrons, and DNA rearrangement. *Methods in Molecular Biology,* **266**, pp. 71-113.

BENNETT, P.M., 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology,* **153 Suppl 1**, pp. S347-57.

BERENS, C. and HILLEN, W., 2003. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *European Journal of Biochemistry,* **270**(15), pp. 3109-3121.

BIYELA, P.T., LIN, J. and BEZUIDENHOUT, C.C., 2004. The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Science and Technology,* **50**(1), pp. 45-50.

BLAHNA, M.T., ZALEWSKI, C.A., REUER, J., KAHLMETER, G., FOXMAN, B. and MARRS, C.F., 2006. The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. *Journal of Antimicrobial Chemotherapy,* **57**(4), pp. 666-672.

BLOT, M., HAUER, B. and MONNET, G., 1994. The Tn5 bleomycin resistance gene confers improved survival and growth advantage on *Escherichia coli. Molecular & General Genetics,* **242**(5), pp. 595-601.

BOERLIN, P. and REID-SMITH, R.J., 2008. Antimicrobial resistance: its emergence and transmission. *Animal Health Research,* **9**(2), pp. 115-126.

BOUCHER, H.W. and COREY, G.R., 2008. Epidemiology of methicillin-resistant *Staphylococcus aureus. Clinical Infectious Diseases,* **46 Suppl 5**, pp. S344-9.

BRUANT, G., MAYNARD, C., BEKAL, S., GAUCHER, I., MASSON, L., BROUSSEAU, R. and HAREL, J., 2006. Development and validation of an oligonucleotide microarray for detection of multiple virulence and antimicrobial resistance genes in *Escherichia coli. Applied and Environmental Microbiology,* **72**(5), pp. 3780-3784.

BRUSSOW, H., CANCHAYA, C. and HARDT, W.D., 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews,* **68**(3), pp. 560-602.

BRYAN, L. E., 1984. Antimicrobial Drug Resistance. Academic. Press, Inc., Orlando, FL.

BUTAYE, P., CLOECKAERT, A. and SCHWARZ, S., 2003. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. *International Journal of Antimicrobial Agents,* **22**(3), pp. 205-210.

BYWATER, R., DELUYKER, H., DEROOVER, E., DE JONG, A., MARION, H., MCCONVILLE, M., ROWAN, T., SHRYOCK, T., SHUSTER, D., THOMAS, V., VALLE, M. and WALTERS, J., 2004. A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. *The Journal of Antimicrobial Chemotherapy,* **54**(4), pp. 744-754.

CALL, D.R., BAKKO, M.K., KRUG, M.J. and ROBERTS, M.C., 2003. Identifying antimicrobial resistance genes with DNA microarrays. *Antimicrobial Agents and Chemotherapy,* **47**(10), pp. 3290-3295.

CANTON, R., COQUE, T.M. and BAQUERO, F., 2003. Multi-resistant Gram-negative bacilli: from epidemics to endemics. *Current Opinion in Infectious Diseases,* **16**(4), pp. 315-325.

CHAPIN, A., RULE, A., GIBSON, K., BUCKLEY, T. and SCHWAB, K., 2005. Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. *Environmental Health Perspectives,* **113**(2), pp. 137-142.

CHEE-SANFORD, J.C., AMINOV, R.I., KRAPAC, I.J., GARRIGUES-JEANJEAN, N. and MACKIE, R.I. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology,* **67**(4), pp. 1494-1502.

CHO, J.C. and TIEDJE, J.M., 2001. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Applied and Environmental Microbiology,* **67**(8), pp. 3677-3682.

CHOPRA, I. and ROBERTS, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews,* **65**(2), pp. 232-60.

CLARK, D.P. 2005. Molecular biology. Understanding the genetic revolutions. Academic Press, Chapter 20, pp, 533-566.

CLAVEL, T. and HALLER, D., 2007. Molecular interactions between bacteria, the epithelium, and the mucosal immune system in the intestinal tract: implications for chronic inflammation. *Current Issues in Intestinal Microbiology,* **8**(2), pp. 25-43.

CLOUGH, H.E., CLANCY, D., O'NEILL, P.D. and FRENCH, N.P., 2003. Bayesian methods for estimating pathogen prevalence within groups of animals from faecal-pat sampling. *Preventive Veterinary Medicine*, 58(3-4), pp. 145-169.

COLE, D., DRUM, D.J., STALKNECHT, D.E., WHITE, D.G., LEE, M.D., AYERS, S., SOBSEY, M. and MAURER, J.J., 2005. Free-living Canada geese and antimicrobial resistance. *Emerging Infectious Diseases,* **11**(6), pp. 935-938.

COSTA, D., POETA, P., SAENZ, Y., VINUE, L., COELHO, A.C., MATOS, M., ROJO-BEZARES, B., RODRIGUES, J. and TORRES, C., 2008. Mechanisms of antibiotic resistance in *Escherichia coli* isolates recovered from wild animals. *Microbial Drug Resistance,* **14**(1), pp. 71-77.

DANTAS, G., SOMMER, M.O., OLUWASEGUN, R.D. and CHURCH, G.M., 2008. Bacteria subsisting on antibiotics. *Science,* **320**(5872), pp. 100-103.

DAVIES, J.E., 1997. Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Foundation Symposium,* **207**, pp. 15-27; discussion 27-35.

DAVIS, J.G., TRUMAN, C.C., KIM, S.C., ASCOUGH, J.C. and CARLSON, K., 2006. Antibiotic transport via runoff and soil loss. *Journal of Environmental Guality,* **35**(6), pp. 2250-2260.

DEFRA. (2009). EU 2136 FOI SAPO. License Application forms.

www.defra.gov.uk/corporate/opengov/defra/available/inforelease/pdf/

DIONISIO, F., CONCEICAO, I.C., MARQUES, A.C., FERNANDES, L. and GORDO, I., 2005. The evolution of a conjugative plasmid and its ability to increase bacterial fitness. *Biology Letters,* **1**(2), pp. 250-252.

DOLEJSKA, M., CIZEK, A. and LITERAK, I., 2007. High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from Black-headed Gulls in the Czech Republic. *Journal of Applied Microbiology,* **103**(1), pp. 11-19.

DOLEJSKA, M., SENK, D., CIZEK, A., RYBARIKOVA, J., SYCHRA, O. and LITERAK, I., 2008. Antimicrobial resistant *Escherichia coli* isolates in cattle and house sparrows on two Czech dairy farms. *Research in Veterinary Science,* **85**(3), pp. 491-494.

DONNENBERG, M.S. AND WHITTAM, T.S., 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli. Journal of Clinical Investigation,* **107**(5), pp. 539-48.

DOUGHERTY, J.D. and GESCHWIND, D.H., 2002. Subtraction-coupled custom microarray analysis for gene discovery and gene expression studies in the CNS. *Chemical Senses,* **27**(3), pp. 293-298.

DRLICA, K. and MALIK, M., 2003. Fluoroquinolones: action and resistance. *Current Topics in Medicinal Chemistry,* **3**(3), pp. 249-282.

ENNE, V.I., BENNETT, P.M., LIVERMORE, D.M. and HALL, L.M., 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. *Journal of Antimicrobial Chemotherapy,* **53**(6), pp. 958-963.

ENNE, V.I., DELSOL, A.A., ROE, J.M. and BENNETT, P.M., 2006. Evidence of antibiotic resistance gene silencing in *Escherichia coli. Antimicrobial Agents and Chemotherapy,* **50**(9), pp. 3003-3010.

ESCHERICH, T., 1885. Die darm bakterien des neugeborenen und sauglings. *Fortshr. Medicine.* **3**(5), pp. 547-554.

FLEMING., 1929. The antibacterial action of culture of pencilliium with special references to their use in the isolation of B. Influenza. *British Journal of Experimental pathology.* **10** pp. 226-236.

FLUIT, A.C. and SCHMITZ, F.J., 2004. Resistance integrons and super-integrons. *Clinical Microbiology and Infection,* **10**(4), pp. 272-288.

FRANKLIN, T.J and SNOW, G.A., 2005. Biochemestry and molecular biology of antimicrobial drug action. Vulnerable shields-The cell wall of bacteria and fungi. Springer-Verlag New York, LLC. Sixth Eddition, pp. 17-41.

FRYE, J.G., JESSE, T., LONG, F., RONDEAU, G., PORWOLLIK, S., MCCLELLAND, M., JACKSON, C.R., ENGLEN, M. and FEDORKA-CRAY, P.J., 2006. DNA microarray detection of antimicrobial resistance genes in diverse bacteria. *International Journal of Antimicrobial Agents,* **27**(2), pp. 138-151.

FULLER, R., 1989. Probiotics in man and animals. *Journal of Applied Bacteriology,* **66**(5), pp. 365-378.

GARROD, L. P., AND F. O' GRADY., 1971. Antibiotic and chemotherapy, Third Eddition, Livingstone. Edinburgh.

GHUYSEN, J.M., 1991. Serine beta-lactamases and penicillin-binding proteins. *Annual Review of Microbiology,* **45**, pp. 37-67.

GIBBS, P.S., KASA, R., NEWBREY, J.L., PETERMANN, S.R., WOOLEY, R.E., VINSON, H.M. and REED, W., 2007. Identification, antimicrobial resistance profiles, and virulence of members from the family *Enterobacteriaceae* from the feces of yellow-headed blackbirds (*Xanthocephalus xanthocephalus*) in North Dakota. *Avian Diseases,* **51**(3), pp. 649-655.

GILLIVER, M.A., BENNETT, M., BEGON, M., HAZEL, S.M. and HART, C.A., 1999. Antibiotic resistance found in wild rodents. *Nature,* **401**, pp. 233-234.

GIRAUD, A., MATIC, I., RADMAN, M., FONS, M. and TADDEI, F., 2002. Mutator bacteria as a risk factor in treatment of infectious diseases. *Antimicrobial Agents and Chemotherapy,* **46**(3), pp. 863-865.

GOERING, R.V., 2004. Pulsed-field gel electrophoresis. In: Persing, D.H., TENOVER, F.C., VERSALOVIC, J., TANG, Y.W., UNGER, E.R., RELMAN, D.H., and WHITE, T.J., editors. Molecular Microbiology; Diagnostic Principles and Practice. Washington, D.C.: *American Society for Microbiology*: 185-196.

GOPEE, N.V., ADESIYUN, A.A. and CAESAR, K., 2000. A longitudinal study of *Escherichia coli* strains isolated from captive mammals, birds, and reptiles in Trinidad. *Journal of Zoo and Wildlife Medicine,* **31**(3), pp. 353-360.

GOW, S.P., WALDNER, C.L., HAREL, J. and BOERLIN, P., 2008. Associations between antimicrobial resistance genes in fecal generic *Escherichia coli* isolates from cow-calf herds in western Canada. *Applied and Environmental Microbiology,* **74**(12), pp. 3658-3666.

GREENWOOD, D., 2000. Detection of antibiotic resistance in vitro. *International Journal of Antimicrobial Agents,* **14**(4), pp. 303-306.

GRIMM, V., EZAKI, S., SUSA, M., KNABBE, C., SCHMID, R.D. and BACHMANN, T.T., 2004. Use of DNA microarrays for rapid genotyping of TEM beta-lactamases that confer resistance. *Journal of Clinical Microbiology,* **42**(8), pp. 3766-3774.

GROH, J.L., LUO, Q., BALLARD, J.D. and KRUMHOLZ, L.R., 2007. Genes that enhance the ecological fitness of *Shewanella oneidensis* MR-1 in sediments reveal the value of antibiotic resistance. *Applied and Environmental Microbiology,* **73**(2), pp. 492-498.

GUERRA, B., JUNKER, E., SCHROETER, A., MALORNY, B., LEHMANN, S. and HELMUTH, R., 2003. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *Journal of Antimicrobial Chemotherapy,* **52**(3), pp. 489-492.

HAMELIN, K., BRUANT, G., EL-SHAARAWI, A., HILL, S., EDGE, T.A., BEKAL, S., FAIRBROTHER, J.M., HAREL, J., MAYNARD, C., MASSON, L. and BROUSSEAU, R., 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in Escherichia coli isolates from Great Lakes recreational waters. *Applied and Environmental Microbiology,* **72**(6), pp. 4200-4206.

HAMMERUM, A.M. and HEUER, O.E., 2009. Human Health Hazards from Antimicrobial-Resistant *Escherichia coli* of Animal Origin. *Clinical Infectious Diseases,* **48**(7), pp. 916-921.

HERSHBERGER, E., OPREA, S.F., DONABEDIAN, S.M., PERRI, M., BOZIGAR, P., BARTLETT, P. and ZERVOS, M.J., 2005. Epidemiology of antimicrobial resistance in *enterococci* of animal origin. *Journal of Antimicrobial Chemotherapy,* **55**(1), pp. 127-130.

HOFMANN, W. K., 2006. Gene expression profiling by microarrays – clinical implications. Cambridge University. Press.

HOOPER, D.C., 2001. Emerging mechanisms of fluoroquinolone resistance. *Emerging Infectious Diseases,* 7(2), pp. 337-341.

HOYLE, D.V., YATES, C. M., CHASE-TOPING, M.E., TURNER, E.J., DAVIES, S.E., LOW, J.C., GUNN, G.J., WOOLHOUSE, M.E. AND AMYES, S.G., 2005. Molecular epidemiology of antimicrobial-resistant commensal *Escherichia coli* strain in a cohort of newborn calves. *Applied and Environmental Microbiology*, **71**(11), pp. 6680-6688.

INFANTE, B., GRAPE, M., LARSSON, M., KRISTIANSSON, C., PALLECCHI, L., ROSSOLINI, G.M. and KRONVALL, G., 2005. Acquired sulphonamide resistance genes in faecal *Escherichia coli* from healthy children in Bolivia and Peru. *International Journal of Antimicrobial Agents,* **25**(4), pp. 308-312.

JACOBY, G.A. and MUNOZ-PRICE, L.S., 2005. The new beta-lactamases. *New England Journal of Medicine,* **352**(4), pp. 380-391.

JEURISSEN, S.H., LEWIS, F., VAN DER KLIS, J.D., MROZ, Z., REBEL, J.M. and TER HUURNE, A.A., 2002. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Intestinal Microbiology*, **3**(1), pp. 1-14.

JOHNSON, J.R., SANNES, M.R., CROY, C., JOHNSTON, B., CLABOTS, C., KUSKOWSKI, M.A., BENDER, J., SMITH, K.E., WINOKUR, P.L. and BELONGIA, E.A., 2007. Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002-2004. *Emerging Infectious Diseases,* **13**(6), pp. 838-846.

JONES, C.S., OSBORNE, D.J. and STANLEY, J., 1992. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. *Molecular and Cellular Probes,* **6**(4), pp. 313-317.

KATSUNUMA, Y., HANAZUMI, M., FUJISAKI, H., MINATO, H., KATAOKA, Y., SAWADA, T., HASHIMOTO, Y. and YONEMOCHI, C., 2008. Comparison of pulsed-field gel electrophoresis patterns of antimicrobial-resistant *Escherichia coli* and *enterococci* isolates from the feces of livestock and livestock farmers in Japan. *Journal of General and Applied Microbiology,* **54**(1), pp. 39-50.

KAYSER, F.H., BIENZ, K.A., ECKERT, J. AND ZINKERNAGEL, R.M., 2005. Bacteriology, Medical Microbiology. Thieme. New York, pp. 146-345.

KHACHATRYAN, A.R., HANCOCK, D.D., BESSER, T.E. and CALL, D.R., 2006. Antimicrobial drug resistance genes do not convey a secondary fitness advantage to calf-adapted *Escherichia coli. Applied and Environmental Microbiology,* **72**(1), pp. 443-448.

KHACHATRYAN, A.R., HANCOCK, D.D., BESSER, T.E. and CALL, D.R., 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. *Applied and Environmental Microbiology,* **70**(2), pp. 752-757.

KNEZEVIC, P. and PETROVIC, O., 2008. Antibiotic resistance of commensal *Escherichia coli* of food-producing animals from three Vojvodinian farms, Serbia. *International Journal of Antimicrobial Agents,* **31**(4), pp. 360-363.

KOUTSOS, E.A and ARIAS, V.J., 2006. Intestinal ecology: infections among the gastrointestinal tract nutrition, and the microflora. *Journal of Applied Poultry Research.* **15**: pp. 161-173.

KOZAK, G.K., BOERLIN, P., JANECKO, N., REID-SMITH, R.J. and JARDINE, C., 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Applied and Environmental Microbiology,* **75**(3), pp. 559-566.

KULLAS, H., COLES, M., RHYAN, J. and CLARK, L., 2002. Prevalence of *Escherichia coli* serogroups and human virulence factors in faeces of urban Canada geese (*Branta canadensis*). *International Journal of Environmental Health Research,* **12**(2), pp. 153-162.

LANZ, R., KUHNERT, P. and BOERLIN, P., 2003. Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Veterinary Microbiology,* **91**(1), pp. 73-84.

LESCHER, G.Y., FROELICH, E.D., GRUET, M.D., BAILEY, J.H AND BRUNDAGE, R.P., 1962. 1, 8-Naphthyridine derivatives. A new class of chemotherapeutic agents. *Journal of Medicinal pharmaceutical Chemistry* **5**: pp. 1063-1068.

LEVIN, B.R., LIPSITCH, M., PERROT, V., SCHRAG, S., ANTIA, R., SIMONSEN, L., WALKER, N.M. and STEWART, F.M., 1997. The population genetics of antibiotic resistance. *Clinical Infectious Disease,* **24 Suppl 1**, pp. 9-16.

LEVY, S., 1987.Antibiotic use for growth promotion in an animals: Ecological and public health consequences. *Journal of Food Protection*, **50**; pp. 616-620

LEVY, S.B. and MARSHALL, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine,* **10**(12 Suppl), pp. S122-9.

LEVY, S.B., 1998. The challenge of antibiotic resistance. *Scientific American,* **278**(3), pp. 46-53.

LIPSITCH, M. and SAMORE, M.H., 2002. Antimicrobial use and antimicrobial resistance: a population perspective. *Emerging Infectious Diseases,* **8**(4), pp. 347-354.

LITERAK, I., VANKO, R., DOLEJSKA, M., CIZEK, A. and KARPISKOVA, R., 2007. Antibiotic resistant *Escherichia coli* and *Salmonella* in Russian rooks (*Corvus frugilegus*) wintering in the Czech Republic. *Letters in Applied Microbiology,* **45**(6), pp. 616-621.

LIU, Y.M., CAO, B., WANG, H., LI, B.B., YANG, C.X., WANG, Q.T., DU, X.L., LI, F., WANG, S.S. and QU, S.S., 2008. Molecular characterization of vancomycin-resistant *enterococci* isolates. *Chinese Journal of Medical genetics,* **88**(11), pp. 760-763.

LIVERMORE, D.M. and WOODFORD, N., 2006. The beta-lactamase threat in *Enterobacteriaceae, Pseudomonas* and *Acinetobacter. Trends in Microbiology,* **14**(9), pp. 413-420.

LIVERMORE, D.M., 1996. Are all beta-lactams created equal? *Scandinavian Journal of Infectious Diseases. Supplementum,* **101**, pp. 33-43.

LIVERMORE, D.M., WARNER, M., HALL, L.M., ENNE, V.I., PROJAN, S.J., DUNMAN, P.M., WOOSTER, S.L. and HARRISON, G., 2001. Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. *Environmental Microbiology,* **3**(10), pp. 658-661.

MALATHUM, K. and MURRAY, B.E., 1999. Vancomycin-resistant *enterococci*: recent advances in genetics, epidemiology and therapeutic options. *Drug Resistance Update,* **2**(4), pp. 224-243.

MASON, I.S AND KITZMANN, M., 1999. Cephalosporine-pharmacological basis of clinical use in veterinary dermatology. Volume 10. Issue **3**, pp. 187-192.

MEDEIROS, A.A., 1997. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. *Clinical Infectious Diseases,* **24 Suppl 1**, pp. 19-45.

MITCHELL, M. A., 2003. Convention notes. In American Veterinary Medical Association. Nashville, Tennessee.

MITSUHASHI, S., 1993. Drug resistance in bacteria: history, genetics and biochemistry. *Journal of International Medical Research,* **21**(1), pp. 1-14.

MORRISON, D., WOODFORD, N., BARRETT, S.P., SISSON, P. and COOKSON, B.D., 1999. DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. *Journal of Clinical Microbiology,* **37**(4), pp. 1084-1091.

MUBITA, C., SYAKALIMA, M., CHISENGA, C., MUNYEME, M., BWALYA, M., CHIFUMPA, G., HANG'OMBE, B. M., SINKALA, P., SIMUNZA, M., FUKUSHI, H., ISOGAI, H., YASUDA, J., ISOGAI. E., 2008. Antibiograms of faecal *Escherichia coli* and *Enterococci* species isolated from pastoralist cattle in the interface areas of the Kafue basin in Zambia - short communication. *Veterinary Archive, 78* (2), pp.179-185.

MURRAY, B.E., 1991. New aspects of antimicrobial resistance and the resulting therapeutic dilemmas. *Journal of Infectious Diseases,* **163**(6), pp. 1184-1194.

NIELD, B.S., HOLMES, A.J., GILLINGS, M.R., RECCHIA, G.D., MABBUTT, B.C., NEVALAINEN, K.M. and STOKES, H.W., 2001. Recovery of new integron classes from environmental DNA. *FEMS Microbiology Letters,* **195**(1), pp. 59-65.

NILSSON, A.I., ZORZET, A., KANTH, A., DAHLSTROM, S., BERG, O.G. and ANDERSSON, D.I., 2006. Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proceedings of the National Academy of Sciences,* **103**(18), pp. 6976-6981.

NORMARK, B.H. and NORMARK, S., 2002. Evolution and spread of antibiotic resistance. *Journal of Internal Medicine,* **252**(2), pp. 91-106.

OSTERBLAD, M., NORRDAHL, K., KORPIMAKI, E. and HUOVINEN, P., 2001. Antibiotic resistance. How wild are wild mammals? *Nature,* **409**, pp. 37-38.

PALANIAPPAN, R.U., ZHANG, Y., CHIU, D., TORRES, A., DEBROY, C., WHITTAM, T.S. and CHANG, Y.F., 2006. Differentiation of *Escherichia coli* pathotypes by oligonucleotide spotted array. *Journal of Clinical Microbiology,* **44**(4), pp. 1495-1501.

PATERSON, D.L. and BONOMO, R.A., 2005. Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews,* **18**(4), pp. 657-686.

PETERSEN, A., CHRISTENSEN, J.P., KUHNERT, P., BISGAARD, M. and OLSEN, J.E., 2006. Vertical transmission of a fluoroquinolone-resistant *Escherichia coli* within an integrated broiler operation. *Veterinary Microbiology,* **116**(1-3), pp. 120-128.

PETERSON, L.R., 2008. Currently available antimicrobial agents and their potential for use as monotherapy. *Clinical Microbiology and Infection,* **14 Suppl 6**, pp. 30-45.

PRICE, L.B., GRAHAM, J.P., LACKEY, L.G., ROESS, A., VAILES, R. and SILBERGELD, E., 2007. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. *Environmental Health Perspectives,* **115**(12), pp. 1738-1742.

PUPO, G.M., LAN, R., REEVES, P.R. and BAVERSTOCK, P.R., 2000. Population genetics of *Escherichia coli* in a natural population of native Australian rats. *Environmental Microbiology,* **2**(6), pp. 594-610.

RICE, D.H., MCMENAMIN, K.M., PRITCHETT, L.C., HANCOCK, D.D. and BESSER, T.E., 1999. Genetic subtyping of *Escherichia coli* O157 isolates from 41 Pacific Northwest USA cattle farms. *Epidemiology and Infection,* **122**(3), pp. 479-484.

RICHARD, K., 2005. The epidemiology of VTEC 0157, non – 0157 VTEC and *campylobacter spp.* In 100Km^2 dairy farming area in Northwest area. Liverpool: Thesis Ph.D.

ROBINSON, S. E., 2004.Temporal characteristics of shedding of *E. coli* 0157 in UK dairy cattle. Liverpool: Thesis Ph.D.

ROE, M.T. and PILLAI, S.D., 2003. Monitoring and identifying antibiotic resistance mechanisms in bacteria. *Poultry Science,* **82**(4), pp. 622-626.

RUBENS, C.E., MCNEILL, W.F. and FARRAR, W.E., 1979. Evolution of multiple-antibiotic-resistance plasmids mediated by transposable plasmid deoxyribonucleic acid sequences. *Journal of Bacteriology,* **140**(2), pp. 713-719.

RUIZ, J., 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy,* **51**(5), pp. 1109-1117.

SALMINEN, S., ISOLAURI, E. and ONNELA, T., 1995. Gut flora in normal and disordered states. *Chemotherapy,* **41 Suppl 1**, pp. 5-15.

SALYERS, A.A., GUPTA, A. and WANG, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology,* **12**(9), pp. 412-416.

SANDER, P., SPRINGER, B., PRAMMANANAN, T., STURMFELS, A., KAPPLER, M., PLETSCHETTE, M. and BOTTGER, E.C., 2002. Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrobial Agents and Chemotherapy,* **46**(5), pp. 1204-1211.

SARMAH, A.K., MEYER, M.T. and BOXALL, A.B., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere,* **65**(5), pp. 725-759.

SAVAGE, D.C., 1986. Gastrointestinal microflora in mammalian nutrition. *Annual Review of Nutrition,* **6**, pp. 155-178.

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

SCHATZ, A., E. BUGIE AND S. A, WAKSMAN., 1944. Streptomycine, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc. Soc. *Experimental Biology and Medicine*. **55**: pp. 66-69

SCHWARZ, S. and CHASLUS-DANCLA, E., 2001. Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Veterinary Research,* **32**(3-4), pp. 201-225.

SCHWARZ, S., KEHRENBERG, C., DOUBLET, B. and CLOECKAERT, A., 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews,* **28**(5), pp. 519-542.

SENNA, J.P., PINTO, C.A., CARVALHO, L.P. AND SANTOS, D.S., 2002. Comparison of pulsed-field gel electrophoresis and PCR analysis of polymorphism on the mec hypervariable region for typing Methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 40(6), pp. 2254-2256.

SHERLEY, M., GORDON, D.M. and COLLIGNON, P.J., 2000. Variations in antibiotic resistance profile in *Enterobacteriaceae* isolated from wild Australian mammals. *Environmental Microbiology,* **2**(6), pp. 620-631.

SHIMA, K., YOSHII, N., AKIBA, M., NISHIMURA, K., NAKAZAWA, M. and YAMASAKI, S., 2006. Comparison of PCR-RFLP and PFGE for determining the clonality of enterohemorrhagic *Escherichia coli* strains. *FEMS Microbiology Letters,* **257**(1), pp. 124-131.

SILBERGELD, E.K., GRAHAM, J. and PRICE, L.B., 2008. Industrial food animal production, antimicrobial resistance, and human health. *Annual Review of Public Health,* **29**, pp. 151-169.

SINGER, R.S., PATTERSON, S.K., MEIER, A.E., GIBSON, J.K., LEE, H.L. and MADDOX, C.W., 2004. Relationship between phenotypic and genotypic florfenicol resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy,* **48**(10), pp. 4047-4049.

SINGH, R., SCHROEDER, C.M., MENG, J., WHITE, D.G., MCDERMOTT, P.F., WAGNER, D.D., YANG, H., SIMJEE, S., DEBROY, C., WALKER, R.D. and ZHAO, S., 2005. Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *Escherichia coli* recovered from humans and food animals. *Journal of Antimicrobial Chemotherapy,* **56**(1), pp. 216-219.

SJOLUND, M., BONNEDAHL, J., HERNANDEZ, J., BENGTSSON, S., CEDERBRANT, G., PINHASSI, J., KAHLMETER, G. and OLSEN, B., 2008. Dissemination of multidrug-resistant bacteria into the Arctic. *Emerging Infectious Diseases,* **14**(1), pp. 70-72.

SKOLD, O., 2001. Resistance to trimethoprim and sulfonamides. *Veterinary Research,* **32**(3-4), pp. 261-273.

SKURNIK, D., RUIMY, R., ANDREMONT, A., AMORIN, C., ROUQUET, P., PICARD, B. and DENAMUR, E., 2006. Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *Journal of Antimicrobial Chemotherapy,* **57**(6), pp. 1215-1219.

SORUM, H. and SUNDE, M., 2001. Resistance to antibiotics in the normal flora of animals. *Veterinary Research,* **32**(3-4), pp. 227-241.

SOULSBY, 2008. Antimicrobial resistance-animals and the environment. *Journal of Antimicrobial Chemotherapy,* **62**(2), pp. 229-233.

STOKES, H.W. and HALL, R.M., 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Molecular Microbiology,* **3**(12), pp. 1669-1683.

SWAMINATHAN B., BARRETT, T.J., HUNTER, S.B., TAUXE, R.V., 2001. PulseNet Task force. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious* Disease. **7**, pp. 382-389

TELFER, S., BENNETT, M., BOWN, K., CAVANAGH, R., CRESPIN, L., HAZEL, S., TREVOR, J., 2002. The Effects of Cowpox Virus on Survival in Natural Rodent Populations: Increases and Decreases. *Journal of Animal Ecology,* **71**, pp. 558-568.

TODAR,'S., 2008. Introduction in the past 60 years. Bacterial resistance to antibiotics (46). Online Textbook of Bacteriology, University of Wisconsin. http://www.textbookofbacteriology.net

TURNIDGE, J., 2004. Antibiotic use in animals--prejudices, perceptions and realities. *The Journal of antimicrobial chemotherapy,* **53**(1), pp. 26-27.

VAN BELKUM, A. and VERBRUGH, H., 2001. 40 years of methicillin resistant *Staphylococcus aureus. British Medical Journal,* **323**(7314), pp. 644-645.

WASTESON, Y., JOHANNESSEN, G.S., BRUHEIM, T., URDAHL, A.M., O'SULLIVAN, K. and RORVIK, L.M., 2005. Fluctuations in the occurrence of *Escherichia coli* O157:H7 on a Norwegian farm. *Letters in Applied Microbiology,* **40**(5), pp. 373-377.

WATERS, B. and DAVIES, J., 1997. Amino acid variation in the GyrA subunit of bacteria potentially associated with natural resistance to fluoroquinolone antibiotics. *Antimicrobial Agents and Chemotherapy,* **41**(12), pp. 2766-2769.

WIENER, P., EGAN, S., HUDDLESTON, A.S. and WELLINGTON, E.M., 1998. Evidence for transfer of antibiotic-resistance genes in soil populations of *streptomycetes. Molecular Ecology,* 7(9), pp. 1205-1216.

WILLIAMS, J.B., 1996. Drug efflux as a mechanism of resistance. *British Journal of Biomedical Science,* **53**(4), pp. 290-293.

WRIGHT, M.S., LOEFFLER PELTIER, G., STEPANAUSKAS, R. and MCARTHUR, J.V., 2006. Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. *Microbiology Ecology,* **58**(2), pp. 293-302.

YAN, J.J., KO, W.C., CHIU, C.H., TSAI, S.H., WU, H.M. and WU, J.J., 2003. Emergence of ceftriaxone-resistant *Salmonella* isolates and rapid spread of plasmid-encoded CMY-2-like cephalosporinase, Taiwan. *Emerging Infectious Diseases,* **9**(3), pp. 323-328.

YATES, C.M., SHAW, D.J., ROE, A.J., WOOLHOUSE, M.E. and AMYES, S.G., 2006. Enhancement of bacterial competitive fitness by apramycin resistance plasmids from non-pathogenic *Escherichia coli. Biology Letters,* **2**(3), pp. 463-465.

Appendix

Appendices

Appendix one

Abbreviations

Media abbreviation

BGB	Brilliant green bile broth
NA	Nutrient agar
EMBA	Eosin Methylene Blue agar
Iso	Iso-sensitest agar
Mac	MacConkey agar
SCA	Simmon citrate agar
TSA	Trypton soy agar

Antibiotic abbreviation

ABs	Antibiotics
AMB	Ampicillin
AUG	Augmentin
CHL	Chloramphenicol
FLO	Florfenicol
TET	Tetracycline
TRI	Trimethoprim
NAL	Nalidixic acid
CIP	Ciprofloxacin

Resistance abbreviation:

AR	Antibiotic-resistance
BSAC	British Society for Antimicrobials Chemotherapy
MDR	Multidrug-resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

Molecular abbreviation:

PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
DNA	Deoxyribo-nucleic acid
RNA	Ribo-nucleic acid

Bacteriological materials

Media

All media described are prepared in accordance with the manufactures' instructions and unless stated otherwise obtained from LabM (IDG)

Brain heart infusion broth (LAB51, Bury, UK)

Eosin Methylene Blue agar (LAB61)

Nutrient agar (LAB8)

Tryptone Soy agar (LAB11)

MacConkey agar (LAB30)

Simmon's Citrate agar (LAB69)

Iso-sensitest agar (LAB170)

Chemical reagents

Hydrogen peroxide (Sigma)

Kovac's reagent (bioMerieux, Basingstoke, UK)

Antibiotic related materials

Antibiotic discs (Mast Diagnostics)

Preparation of antibiotic stock solution for breakpoint determination (NCCLS breakpoint)

Ampicillin – 8µg/ml in plates

To prepare a stock solution (8mg/ml)

Add 0.16g to 20 ml of sterile PBS (shake well until dissolved).

Chloramphenicol – 8µg/ml in plates

To prepare a stock solution (8mg/ml)

Add 0.16g to 20 ml of 95% ethanol (shake well until dissolved).

Naladixic acid 16µg /ml in plates

To prepare a stock solution (16mg/ml)

Add 0.32g to 10 ml of sterile distilled water . Once dissolved add NaOH (1mol/L) dropwise to solution. Once dissolved make up to 20 ml with water.

Tetracycline 4µg /ml in plates

To prepare a stock solution (4mg/ml)

Add 0.08g of tetracycline to 20ml of sterile distilled water (mix well until dissolved).

Trimethoprim 4µg /ml in plates

To prepare a stock solution (4mg/ml)

Add 0.08g of trimethoprim to 2ml of 0.05 mol/L HCI, and 18ml of sterile distilled water and mix well to dissolve (trimethoprim will not always dissolve, gentle heating may help), otherwise it is acceptable to use the trimethoprim solution providing that it is well mixed prior to adding it to the agar.

Preparation of agar

To 500ml of cooled EMBA add 0.5ml of antibiotic solution and pour plates and ensure that each petri dish is labelled appropriately with the antibiotic that has been added.

When using the antibiotic agar plates, add the following volumes of antibiotic for the different batch sizes;

1L of agar – 1 ml of antibiotic solution

2L of agar – 2 ml of antibiotic solution

Molecular Materials

Reagents used in pulsed-field gel electrophoresis

Cell lysis buffer (CLB)

50mM Tris, 50mM EDTA, pH8.0 = 1% Sarcosyl

Tris-base 6.055g

EDTA 14.6g

N-lauroyl sarcosine 10g

Mix all reagents in 900ml water and dissolve on a heated stirring plate. Adust to pH8,

Lysozyme (Sigma)

20mg/ml

Make up to the correct concentration with molecular grade water and stored -20^{0}C in 1ml allequots.

SDS

2g of SDS was added to 10ml 1XTE buffer (sterile) in a sterile flask and mixed.

Proteinase K (Sigma)

25mg/ml

Make up to the correct concentration with molecular grade water and stored at -20^{0}C in 1ml allequts.

E. coli pulsed field – agarose mixture

	Agarose	20% SDS	1XTE
Small gel (13 isolates)	0.04g	200µl	3.8ml
Large gel (27 isolates)	0.09g	300µl	5.7ml

Lab Form Data

Sample NO: ---------- Animal Species: ----------

Date Collected: ---------- Date processed: ----------

Origin of sample: ----------

Typical *E. coli* present on EMBA + Ampicillin: Yes / No

Isolate	Indole	Lact	Citrate	Amp	Aug	Chl	Nal	Cipro	Tet	Trim	CCNO
A											
B											
C											

Typical *E. coli* present on EMBA + Chloramphenicol: Yes / No

Isolate	Indole	Lact	Citrate	Amp	Aug	Chl	Nal	Cipro	Tet	Trim	CCNO
A											
B											
C											

Typical *E. coli* present on EMBA + Naladixic acid: Yes / No

Isolate	Indole	Lact	Citrate	Amp	Aug	Chl	Nal	Cipro	Tet	Trim	CCNO
A											
B											
C											

Typical *E. coli* present on EMBA + Tetracycline: Yes / No

Isolate	Indole	Lact	Citrate	Amp	Aug	Chl	Nal	Cipro	Tet	Trim	CCNO
A											
B											
C											

Typical *E. coli* present on EMBA + Trimethoprim: Yes / No

Isolate	Indole	Lact	Citrate	Amp	Aug	Chl	Nal	Cipro	Tet	Trim	CCNO
A											
B											
C											

Table 1. The number of samples collected and tested from each species and farm

Species	BHF	BGF	CLF	GF	MF	PHF
heifer-bovine[1]	14					
lactating-bovine	36	39	18		2	29
young-bovine[2]	3				3	3
bull-bovine		1				
finishers-bovine					24	
Cow[3]	29	30	35	63	50	12
Calf[4]	15	8	9	11	6	9
vole-bank	13	37	44	43	21	30
vole-field	8	5	8	3	1	
mouse-wood	79	129	126	71	80	126
mouse-house	9	10	19		2	25
rodent-unknown	1	5	3	15	3	2
rat	1	1	5		6	3
fox	2	2	1	3		4
badger	12			1	1	11
rabbit	6		12		9	5
dog domestic	2	1	2			
buzzard	1		2		1	
pheasant			4		6	
magpie					1	
raven			6			
redwing		3	1	1	4	
robin	3	10	11	2	6	1
house sparrow	29	14				2
jackdaw	4					
jay				1		
crow	2		2		3	
large bird (corvid)	10	3			8	
long tailed tit	17		6		5	
greenfinch		3	3		4	2
meadow pipit		4		1		
black bird	5	3	4	1	6	1
blue tit	37	12	40	13	18	9
nuthatch	3			1		
bullfinch			2			
pied wagtail				1		
shrew	6	1		1		1
song thrush		2	2		5	
spreo starling						5
swallow pullus					4	
tree sparrow						13

Species	BHF	BGF	CLF	GF	MF	PHF
treecreeper	2					
unknown bird	3				8	
willow warbler			1			
wood pigeon	1					
wren	11	3	5	4	3	3
chaffinch	11	4	7		20	5
chiffchaff						1
coal tit	6			1		
dunnock	2	8	13	3	5	5
feral pigeon		6	2		2	
fieldfare		1				
gold crest	2			1	2	
goldfinch	1					
great spotted	1					
great tit	41	9	2	9	22	9

[1] Heifer-bovine; a young female cow before she has had her first calf

[2]Bull-bovine; an adult male of cattle

[3]Cow; is a fully mature adult female

[4]Calf; young cattle of both sexes until they are weaned

Appendix1. Table 2. Antibiotic resistance profiles encountered in *E.coli*, and their distribution by host and farm.

FARMS	Host	trim	tet	tet, trim	nal, tet	nal, cip	chl	chl, trim	chl, tet	amp	amp, trim	amp, tet	amp, tet, trim	amp, cip, trim	amp, nal, tet	amp, nal, tet, trim	amp, nal, cip, trim	amp, chl, trim	amp, chl, tet	amp, chl, tet, trim	amp, chl, nal, tet, trim	amp, aug	amp, aug, tet	amp, aug, tet, trim	amp, aug, nal, trim	amp, aug, nal, tet	amp, aug, nal, tet, trim	amp, aug, nal, cip tet	amp, aug, chl	amp, aug, chl, tet	amp, aug, chl, tet, trim	amp, aug, chl, nal, tet	amp, aug, chl, nal, cip, tet, trim
M F	Cattle	1	29	5					6	2	6	21	31						10	8			1	8						1			
	Rodents		2	5	2				1			3	7			1			3	2	2		1										
	Birds	1	3				1		1			2	1								1		1										1
	O.W.M		4	1	2							2	4						1	2													
PHF	Cattle		13	1					2	2		18	4						13	1			6	14				5	1	5	7		
	Rodents	1	12				1		2			8							3			3	7	1						10	9		
	W. Birds		3						2			2	1						3											2			
	O.W.M		2						2			2	1							1		1	3	14				1		3	6		1

FARMS	Host	trim	tet	tet, trim	nal, tet	nal, cip	chl	chl, trim	chl, tet	amp	amp, trim	amp, tet	amp, tet, trim	amp, cip, trim	amp, nal, tet	amp, nal, tet, trim	amp, nal, cip, trim	amp, chl, trim	amp, chl, tet	amp, chl, tet, trim	amp, chl, nal, tet, trim	amp, aug	amp, aug, tet	amp, aug, tet, trim	amp, aug, nal, trim	amp, aug, nal, tet	amp, aug, nal, tet, trim	amp, aug, nal, cip tet	amp, aug, chl	amp, aug, chl, tet	amp, aug, chl, tet, trim	amp, aug, chl, nal, tet	amp, aug, chl, nal, cip, tet, trim
GF	Cattle	9	13							5	1	11	4					1	25	3				2						6	1		
	W. Rodents	1	1							3																							
	W. Birds		2	1						4		1	2																				
	O.W.M		1							1	1	5	1						2														
BGF	Cattle	1	20	1			1		19			16	1										15	4						1			1
	W. Rodents			3	1		1			2		2	1										1	5						1			
	W. Birds		3	2						2													2	3						3			
	O.W.M	1					1			2			1						1	1				1									

FARMS	Host	trim	tet	tet, trim	nal, tet	nal, cip	chl	chl, trim	chl, tet	amp	amp, trim	amp, tet	amp, tet, trim	amp, cip, trim	amp, nal, tet	amp, nal, tet, trim	amp, nal, cip, trim	amp, chl, trim	amp, chl, tet	amp, chl, tet, trim	amp, chl, nal, tet, trim	amp, aug	amp, aug, tet	amp, aug, tet, trim	amp, aug, nal, trim	amp, aug, nal, tet	amp, aug, nal, tet, trim	amp, aug, nal, cip tet	amp, aug, chl	amp, aug, chl, tet	amp, aug, chl, tet, trim	amp, aug, chl, nal, tet	amp, aug, chl, nal, cip, tet, trim
CLF	Cattle	1	1	1			1		9			7	22		1					11				5					2				
	W. Rodents		15	11	4				14			2	7					3	2	11	3			10		3	4			1	5	1	
	W. Birds			1					3				2						2	2											2		
	O.W.M		2	2					4			3	3		3	3		3	3	5	4			2			1			1	7	1	
BHF	Cattle	2	34	4	4		1	5	5	3	6	13	27	1	8	9	1	2	5	10	5	1	14	9		1	2	1	2	4	4		1
	W. Rodents	1	6									5	2																				1
	W. Birds								3			2	1																				
	O.W.M	2	8	4		2	1	1	3	1	4	3	3		2	5			5	7			1	3	1		3					3	

Appendix 2.

Table 1. Oligonucleotide primers used for PCR validation of array results.

Target	Primer sequence	
qnr	F- GATAAAGTTTTTCAGCAAGAGG	
	R- ATCCAGATCGGCAAAGGTTA	
sulI	F- TCACCGAGGACTCCTTCTTC	
	R- AATATCGGGATAGAGCGCAG	
sulII	F- GATATTCGCGGTTTTCCAGA	
	R- CGAATTCTTGCGGTTTCTTT	
sul3	F- ACCGATAGTTTTTCCGATGG	
	R- TGCGGAGATAATCTGCACCT	
tet(A)	F- GCTACATCCTGCTTGCCTTC	
	R- CATAGATCGCCGTGAAGAGG	
tet(B)	F- TTGGTTAGGGGCAAGTTTTG	
	R- GTAATGGGCCAATAACACCG	
tet(C)	F- CTTGAGAGCCTTCAACCCAG	
	R- ATGGTCGTCATCTACCTGCC	
tet(D)	F- AAACCATTACGGCATTCTGC	
	R- GACCGGATACACCATCCATC	
tet(E)	F- AAACCACATCCTCCATACGC	
	R- AAATAGGCCACAACCGTCAG	
tet(G)	F- CCGGTCTTATGGGTGCTCTA	
	R- CCAGAAGAACGAAGCCAGTC	
IntI1	F- GGCATCCAAGCAGCAAG	
	R- AAGCAGACTTGACCTGA	
IntI2	F- TTATTGCTGGGATTAGGC	
	R- ACGGCTACCCTCTGTTATC	
aadA1-like	F- TATCAGAGGTAGTTGGCGTCAT	

	R- GTTCCATAGCGTTAAGGTTTCATT	
aadA2-like	F- TGTTGGTTACTGTGGCCGTA	
	R- GATCTCGCCTTTCACAAAGC	
aadA4-like	F- CAATCCACCTGTTCGGATCT	
	R- AGCAACGTCCTTAGGAGCAA	
*bla*PSE-1-like	F- GCTTCGCAACTATGACTAC	
	R- GTTCACCATCCAAGACTC	
cmlA1-like	F- TGTCATTTACGGCATACTCG	
	R- ATCAGGCATCCCATTCCCAT	
catA1	F- CGCCTGATGAATGCTCATCCG	
	R- CCTGCCACTCATCGCAGTAC	
catIII	F- CCTGGAACCGCAGAGAAC	
	R- CCTGCTGAAACTTTGCCA	
catB3-like	F- GGTACGACTGGGCATCATCT	
	R- TCGAGCCAATACTTGTGCAG	
floR	F- GGAGCAGCTTGGTCTTCAAC	
	R- AATGAATATCGCCTGCCATC	
dfrA1	F- GTGAAACTATCACTAATGG	
	R- TTAACCCTTTTGCCAGATTT	
dfrA7	F- CAGAAAATGGCGTAATCG	
	R- TCAACGTGAACAGTAGACAAA	
dfr12	F- GGTG(G/C)GCAGAAGATTTTTCGC	
	R- TGGGAAGGCGTCACCCTC	
dfrA14	F- ATAGCTGCGAAAGCGAAAAA	
	R- CCCTTTTTCCAAATTTGATAGC	
dfrA17	F- CAGAAAATGGCGTAATCG	
	R- TCAACGTGAACAGTAGACAAA	
dfrA19	F- GCGATTTACGCGGATTTCTA	

	R- CAAAGTGAATGCGCTCTTGA	
aac(3)-*Ia*	F- TTGATCTTTTCGGTCGTGAGT	
	R- TAAGCCGCGAGAGCGCCAACA	
aac(3)-*IVa*	F- TCGGTCAGCTTCTCAACCTT	
	R- ACCGACTGGACCTTCCTTCT	
aac(6')-*Ib*	F- GTTACTGGCGAATGCATCACA	
	R- TGTTTGAACCATGTACACGGC	
ant(2")-*Ia*	F- GGGCGCGTCATGGAGGAGTT	
	R- TATCGCGACCTGAAAGCGGC	
*bla*DHA-1	F- AACTTTCACAGGTGTGCTGGGT	
	R- CCGTACGCATACTGGCTTTGC	
*bla*ACC	F- AACAGCCTCAGCAGCCGGTTA	
	R- TTCGCCGCAATCATCCCTAGC	
*bla*MOX	F- GCTGCTCAAGGAGCACAGGAT	
	R- CACATTGACATAGGTGTGGTGC	
*bla*CMY	F- TGGCCAGAACTGACAGGCAAA	
	R- TTTCTCCTGAACGTGGCTGGC	
*bla*FOX	F- AACATGGGGTATCAGGGAGAT	
	R- CAAAGCGCGTAACCGGATTGG	
*bla*SHV	F- CGGCCCCGCAGGATTGACT	
	R- TCCCGGCGATTTGCTGATTTC	
*bla*TEM-1	F- TCGTGTCGCCCTTATTCCCTTTTT	
	R- GCGGTTAGCTCCTCCGGTCCTC	
*bla*OXA-1	F- TTGATGCGGAAATAATAGAT	
	R- TGCGGACACAAAAACATA	
*bla*OXA-2	F- TTCAAGCCAAAGGCACGATAG	
	R- TCCGAGTTGACTGCCGGGTTG	
*bla*OXA-7	F- CGTGCTTTGTAAAAGTAGCAG	

	R- CATGATTTTGGTGGGAATGG	
*bla*CTX-M1	F- CGATGTGCAGTACCAGTAA	
	R- TTAGTGACCAGAATCAGCGG	
*bla*CTX-M2	F- CGATGTGCAGTACCAGTAA	
	R- TTAGTGACCAGAATCAGCGG	
*bla*CTX-M9	F- CGATGTGCAGTACCAGTAA	
	R- TTAGTGACCAGAATCAGCGG	

Second probe was designed for several genes due to improve discrimination between closely related genes.

Nummer SPOT_ID	Well SPOT_NAME	geneName NAME	probeSeq parameter2
35	C3	prob_*qnr_11*	CAGTGTGACTTCAGCCACTGTCAGC
36	D3	prob_*sul2_11*	TCGATTTGCCGGTGCTTCTGTCTGT
37	E3	prob_*sul3_11*	GCTCTGCATTTGGTTGAAGATGGAGCA
38	F3	prob_*tetA_11*	CTCATGCTCGGAATGATTGCCGACG
39	G3	prob_*tetB_11*	CGTTTGCTTTCAGGGATCACAGGAGC
40	H3	prob_*tetC_11*	CTCGCTCAAGCCTTCGTCACTGGT
41	I3	prob_*tetD_1*	GCACTGTCCAATGTGCTGTGGATGT
42	J3	prob_*tetE_11*	GGTACAGGCACCGTTTATGTTCGCTG
43	K3	prob_*tetG_11*	GCTTCACAGCACTCTATTCTGCCACC
44	L3	prob_*tetG_12*	GCTTCACGGCACTCTATTCTGCCAC
45	M3	prob_*intI1_1*	CCATTCCGACGTCTCTACGACGATGA
46	N3	prob_*intI2_11*	GCAAGCCTAGACGGCTACCCTCTG
51	C4	prob_*aadA1_1*	AGATTCTCCGCGCTGTAGAAGTCACC
52	D4	prob_*aadA2_1*	ACGCTCCGCGCTATAGAAGTCACC
53	E4	prob_*aadA4_1*	CTGGATCACGATCTTGCGATTTTGCTGA
54	F4	prob_*ctxM1_11*	CGTCACGCTGTTGTTAGGAAGTGTGC
55	G4	prob_*ctxM1_12*	GCCACGCTGTCGTTAGGAAGTGTG
56	H4	prob_*cmlA1_11*	GACCATGTTGCTGGAACGGTCACG
57	I4	prob_*catA1_11*	CGTCTCAGCCAATCCCTGGGTGAG
58	J4	prob_*catIII_1*	TGGGTTCGCCGTGAGCATTTTGAG
59	K4	prob_*catB3_11*	GCAATGACGTTTGGATCGGCTCTGAG
60	L4	prob_*floR_11*	GCTTTCGTCATTGCGTCTCTGGGAG
61	M4	prob_*dfrA1_21*	CAATAGACATCGAGCCGGAAGGTGATG
62	N4	prob_*dfrA1_22*	CAATCGACATTGAGCCAGAAGGTGATGT
67	C5	prob_*dfrA7_11*	GGTAATGGCCCTGATCTCCCATGGTC
68	D5	prob_*dfrA7_12*	GGTAATGGCCCTGATATCCCATGGTCAG

69	E5	prob_*dfr12_11*	CAGTACGCATTTATCTCGTTGCTGCGA
70	F5	prob_*dfr13_11*	GGTCCGCATTTATCTGGTCGCTGC
71	G5	prob_*dfrA14_21*	GCCATGGACAGGCTAGCTGAATTCAC
72	H5	prob_*dfrA15_1*	CGTTCAAGCTTCACTTCCAGTGATGAGA
73	I5	prob_*dfrA17_11*	GGTAGTGGTCCTGATATCCCGTGGTCA
74	J5	prob_*dfrA19_1*	TGTCATAGGCGCTACTGTCGTTCCTG
75	K5	prob_*aac3Ia_1*	CGTGAGTTCGGAGACGTAGCCACC
76	L5	prob_*aac3IVa_1*	CGTTACACCGGACCTTGGAGTTGTCT
77	M5	prob_*aac6Ib_1*	CGTCACACTGCGCCTCATGACTGA
78	N5	prob_*ant2Ia_1*	TGGACTATGGATTCTTAGCGGAGATCGG
83	C6	prob_*act1_11*	GCAAGCTGGGTGATGGTCAACATGA
84	D6	prob_*dha1_1*	TGGTGGACAGCACCATTAAACCGCT
85	E6	prob_*acc2_11*	TGCCGAATTTGCTCACCGGTAACG
86	F6	prob_mox_1pm	GCTGCTCAAGGAGCACAGGATCCC
87	G6	prob_mox_1mm	CGATGCTCAAGGCGTATCGGATCCC
88	H6	prob_*cmy_11*	ACGAAGAGGCAATGACCAGACGCG
89	I6	prob_*fox_11*	AGCTTCCAGGCCAATCCGGTTACG
90	J6	prob_*tem1_1*	CGAACTACTTACTCTAGCTTCCCGGCAA
91	K6	prob_*oxa1_21*	ACAACGGATTAACAGAAGCATGGCTCG
92	L6	prob_*oxa2_11*	CGATAGTTGTGGCAGACGAACGCC
93	M6	prob_*oxa7_11*	CGCAATTATCGGCCTAGAAACTGGTGTC
94	N6	prob_*ctxM9_11*	GCGATGAGACGTTTCGTCTGGATCG
99	C7	prob_*ctxM9_12*	GGTGATGAGACCTTCCGTCTGGACAG
100	D7	prob_*shv1_11*	ACAGCTGGAGCGAAAGATCCACTATCG
101	E7	prob_*oxa9_11*	GACTCTGTTGTCTGGTTCTCGCAGCA
102	F7	prob_*len1_11*	ACAACTGGATCGGCGGATCCACTAC
103	G7	prob_*acc1_11*	TGTCTGGCAGCAACTGTCCAAGGT
104	H7	prob_*ctxM2_11*	GCATTCGCCGCTCAATGTTAACGGT
105	I7	prob_*per2_1*	CGGTGTCACACAGCGACAATGTGG

106	J7	prob_*dfrV_21*	CCATGTACGGGCTGGCTGAACTCA
107	K7	prob_*sul1_11*	CCTTCCTGTAAAGGATCTGGGTCCAGC
108	L7	prob_*pse1_1pm*	AGCAGATCTTGTGACCTATTCCCCTGT

Appendix 2. Table 2. reagents used in microarrays

Reagent	Order from	Cat no.	Volume	Notes
Therminator buffer and polymerase	NEB	M0261S	200 units	Buffer and polymerase supplied together. Store at -20°C
Primer mix	Clondiag	-	-	Store at -20°C
dNTPs				Store at -20°C
Biotin-16-dUTP	Roche	11093070910	50µl/50nmol	Store at -20°C
Sodium phosphate dibasic (anhydrous)	Sigma	71636	250g	Only use once, then discard
0.5M EDTA (pH 8)	Sigma	E7889	100ml	Also available from RPU
Triton x 100	Sigma	T8787	100ml	
Poly-HRP-streptavidin	Pierce	N200	250µl	Aliquot and store at -20°C. Do not freeze-thaw
Seramun green	Seramun diagnostica GmbH	S-011-1-ODI	100ml	Store at 4°C, discard after 6 months
True blue	Insight biotechnology	71-00-67	10ml	Only stable for 1 year at room temperature (22-28°C)
		71-00-64	50ml	
		50-78-02	2 x 100ml	
20% SDS	RPU			
20 x SSC (pH 7)	RPU			
1 x PBS	RPU			
10 x SSPE (pH 7.4)	RPU			

Appendix2. Table3. Resistance of phenotypes and genotypes as identified by disc diffusion tests and microarray. Positive hybridization was indicated by (1) squares; Amp, ampicillin; aug, augmentin; chl, chloramphenicol; nal, nalidixic acid; cip, ciprofloxacin; tet, tetracycline; trim, trimethoprim.

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intI1_1	aadA1_1	catA1_11	dfrA1_21	temI_1	dfrV_21	sulI_11
MF	cattle	374	1	0	1	0	0	1	1	0	1	1	1	1	1	1	0	1
MF	rodents	271	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
MF	rodents	278	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
MF	rodents	283	1	0	0	0	0	1	1	0	1	1	1	0	1	1	0	0
MF	wild-birds	300	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
MF	cattle	317	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
MF	cattle	324	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
MF	rodents	268	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0
MF	cattle	132	1	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0
MF	cattle	216	1	0	1	0	0	1	1	0	1	0	0	0	1	1	0	0
MF	cattle	221	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
MF	cattle	225	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
MF	cattle	228	1	0	0	0	0	1	1	0	1	1	1	0	1	1	0	0
MF	cattle	320	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
MF	cattle	363	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
MF	cattle	783	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0
MF	cattle	778	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0
MF	wild-mammals	305	1	0	1	0	0	1	1	1	0	1	0	1	0	1	0	1
MF	wild-birds	298	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1
PHF	rodents	847	1	1	1	0	0	1	1	0	0	0	0	0	1	0	0	0
MF	wild-birds	162	1	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intl1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
PHF	rodents	857	1	0	0	1	1	1	0	1	0	1	1	1	1	0	0	1
PHF	rodents	846	1	1	1	0	0	1	1	1	0	0	1	1	1	0	0	1
PHF	rodents	1329	1	0	0	1	1	1	0	0	0	0	1	0	1	1	0	1
PHF	wild-mammals	731	1	1	1	0	0	1	1	0	1	0	1	0	1	1	0	0
PHF	wild-mammals	715	0	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0
PHF	wild-mammals	724	1	1	1	0	0	1	1	0	0	0	0	0	1	1	0	0
PHF	rodents	1251	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0
PHF	rodents	853	0	0	1	0	0	1	0	1	0	1	1	1	0	1	0	0
MF	wild-mammals	315	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
MF	cattle	327	1	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0
PHF	wild-mammals	1248	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	757	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
PHF	wild-mammals	92	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0
PHF	wild-mammals	1245	1	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0
PHF	wild-mammals	730	1	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0
PHF	cattle	97	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	817	0	0	1	0	0	1	0	1	0	1	1	1	0	1	0	0
PHF	wild-birds	700	0	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1
PHF	cattle	1312	1	1	1	0	0	1	1	0	0	0	1	1	1	1	0	1
PHF	rodents	749	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0
PHF	rodents	1252	0	0	1	0	0	1	0	1	0	1	1	1	0	0	0	0
PHF	wild-birds	702	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	761	1	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0
PHF	cattle	764	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0
PHF	cattle	792	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intl1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
PHF	wild-mammals	733	1	0	0	1	1	1	0	0	1	0	0	0	0	1	0	0
PHF	wild-mammals	717	1	0	0	1	1	1	0	0	1	0	0	0	0	1	0	0
CLF	rodents	504	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0
CLF	rodents	509	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
CLF	rodents	580	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0
PHF	wild-birds	706	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	1
CLF	wild-mammals	460	0	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0
CLF	cattle	178	0	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0
CLF	cattle	182	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0
CLF	cattle	205	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CLF	cattle	380	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
CLF	cattle	382	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
CLF	cattle	386	1	0	1	0	0	1	1	0	0	1	1	1	1	1	0	1
CLF	cattle	212	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0
PHF	wild-birds	701	0	0	1	0	0	1	0	1	0	0	1	1	0	1	0	0
CLF	wild-mammals	424	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0
CLF	rodents	488	1	0	1	0	0	1	1	0	0	0	1	1	1	1	0	1
CLF	rodents	494	0	0	1	0	0	1	0	1	0	1	1	0	0	0	0	0
CLF	wild-birds	526	1	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0
PHF	rodents	862	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
PHF	wild-birds	703	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	769	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
CLF	wild-birds	531	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0
CLF	rodents	1170	1	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0
CLF	cattle	403	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intI1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
CLF	wild-mammals	426	1	0	1	0	0	1	1	0	0	0	1	1	1	1	0	1
CLF	wild-mammals	456	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0
BHF	rodents	998	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0
BHF	rodents	1003	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1
BHF	rodents	1006	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
CLF	wild-mammals	433	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
PHF	cattle	1314	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	1320	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0
PHF	cattle	816	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0
CLF	rodents	502	1	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1
PHF	wild-mammals	737	1	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0
BHF	wild-mammals	982	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
BHF	cattle	174	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
BHF	wild-birds	973	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
CLF	wild-birds	1176	0	0	1	0	0	1	0	1	1	0	1	0	0	0	0	0
BHF	wild-mammals	1049	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
BHF	cattle	1093	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1
CLF	wild-mammals	459	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0
BHF	wild-mammals	1139	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
CLF	wild-mammals	437	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
CLF	wild-mammals	457	1	1	1	0	0	1	1	1	0	1	1	1	1	1	0	1
BHF	cattle	1116	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0
BHF	rodents	1007	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
CLF	wild-mammals	475	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intl1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
BHF	cattle	955	1	0	1	0	0	1	1	0	1	1	1	0	1	1	1	1
PHF	rodents	868	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0
BHF	cattle	1016	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
BHF	cattle	1302	1	0	0	0	0	1	0	1	1	1	1	0	1	1	0	1
BGF	cattle	616	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0
CLF	cattle	184	1	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0
BHF	cattle	972	1	0	0	0	0	0	1	0	0	1	1	0	1	1	0	1
CLF	cattle	385	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0
BGF	wild-birds	87	1	1	1	0	0	1	1	1	0	1	1	0	1	1	0	0
BGF	wild-birds	663	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0
BGF	wild-birds	1135	1	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1
BHF	cattle	916	1	0	0	0	0	1	0	0	1	1	1	0	0	1	1	1
CLF	cattle	413	1	0	1	0	0	1	1	1	0	1	1	0	1	1	0	0
BHF	wild-mammals	935	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0
CLF	wild-mammals	438	1	0	1	0	0	1	1	0	0	0	0	0	1	1	0	1
BGF	cattle	619	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
GF	cattle	41	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
GF	wild-birds	882	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
MF	wild-mammals	313	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
MF	wild-birds	665	1	0	0	0	0	1	1	0	0	1	0	0	0	1	1	0
PHF	rodents	46	1	1	1	0	0	1	1	1	0	1	1	1	1	0	0	1
PHF	wild-mammals	708	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0
BGF	wild-birds	32	1	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intl1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
PHF	rodents	1331	1	1	1	0	0	1	1	0	0	0	0	1	1	1	0	1
BGF	cattle	1210	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
PHF	rodents	866	1	1	1	0	0	1	1	0	0	0	0	1	1	0	0	0
PHF	wild-mammals	1250	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0
GF	wild-mammals	17	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
GF	cattle	40	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
GF	cattle	47	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
CLF	rodents	1169	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0
CLF	rodents	482	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0
PHF	cattle	39	1	1	1	0	0	1	1	1	0	1	1	1	1	0	0	1
BHF	cattle	1019	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
CLF	wild-birds	928	1	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1
PHF	cattle	845	1	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0
BHF	cattle	951	0	0	1	0	0	0	1	0	0	1	1	0	0	0	1	1
BHF	wild-birds	919	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
BGF	rodents	1178	1	1	0	0	0	1	0	1	0	1	1	0	0	1	0	1
BGF	cattle	622	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	0
BHF	wild-mammals	984	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0
BHF	wild-mammals	988	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0
MF	wild-mammals	314	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1
GF	wild-birds	883	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	1
MF	cattle	230	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
BHF	rodents	1008	1	0	0	0	0	1	1	1	0	0	1	0	1	1	0	1
BHF	rodents	994	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intI1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
CLF	wild-birds	528	1	1	1	0	0	1	1	1	0	1	1	0	1	1	0	0
BHF	wild-mammals	1048	1	0	1	0	0	1	1	0	1	1	1	0	0	1	0	0
BGF	wild-birds	1349	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0
BGF	wild-birds	664	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0
BHF	rodents	1005	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1
BHF	wild-mammals	978	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
BHF	wild-mammals	1343	1	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0
BHF	wild-mammals	1052	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
BHF	wild-mammals	15	1	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0
BHF	cattle	949	1	0	0	0	0	1	1	0	1	1	0	0	0	1	0	1
BHF	wild-birds	920	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
BHF	cattle	962	1	0	1	0	0	1	1	0	1	1	1	0	1	1	1	1
MF	wild-birds	680	1	1	0	0	0	1	1	1	0	0	1	0	1	1	0	0
BHF	cattle	1013	0	0	1	0	0	1	0	1	0	0	1	1	0	0	0	1
BHF	cattle	116	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1
BHF	cattle	1077	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	0
BHF	cattle	1119	1	0	1	0	0	1	1	0	1	1	0	1	0	1	0	1
BGF	rodents	609	1	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0
BHF	cattle	1029	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0
BHF	wild-birds	922	1	0	0	0	0	1	1	1	0	1	0	0	0	1	0	0
BGF	cattle	657	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	0
GF	wild-mammals	9	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0
GF	wild-mammals	19	1	0	0	0	0	0	1	0	0	1	1	0	0	1	1	1

Location	group_name	ccno	amp	aug	chl	nal	cipro	tet	trim	tetA_11	tetB_11	intl1_1	aadA1_1	catA1_11	dfrA1_21	tem1_1	dfrV_21	sul1_11
GF	cattle	49	1	0	0	0	0	1	1	1	0	0	0	1	1	0	0	1
BHF	wild-mammals	942	1	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1
GF	cattle	904	1	0	0	0	0	0	1	0	1	0	1	0	1	1	1	0
BHF	wild-mammals	1344	1	0	0	0	0	1	1	1	0	1	1	0	1	1	1	1
BHF	cattle	1067	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
GF	cattle	53	1	0	1	0	0	1	1	0	1	1	1	0	1	1	0	0
BGF	rodents	1207	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1
BGF	rodents	1180	1	1	1	0	0	1	1	0	0	0	0	0	1	1	0	0
BGF	cattle	617	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0
MF	rodents	266	1	0	0	0	0	1	1	1	0	1	1	0	1	1	0	1
GF	wild-birds	885	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1
BGF	wild-mammals	1183	1	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0
GF	cattle	910	1	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0
BHF	cattle	1092	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0
MF	wild-mammals	309	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0
BGF	wild-birds	90	1	1	1	0	0	1	1	1	0	1	1	0	1	1	0	0
MF	rodents	258	1	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0
GF	wild-mammals	10	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0
GF	cattle	55	1	0	1	0	0	1	1	0	1	1	1	1	1	1	0	1
MF	wild-mammals	315	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
MF	cattle	327	1	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0
MF	pheasant	673	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0