From the Faculty of Medicine, University of Iceland

Antimicrobial resistant bacteria in production animals in Iceland

– possible transmission to humans?

Þórunn Rafnar Þorsteinsdóttir

Supervisor:

Eggert Gunnarsson

Co-supervisor:

Karl G. Kristinsson

PhD committee:

Vala Friðriksdóttir (chair), Institute for Experimental Pathology, University of Iceland, Keldur

Eggert Gunnarsson, Institute for Experimental Pathology, University of Iceland, Keldur

Karl G. Kristinsson, Faculty of Medicine, University of Iceland; Department of Clinical Microbiology, Landspitali University Hospital

Frank M. Aarestrup, National Food Institute, Technical University of Denmark

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ÁGRIP

Tíðni baktería sem eru ónæmar fyrir sýklalyfjum fer vaxandi, bæði meðal manna og dýra og getur valdið vandkvæðum við meðhöndlun sýkinga. Víða erlendis hefur verið fylgst með tíðni ónæmra baktería í sláturdýrum og matvælum en lítið hefur verið vitað um algengi hér á Íslandi. Megin markmið þessarar rannsóknar var að ákvarða tíðni ónæmra baktería (*Salmonella* spp., *Campylobacter* spp. og *Escherichia coli*) í sláturdýrum á Íslandi og meta hvort og í hve miklum mæli þessar bakteríur berast frá dýrum til manna, þá aðallega með matvælum.

Rannsakaðir voru 163 stofnar af *Salmonellu* og 362 stofnar af *Campylobacter*, sem einangraðir voru í reglulegu eftirliti með sláturdýrum á árunum 2001-2005. Einnig voru rannsakaðir 482 stofnar af *E. coli*, sem einangraðir voru úr svínum, kjúklingum, svínakjöti, kjúklingakjöti, kjúklingafóðri, starfsmönnum sláturhúsa og sjúklingum utan sjúkrahúsa á árunum 2005-2008. Val stofna var með tilliti til þess að þeir endurspegluðu þýðið á öllu landinu. Allir stofnar voru næmisprófaðir með míkró-seyðis þynningar aðferð (VetMIC) og skyldleiki ónæmra stofna var borinn saman með Pulsed Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA (RAPD) og faga týpu greiningu.

Tíðni ónæmra *Salmonella* stofna var 13.6% í kjúklingum og 12.8% í svínum. Alls var 21 stofn (12.8%) ónæmur fyrir einu sýklalyfi eða fleirum, þar af 19 af tegundinni *S.* Typhimurium, einn *S.* Infantis stofn og einn *S.* Worthington stofn. Af 19 ónæmum *S.* Typhimurium stofnum voru 16 fjölónæmir (ónæmir fyrir ≥3 sýklalyfjum), þar af voru 15 stofnar með eins eða mjög skyld PFGE mynstur og greindust allir sem faga týpa DT 104.

Tíðni ónæmra *Campylobacter* stofna var 6.9% í kjúklingum, en enginn stofn reyndist fjölónæmur. Algengast var ónæmi fyrir ampicillín (3.6%), því næst kom ónæmi fyrir enrófloxacín (3%), nalidixic sýru (1.9%) og oxytetracyclín (0.3%). Kross-ónæmi var á milli enrófloxacín og nalidixic sýru. Tíðni ónæmis meðal *C. coli* stofna (53.8%) var mun hærri en meðal *C. jejuni* stofna (5.2%) og ónæmismynstrin ólík. Skerðing með skerðiensímunum *Sma*I og *Kpn*I gaf 13 mismunandi PFGE týpur, en engin ráðandi. Vissar PFGE týpur með eins ónæmismynstur komu upp á búum í mismunandi landshlutum, sem bendir til dreifingar klóna.

Tíðni ónæmra *E. coli* stofna var 54.1% og 28% (botnlanga- og kjötsýni) í svínum, 33.6% og 52% í kjúklingum (botnlanga- og kjötsýni), 31.8% í kjúklingafóðri, 39.1% í sláturhúsastarfsmönnum og 23.1% í sjúklingum utan sjúkrahúsa. Tíðni ónæmis fyrir cíprófloxacín og nalidixic sýru jókst milli sýnatöku tímabilanna 2005-2007 og 2008 ($p<0.0001$) en tíðni ónæmis fyrir öðrum sýklalyfjum lækkaði. Meirihluti (78.6%) ónæmra *E. coli* stofna var með ólík PFGE mynstur og lítið var um þyrpingar skyldra stofna. Þó einangruðust stofnar með sama ónæmismynstur og PFGE mynstur úr kjúklingakjöti og starfsmanni sláturhúss og ennfremur fundust mjög skyldir stofnar í mönnum, kjúklingum, kjúklingakjöti og kjúklingafóðri.

Tíðni sýklalyfjaónæmra *Salmonella* og *Campylobacter* stofna í kjúklingum og svínum á Íslandi er frekar lág. Við fundum þó fjölónæman *S*. Typhimurium klón sem veldur áhyggjum. Tíðni sýklalyfjaónæmis meðal *E. coli* stofna úr sláturdýrum og matvælum var meðalhá eða há, sérstaklega í kjúklingum og kjúklingakjöti. Athygli vakti að ónæmi fannst fyrir flúórókínólónum, þar á meðal í stofnum úr kjúklingum og kjúklingakjöti, en engin sýklalyf eru notuð í kjúklingaeldi á Íslandi. Þyrpingar stofna af mismunandi uppruna sem hafa eins eða lík PFGE og ónæmismynstur gefa vísbendingar um flutning sýklalyfjaónæmra *E. coli* stofna frá dýrum til manna. Fjölbreytni í ónæmismynstrum og PFGE mynstrum þykir benda til þess að stórt þýði ónæmra *E. coli* sé að finna í sláturdýrum á Íslandi. Niðurstöður þessarar rannsóknar styðja þá tilgátu að ónæmar bakteríur berist með fóðri í kjúklinga og að kjúklingar og afurðir þeirra séu uppspretta flúórkínólóna ónæmra *E. coli* stofna í mönnum. Áframhaldandi eftirlit er mikilvægt og nauðsynlegt er að rannsaka betur uppruna ónæmra klóna og mögulegan flutning þeirra til manna.

Lykilorð: *Salmonella*, *Campylobacter*, *E. coli*, sýklalyfjaónæmi, flúórkínólón, menn, sláturdýr, dýraafurðir, pulsed field gel electrophoresis.

ABSTRACT

Antimicrobial resistance is a growing problem in human as well as veterinary medicine. Antimicrobial resistance rates of bacteria from production animals and their food products are available for many countries but until now little has been known about the prevalence in Iceland. The main objective of this study was to determine the prevalence of antimicrobial resistant bacteria (*Salmonella* spp., *Campylobacter* spp. and *Escherichia coli*) in production animals in Iceland and if these resistant bacteria were transferred from animals to humans, possibly through food.

A total of 163 *Salmonella* strains and 362 *Campylobacter* strains, isolated from broilers and pigs in the national *Salmonella* and *Campylobacter* surveillance programmes in the years 2001-2005, were available for study. A total of 482 *E. coli* isolates from healthy pigs, broiler chicken, pork, broiler meat, broiler feed, slaughterhouse personnel and outpatients in the years 2005-2008 were collected and tested. Isolates were tested for antimicrobial susceptibility using a microbroth dilution method (VetMIC) and resistant strains were compared using Pulsed Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA (RAPD) and phage typing.

The overall prevalence of resistance among *Salmonella* spp. was 13.6% in chickens and 12.8% in pigs. Twenty one isolates (12.8%) were resistant to one or more antimicrobials, 19 *S.* Typhimurium strains, one *S.* Infantis strain and one *S.* Worthington strain. Of the 19 resistant *S.* Typhimurium strains, 16 were multiresistant (to ≥3 antimicrobial agents) and of these 15 had identical or closely related PFGE patterns and were of phage type DT 104.

The prevalence of resistance among *Campylobacter* isolates was 6.9%, although none were multiresistant. Resistance to ampicillin was most commonly observed (3.6%) followed by resistance to enrofloxacin (3%), nalidixic acid (1.9%) and oxytetracyclin (0.3%), with cross-resistance between enrofloxacin and nalidixic acid. Resistance rates among *C. coli* isolates (53.8%) were much higher than among *C. jejuni* isolates (5.2%) and resistance patterns differed. Macrorestriction with *Sma*I and *Kpn*I restriction enzymes yielded 13 different pulsotypes, none of wich indicated a predominant genotype. Specific

pulsotypes with uniform resistance patterns arising on geographically separated farms indicates clonal dissemination.

The resistance rates among *E. coli* isolates were 54.1% and 28% (cecal and meat samples) among pigs, 33.6% and 52% (cecal and meat samples) among broilers, 31.8% in broiler feed, 39.1% among slaughterhouse personnel and 23.1% among outpatients. Prevalence of resistance to ciprofloxacin and nalidixic acid increased significantly between sampling in 2005-2007 and in 2008 ($p<0.0001$) but decreased for other antimicrobials. The majority (78.6%) of the resistant *E. coli* isolates was genotypically diverse based on PFGE fingerprint analyses and clustering was limited. The same resistance pattern and pulsotype was found among isolates from broiler meat and a slaughterhouse worker and closely related isolates were found in humans, broilers, broiler meat and broiler feed.

The prevalence of antimicrobial resistance in *Salmonella* spp. and *Campylobacter* spp. in pigs and poultry in Iceland is low. However we found a multiresistant *S.* Typhimurium clone that causes concern. Prevalence of antimicrobial resistant *E. coli* from production animals and their food products in Iceland was moderate to high, especially in broilers and broiler meat. Of notice was resistance to quinolones, particularly among broiler and broiler meat isolates as there is no known antimicrobial selection pressure in the broiler production in Iceland. There was some clustering of isolates of different origin, indicating spread of antimicrobial resistant *E. coli* from animals to humans. Diverse resistance patterns and pulsotypes suggest the presence of a large population of resistant *E. coli* in production animals in Iceland. Data presented in this thesis support the hypothesis that feed is a source of antimicrobial resistant bacteria in chicken and that chicken and their products may be a source of fluoroquinolone resistant *E. coli* in humans. Continuous resistance surveillance in Iceland is important and further research on the source of resistant clones and possible transmission to humans is needed.

Keywords: *Salmonella*, *Campylobacter*, *E. coli*, antimicrobial resistance, fluoroquinolones, humans, production animals, animal products, pulsed field gel electrophoresis.

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Þórunn Rafnar Þorsteinsdóttir

TABLE OF CONTENTS

ÁGRIP……..... i
ABSTRACT……..... iii
ACKNOWLEDGEMENTS…… v
TABLE OF CONTENTS…… vii
LIST OF FIGURES AND TABLES…….. x
LIST OF ABBREVIATIONS……... xi
LIST OF PAPERS…….. xii
DECLARATION OF CONTRIBUTION…….. xiii
INTRODUCTION…….. 1
Antimicrobials……... …… 2
Classification…….. 2
Mode of action…… 3
Antimicrobial use……..... 3
Antimicrobial use in animals…….. 5
Antimicrobial use in Iceland……... 7
Bacterial resistance to antimicrobials……. 8
Mechanisms of antimicrobial resistance……. 10
Emergence and dissemination of bacterial resistance…… 11
Burden of antimicrobial resistance……..... 13
Antimicrobial resistance in production animals in relation to resistance in humans…….. 14
Salmonella…….. 15
Campylobacter…… 18
Escherichia coli……... 20
Poultry and swine production in Iceland…… 23
Antimicrobial susceptibility testing…… 25
Antimicrobial resistance monitoring…….. 26
Molecular epidemiological analysis……... 27
SUMMARY…… 29

AIMS OF THE STUDY……..... 30
MATERIALS AND METHODS…… 31
Sample collection……..... 31
Salmonella…… 31
Campylobacter……. 31
E. coli……... 33
Bacterial isolation and identification…….. 34
Salmonella…… 34
Campylobacter……. 35
E. coli……... 35
Susceptibility testing…….. 36
Molecular typing……. 37
Pulsed Field Gel Electrophoresis (PFGE)……..…….. 37
Randomly amplified polymorphic DNA (RAPD)…….. 37
Antimicrobial residue screening test……... 38
Statistical analysis……. 38
Ethical considerations…….. 39
RESULTS……..... 40
Salmonella…… 40
Salmonella serovars……. 40
Antimicrobial susceptibility……. 40
PFGE patterns and definitive phage typing…….. 40
Campylobacter……. 42
Antimicrobial susceptibility…….. 43
Pulsed Field Gel Electrophoresis…….. 44
E. coli…… 45
Prevalence……. 45
Antibiotic susceptibility…… 45
PFGE……. 49
RAPD…… 52
Antimicrobial residue screening test……...……. 52

DISCUSSION……..…… 54
Salmonella…… 54
Campylobacter……. 55
E. coli……... 57
Limitations of the study……... 61
GENERAL SUMMARY AND CONCLUSIONS ……..…… 63
REFERENCES…….... 66
PAPERS I – IV…… 87

LIST OF FIGURES AND TABLES

Figures		**Page**
Figure 1	Outpatient antimicrobial use in the Nordic countries	8
Figure 2	Antimicrobial use in veterinary medicine in Iceland	9
Figure 3	Transfer pathways of antimicrobial resistant bacteria	16
Figure 4	Prevalence of *Salmonella* and *Campylobacter* in pigs and broilers in Iceland	24
Figure 5	Geographical distribution of chicken and pig farms	32
Figure 6	Dendrogram of resistant *S*. Typhimurium strains (PFGE analysis)	42
Figure 7	Trends in resistance among *Campylobacter*	44
Figure 8	Dendrogram of resistant *Campylobacter* strains (PFGE analysis)	45
Figure 9	Prevalence of resistance among E. coli to five antimicrobials	49
Figure 10	Dendrogram of resistant *E. coli* strains (PFGE analysis)	51
Figure 11	Dendrogram of resistant *E. coli* strains (RAPD analysis)	53

Tables		
Table 1	Antimicrobial classes and their mode of action	4
Table 2	Antimicrobial resistance of *Salmonella* Typhimurium	41
Table 3	Characteristics of nineteen *S*. Typhimurium strains	41
Table 4	Antimicrobial resistance of *Campylobacter* spp	43
Table 5	Origin and number of *E. coli* isolates	46
Table 6	Antimicrobial resistance of *E. coli*	47
Table 7	Antimicrobial resistance patterns of *E. coli* isolates	48

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AST	Antimicrobial Susceptibiliy Test
DDD	Defined Daily Dose
DID	DDD per 1000 inhabitants per day
EHEC	Enterohaemorrhagic *E. coli*
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
PDR	Pandrug Resistance
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
STEC	Shiga-like Toxin producing *E. coli*
UTI	Urinary Tract Infection
XDR	Extensive Drug Resistance

LIST OF PAPERS

Paper I Thorsteinsdottir TR, Kristinsson KG, Gunnarsson E. Antimicrobial resistance and serotype distribution among Salmonella spp. in pigs and poultry in Iceland, 2001-2005. Microb Drug Resist. 2007 Winter;13(4):295-300.

Paper II Thorsteinsdottir TR, Kristinsson KG, Fridriksdottir V, Gunnarsson E. Antimicrobial resistance of Campylobacter spp. isolated from broiler flocks in Iceland 2001-2005. Microb Drug Resist. 2008 Mar;14(1):49-53.

Paper III Thorsteinsdottir TR, Haraldsson G, Fridriksdottir V, Kristinsson KG, Gunnarsson E. Prevalence and genetic relatedness of antimicrobial resistant Escherichia coli isolated from animals, foods and humans in Iceland. Zoonoses Public Health (In press 2009)

Paper IV Thorsteinsdottir TR, Haraldsson G, Fridriksdottir V, Kristinsson KG, Gunnarsson E. Probable broiler-feed origin of fluoroquinolone resistant Escherichia coli isolated from broilers, broiler meat and humans in Iceland. Emerging Infectious Diseases (Submitted)

Declaration of contribution

Paper I

Thorunn R. Thorsteinsdottir (TRT), Karl G. Kristinsson (KGK) and Eggert Gunnarsson (EG) were responsible for the design of the study. TRT was responsible for the bacteriological studies, susceptibility testing, molecular typing and analysis of the data. TRT wrote the first draft of the paper and together with KGK and EG was responsible for writing the paper. All authors were involved in editing and in the final approval of the paper.

Paper II

TRT, KGK and EG were responsible for the design of the study. TRT was responsible for the bacteriological studies, susceptibility testing, molecular typing and analysis of the data. TRT wrote the first draft of the paper and together with KGK and EG was responsible for writing the paper. All authors were involved in editing and in the final approval of the paper.

Paper III

TRT, Vala Fridriksdottir (VF), KGK and EG were responsible for the design of the study. TRT, KGK and EG were responsible for sample collection. TRT was responsible for the bacteriological studies, susceptibility testing and analysis of the data. TRT together with Gunnsteinn Haraldsson (GH) was responsible for molecular typing. TRT wrote the first draft of the paper and together with KGK and EG was responsible for writing the paper. All authors were involved in editing and in the final approval of the paper.

Paper IV

TRT, VF, KGK and EG were responsible for the design of the study. TRT, KGK and EG were responsible for sample collection. TRT was responsible for the bacteriological studies, susceptibility testing and analysis of the data. TRT together with GH was responsible for molecular typing. TRT wrote the first draft of the paper and together with KGK and EG was responsible for writing the paper. All authors were involved in editing and in the final approval of the paper.

INTRODUCTION

The introduction of antimicrobial agents in the early 20th century is one of the greatest achievements of scientific medicine. Ehrlich's search for the "magic bullet", Flemings discovery of penicillin in 1928 and its mass production in 1943, Domagks synthesis of a sulfonamide in 1932 and the subsequent findings and production of other antimicrobials in the following decades (205) led scientists to believe they were well on the way to treating, preventing or eradicating many of the most deadly infectious diseases. However, ever since the first antimicrobial agent was used for clinical purposes antimicrobial resistance has been on the rise. For many agents it took no longer than three to five years from the introduction of an antimicrobial into clinical use until the first resistant bacteria were found (222, 252). Furhermore, there has been little success in the discovery of new antimicrobials in the last decades and most of the new agents are synthetic relatives of the older ones.

The World Health Organization (WHO) has recognized that antimicrobial resistance is a global problem that calls for a global response. With the issue of the *WHO Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food* and the *WHO Global Strategy for Containment of Antimicrobial Resistance* some interventions were recommended that hopefully will enable local authorities to slow down the emergence and reduce the spread of resistance in diverse settings (280, 281). These guidelines recommend the establishment of surveillance programs for both antimicrobial consumption and resistance, as well as guidelines for prudent use of antimicrobials and further research.

Antimicrobial resistance rates of bacteria from production animals and their food products are available for many countries (44, 53, 69, 250), but until now little has been known about the prevalence in Iceland.

Antimicrobials

Antimicrobial agents are any low molecular weight compounds, of natural, semisynthetic, or synthetic origin that kill or inhibit the growth of microorganisms. Antibiotics are, on the other hand, compounds produced by microorganisms that in dilute solutions can kill or inhibit the growth of other microorganisms. However, these terms are used interchangeably as today the natural antibiotics have been modified and synthesized in the laboratory. Antimicrobials make use of the differences in the structure and biochemical functions between prokaryotic and eukaryotic cells and therefore have greater toxicity against bacteria compared to other microorganisms such as fungi and viruses.

Classification

Various criteria can be used for the classification of antimicrobial agents. They can be classified according to the target microorganisms, as antibacterial, antifungal, antiparasitic or antiviral. However, some antimicrobials, such as sulfonamides and tetracyclines, do have activity against protozoa and ionophores (antiparasitic agents) have some antibacterial activity. Antimicrobials can also be categorized based on their antibacterial activity, that is if they are narrow-spectrum or broad spectrum, bacteriostatic or bacteriocidal. Narrow-spectrum agents are mainly active against specific bacterial groups such as Gram-negative organisms, or anaerobes while broad-spectrum agents have activity against both Gram-positive and Gram-negative organisms. Antimicrobials that inhibit or delay the growth of bacteria at the minimum inhibitory concentration (MIC) are called bacteriostatic, although at higher concentrations (minimum bactericidal concentration, MBC) they might be able to kill the bacteria. Bactericidal drugs, on the other hand, kill the bacteria around the same concentration that inhibits their growth. Furthermore, antimicrobials can be either time or concentration dependent. That is, their activity (microbial killing) increases either with extended exposure of the agent (frequent administration) or increasingly higher concentrations (administration of high doses at long dosing intervals).

The most useful classification of antimicrobials is based on the chemical structure of the agents. Structural classes of antimicrobials generally show similar patterns of activity, pharmacodynamics, toxicity and have similar or identical target sites.

Mode of action

To exert their activity antimicrobials must enter the microbial cell, for example by passive diffusion, self-promoted uptake or through an energy-dependent transport system (254). Antimicrobials act at specific target sites and thereby inhibit biosynthesis of essential components of the microbial cell. There are three main mechanisms by wich antimicrobials exert their activity on bacteria (112, 124, 180): 1) inhibition of cell wall synthesis, 2) inhibition of protein synthesis and 3) inhibition of nucleic acid synthesis. The β-lactam antibiotics (including one of the oldest classes of antimicrobials, penicillins, and novel classes such as cephalosporins, carbapenems, and monobactams) bind to penicillin-binding proteins (PBPs) that are involved in the later stages of peptidoglycan synthesis and thereby inhibit cell wall synthesis. Most protein synthesis inhibitors, such as tetracyclines and chloramphenicols, bind to specific sites on the bacterial ribosome and thereby inhibit mRNA translation. Quinolones inhibit nucleic acid synthesis by interacting with the DNA gyrase (topoisomerase II) and topoisomerase IV and thus interfere with DNA replication, repair and transcription. Sulfonamides and trimethoprim (and other diaminopyrimidines) indirectly affect nucleic acid synthesis by blocking folic acid synthesis, which is a precursor in nucleic acid synthesis. Furthermore, some antimicrobials target the cell membrane of Gram-negative bacteria. Polymyxins act as detergents or surfactants and disrupt the bacterial cell membrane and consequently increase its permeability. The antimicrobial classes and their mode of action are listed in Table 1.

Antimicrobial use

Since the discovery of antimicrobials in the early 20th century, their use, along with other factors such as improved sanitation, housing, and nutrition, has reduced the threat posed by infectious diseases and increased life expectancy dramatically (17).

Table 1. Antimicrobial classes and their mode of action[a]

Target	Antimicrobial Class	Antimicrobial Agents	Activity	Mechanism
Cell wall synthesis	B-lactams:			Bind to Penicillin Binding Proteins (PBP´s) and inhibit cross-linking of polysaccharide chains constituting the peptidoglycan of the bacterial cell wall. Activate cell wall lytic enzymes.
	Penicillins	Ampicillin	Cidal	
	Cephalosporins	Ceftiofur	Cidal	
	Carbapenems	Imipenem	Cidal	
	Monobactams	Aztreonam	Cidal	
	Glycopeptides	Vancomycin	Cidal	Bind to the $_{D}$-Ala-$_{D}$-Ala termini of pentapeptide precursors, thereby inhibiting transpeptidation.
	Polypeptides	Bacitracin	Cidal	Interfere with the lipid carrier, thereby inhibit translocation of pentapeptides through the cell membrane.
Protein synthesis	Aminoglycosides	Streptomycin	Cidal	Bind to the A site on 16S rRNA of the 30S bacterial ribosomal subunit, causing misreading of mRNA and rapid cell death.
	Macrolides	Erythromycin	Static	Bind to the V domain on 23S rRNA of the 50S bacterial ribosomal subunit, inhibitng peptide chain elongation.
	Phenicols	Chloramphenicol	Static	Bind to the V domain on 23S rRNA of the 50S bacterial ribosomal subunit and blocks peptide bond formation.
	Tetracyclines	Oxytetracycline	Static	Bind to the 30S bacterial ribosomal subunit and restrain tRNA movement along the ribosome, inhibiting the first peptide bond.
Nucleic acid synthesis	Rifamycins	Rifampin	Static	Binds to the DNA dependent RNA polymerase, thereby blocking mRNA synthesis.
	Quinolones	Nalidixic acid	Cidal	Interact with the gyrase and topoisomerase IV and block the progress of the replication fork, thereby interfere with DNA replication, repair and transcription.
Intermediary metabolism	Sulfonamides	Sulfamethoxazole	Static	Analogs of PABA (*p*-aminobenzoic acid) and competitively inhibit modification of PABA into dihydrofolate, thus inhibiting folic acid synthesis.
	Diaminopyrimidines	Trimethoprim	Static	Analogs of dihydrofolic acid, competitively inhibit dihydrofolate reductase and therby inhibit folic acid synthesis.
		Trimethoprim/ Sulfamethoxazole	Cidal	
Cell membrane	Polypeptides	Polymyxin B	Cidal	Interact with phospholipids on the cell membrane, disrupt its structure and increase its permeability.

[a] Data adapted from (5, 112, 207)

Nonetheless, infectious diseases represent one-fifth of global deaths (279) and antimicrobial resistant bacteria are on the rise. Antimicrobial therapy of infectious diseases must be initiated early and be sufficiently broad to provide activity against the most likely pathogens, including antimicrobial-resistant strains if they are suspected. When the pathogen has been isolated treatment should shift to a narrow spectrum antimicrobial agent to minimize the risk of the selection of resistant strains. The choice of antimicrobial therapy depends on the pathogens suspected, which can differ among countries and among communities. Therefore, knowledge of local resistance patterns is essential for the appropriate choice of antimicrobial agents.

It is very difficult to estimate the exact volume of antimicrobial consumption in the world, as few countries collect data on antimicrobial sales and use. In Europe it has been estimated that around 52% of total sales volumes of antimicrobials were administered to humans, 33% for veterinary use and 15% for growth promotion in production animals (222). In the USA, where it has been estimated that 50% of the worldwide consumption takes place (233), estimates of antimicrobial use in veterinary medicine range from 40% to 84% (176, 206) and some claim that the nontherapeutic use in livestock alone is 70% (176). Estimates that over 50% of antimicrobials worldwide are used without prescription (48) and that up to 75% of antimicrobial usage is inappropriate or of questionable value (71, 285) are alarming, as nearly 50% of the human use of antimicrobials is based on incorrect indications, such as viral infections (285).

The use of antimicrobials, for any purpose, has been increasing almost everywhere in the world (14, 69, 94, 126, 137). Their use in all fields, human and veterinary medicine, agriculture and aquaculture and even antibacterial household products is selecting for the emergence and dissemination of resistant strains. However, antimicrobial use in animals, particularly in food animals, has during the last decades been under intense debate.

Antimicrobial use in animals

Antimicrobials are used in animal production mainly for three purposes: i) Therapy of diseased animals for short periods, given in high doses to individuals or groups through feed or water; ii) Disease prevention of a group during high risk periods (prophylaxis) or when some individuals in the group present symptoms, but it is expected that most of the

group will be affected (metaphylaxis). Antimicrobials are administered through feed or water in moderate to high doses for a defined period; iii) Growth promotion and enhanced feed efficiency. Antimicrobials are given at subtherapeutic levels to a group (herds and flocks) for a long period of time through feed (27, 172, 223).

It is the use of antimicrobial agents for growth promotion of production animals that has mostly been scrutinized. The growth promoting effect of low levels of antimicrobials in animal feeds was first described in the mid 20th century by Moore et al. (1946) (178) and Stokstad et al. (1949) (240), and since then antimicrobial growth promoters have been shown to improve growth rates and feed conversion in animals (139, 226). Their exact mode of action is not known, although they are assumed to have various effects on the intestinal microflora and the intestines themselves. The commensal bacteria compete with the host for nutrients, secrete toxic compounds into the gut and demand for constant gut epithelial cell turnover (75). Antimicrobial growth promoters are believed to reduce the total number of bacteria, both commensal and potentially harmful bacteria and thereby decrease competition for nutrients, decrease the secretion of bacterial toxins and other metabolites that depress growth and reduce stimulation of the immune system. Furthermore, they are believed to reduce the gut size and intestinal mucosa epithelial cell turnover and consequently increase nutrient absorption (27, 75, 229) The effect of antimicrobial growth promoters are more evident among animals housed under poor hygiene conditions, under stressful conditions such as crowding and shipping and during critical periods of production, such as weaning (229).

Almost immediately after the discovery of the growth promoting effects of antimicrobials came the first reports of antimicrobial resistance in food animals. In 1951 Starr and Reynolds (234) reported the emergence of streptomycin resistant coliform bacteria in turkeys after only a few days of use of streptomycin as a growth promoting supplement and soon other researchers reported the same (24, 166, 173, 259). Concerns about the emergence of transferable antimicrobial resistance in zoonotic bacteria led to the appointment of the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine in the UK. In the committee report (often called the Swann report after the chairman of the committee, Professor Michael Swann) (247) it was concluded that administration of antimicrobial agents to food producing animals at

subtherapeutic levels could pose a hazard to human health. Furthermore, they recommended that only antimicrobial agents that had little or no therapeutic relevance in humans and animals and would not impair the efficacy of therapeutic antimicrobials through cross-resistance should be used for growth promotion. Since the Swann report was published many countries began to individually phase out and ban the use of growth promoters (110) and in 2003 the European Union adopted a Directive to phase out and eventually in January 2006 ban completely the use of antimicrobials as growth promoters in farm animals (208, 209). Despite these recommendations antimicrobial agents were and still are used as growth promoters in many parts of the world, including the United States of America (USA).

Antimicrobial use in Iceland

In Iceland, all antimicrobials are prescription only medicines, both in human and veterinary medicine, and the use of antimicrobials for growth promotion in animals has never been allowed. Drug statistics presented here are based on sales figures from wholesalers. Antimicrobials are commonly used in human medicine in Iceland and their sales figures are higher than in the other Nordic countries. The DDD/1000 inhabitants/day (DID) in Iceland in 2005 was 23.17 (78), compared to 16.6 in Sweden (253), 16.4 in Denmark (68) and 18.2 in Norway (190). When comparing outpatient antimicrobial use in Europe in 2003, Iceland had the 11th highest DID (20.36) of the 25 countries participating in the study (94). The other Nordic countries had somewhat lower outpatient DID than Iceland while Greece, France and Portugal had considerably higher DID (31.40, 28.97 and 27.1, respectively) (94). Figure 1 shows the comparison of outpatient antimicrobial use in the Nordic countries in the years 2003-2008. The use of antimicrobials in humans has been increasing in Iceland since 2001, especially the use of penicillins, macrolides, aminoglycosides and quinolones (78). The use of fluoroquinolones increased by 63% from 1998 to 2006 and consequently the prevalence of fluoroquinolone resistant *E. coli* causing human infections increased from 1% to 9% (137). In 2005 the sales figures for antimicrobials used in veterinary medicine in Iceland were 785.5 kg of active substance, an increase of 2.5% from 2004, and icreased further by 21% to 950 kg in 2006 (78, 79). This increase is mainly due to increased use of

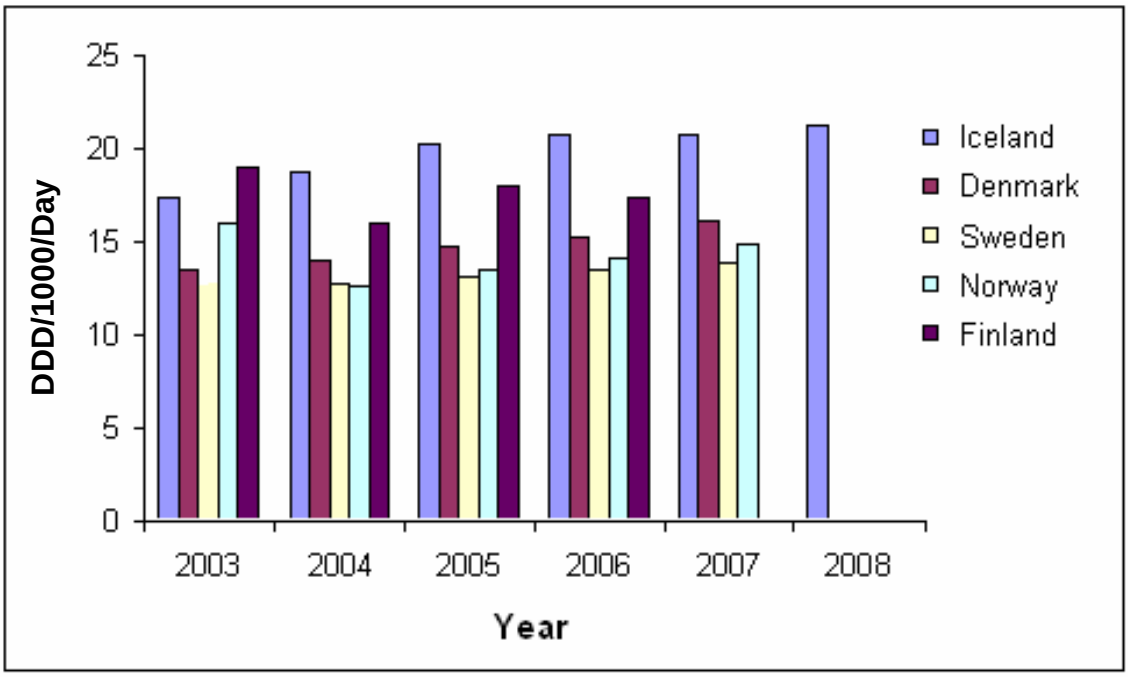

Figure 1: Outpatient antimicrobial use in the Nordic countries 2003-2007 (DID). Reproduced from (80) with permission.

Tiamulin, wich was used in 2006 to eradicate a *Mycoplasma* infections on a single pig farm. The antimicrobials most commonly used were penicillins, streptomycin, oxolinic acid (a quinolone mostly used in aquaculture), oxytetracycline and sulfadiazin (Figure 2).

Bacterial resistance to antimicrobials

Microbiologically, a strain is considered resistant if it grows in the presence of drug concentrations higher than those of phylogenetically related strains. Clinically, a strains is considered resistant if it survives antimicrobial therapy. Studies have shown that there is a relationship between the use of antimicrobials and the prevalence of bacteria resistant to those agents (4, 20, 23, 85, 86, 130). Use of antimicrobial agents exerts a selection pressure that favours the emergence and maintenance of these resistant strains. Misuse (as a result of inadequate dosage, poor adherence and inferior drugs) and abuse of antimicrobials in humans and animals are the major selection forces in antimicrobial resistance (281).

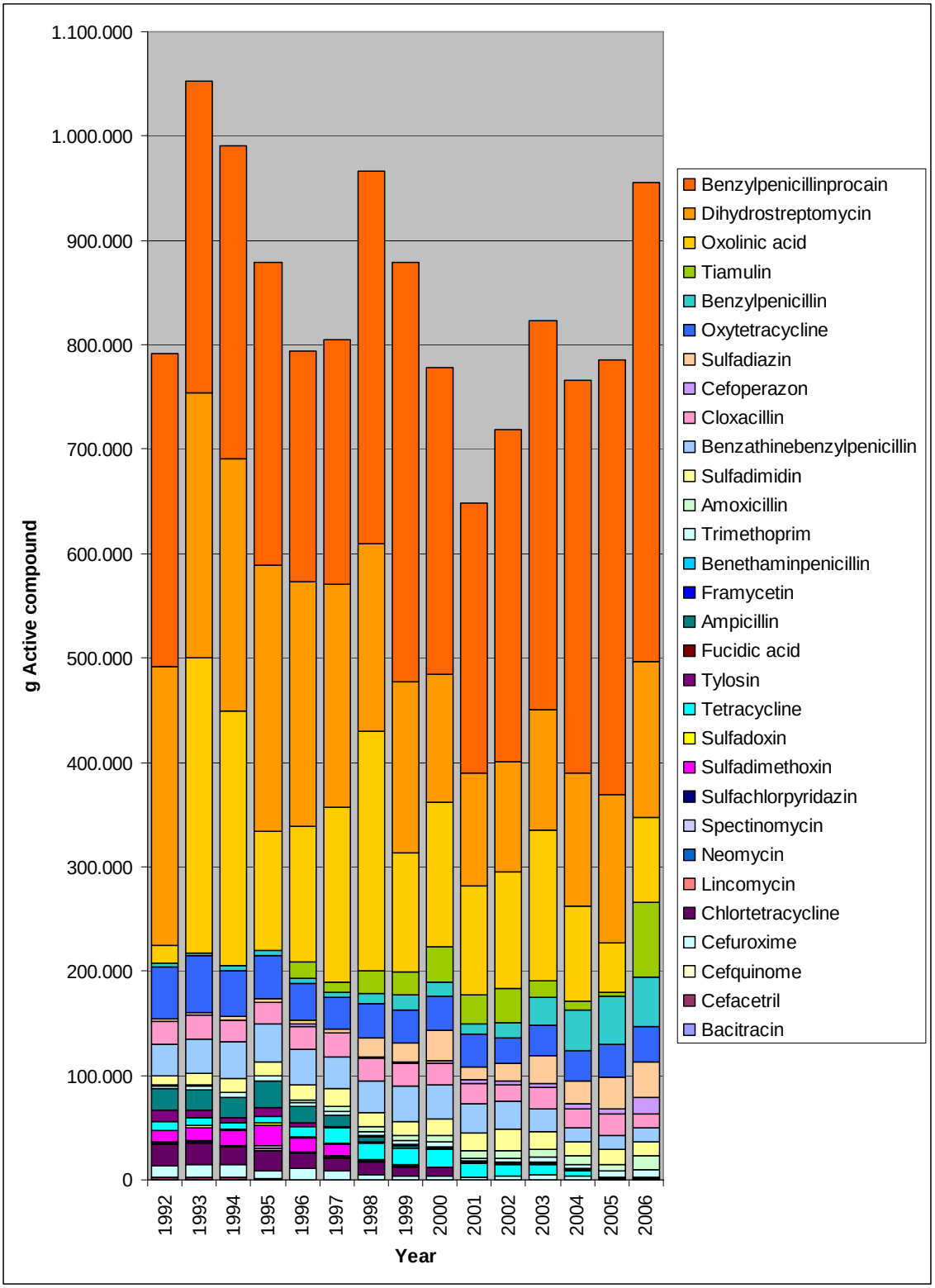

Figure 2: Antimicrobial use in veterinary medicine in Iceland 1992-2006 in grams of active substance. Reproduced from (79) with permission.

Mechanisms of antimicrobial resistance

Bacterial resistance to antimicrobials can be either intrinsic or acquired, that is to say it can be a characteristic (structural or functional trait) associated with an entire species or genus (intrinsic or natural resistance) or it may emerge in strains of a normally susceptible species through mutations or horizontal gene transfer (acquired resistance). The latter is considered to be a major threat as it can lead to therapeutic failure.

Furthermore, a third type of resistance has been described, the so called non-inherited antibiotic resistance, a purely phenotypic resistance type. Certain cells in a bacterial population, called persister cells, display tolerance to antimicrobial agents without expressing resistance mechanisms, they neither die nor grow in the presence of these agents (156, 157). This type is especially significant in the high tolerance of bacterial biofilms to antimicrobials (143, 232).

There are four main known resistance mechanisms (37, 112, 202): 1) Reduced influx of the antimicrobial agent. Mutation of genes regulating or coding for outer membrane porins (OMPs) can reduce or prevent the entry of antimicrobials into the bacterial cell. This generally leads to low level resistance but can also contribute, along with other resistance mechanisms, to a multiresistant phenotype. 2) Increased efflux of the antimicrobial agent. Bacterial efflux pumps transport various structurally unrelated compounds out of the cell in an energy dependent manner. Mutational changes can lead to hyperexpression of these pumps and increased efflux of antimicrobials out of the cell. This is commonly associated with tetracycline resistance and as a contributor to multiresistant phenotypes. 3) Inactivation of the antimicrobial agent. Bacteria can produce enzymes that modify the antimicrobial agent, rendering it unable to bind to its target and thus inactive. An example of this are the aminoglycoside-modifying enzymes and the β-lactamases. 4) Modification of the antimicrobial target. Bacteria can structurally modify or replace the target so that the antimicrobial can not exert its action. This is seen in methicillin resistant *Staphylococcus aureus* (MRSA), which produce a low-affinity penicillin-binding protein (PBPs).

Resistance phenotypes can result from a combination of resistance mechanisms, for exemple from an efflux pump and target modification, leading to higher resistance levels than if only one mechanism was at work. When resistance to two drugs, of the same or

different antimicrobial classes, is caused by a single mechanism it is defined as cross-resistance. Co-resistance, however, is defined as the coexistence of genes or mutations that confer resistance to different classes of drugs. Bacteria resistant to a certain antimicrobial can be selected for by exposure to another antimicrobial, as a consequence of cross-resistance or co-resistance (1). Multi-resistance, resistance to several different antimicrobials, can be caused by clusters of resistance determinants on plasmids or transposons.

Emergence and dissemination of bacterial resistance

Naturally resistant bacteria are thought to have been present long before the discovery of antimicrobial agents, as antimicrobial producing organisms protected themselfs with resistance determinants (37, 138, 203). Other possible origins of resistance determinants are various housekeeping genes (e.g. efflux pumps) and signaling molecules in soil communities (72, 92, 158, 169). Resistant bacteria have been found in culture collections collected prior to the widespread usage of antimicrobials (230), although the prevalence was very low, and resistant coliform bacteria, estimated to be around 2000 years old, were isolated from glaciers in Canada (66). The emergence of resistant bacteria was therefore not surprising. Even Alexander Fleming himself, in his Nobel Prize lecture, warned against the emergence of resistant bacteria as a consequence of the misuse or underuse of penicillin (100).

Bacteria can develop resistance to antimicrobials either by random chromosomal mutation or, most commonly, by acquisition of resistance determinants, such as plasmids, transposons, integrons and naked DNA (34, 180, 218, 241). Plasmids are extrachromosomal elements that can replicate and maintain themselves in a host cell and multiple plasmids can exist within a single bacterial cell. Plasmids can carry various genes, for exemple coding for antimicrobial resistance or virulence, and many plasmids carry systems that promote their transmission to other bacterial cells (conjugative plasmids). Transposons are elements that can translocate (excise and integrate themselves) between chromosomal and plasmid DNA, carrying with them additional genes, e.g. resistance genes. Gene cassettes are small mobile elements consisting of a specfic recombination site and a single gene, often a resistance gene. Integrons are

assemblies of multiple gene cassettes, which can reside on plasmids and transposons and be transferred between cells together with the plasmid, but can also be inserted into the chromosome by recombination. Integrons can not move by themselves, but rather they are in a sense a “gene-capturing” mechanisms, inserting gene cassettes into an insertion site using a site specific recombinase (integrase). Acquisition of resistance determinants occurs by horizontal transfer; by conjugation (plasmids), transduction (through bacteriophages) or by transformation (incorporation of naked DNA into the chromosome) (10, 37, 180, 218). This horizontal transfer is most common between bacteria of the same species, although evidence is increasing that this can occur between bacteria of different species and even of different genera (63, 84, 185, 228, 283). It has been shown that conjugative plasmids were common in enterobacteria of the “pre-antibiotic” era, although they rarely harboured antimicrobial resistance genes at that time (70, 128).

Resistance genes on a plasmid in one strain can transfer to another strain and from there be mobilized to another plasmid that is transferred to the third strain or another species. On the way they can collect and recombine different resistance gene cassettes, rendering the recipient resistant to multiple antimicrobials. These resistance elements can then evolve in the new host to reduce the cost they impose, thereby increasing its likelihood of survival and further dissemination (34, 241). In the presence of a certain antimicrobial, or other substances (e.g. heavy metals or disinfectants) that the plasmid harbours resistance for, all the resistance genes on these multi-resistant plasmids are co-selected for. Not only do antimicrobials select for those strains already resistant, by mutation or acquisition, additionally their presence in subinhibitory concentrations has been shown to increase mutation rates and induce horizontal transfer of resistance elements, by triggering stress responses (32, 147, 255). Multiresistance by itself has furthermore been suggested to increase the possibility of the transfer of resistance elements (242). Therefor, the use of antimicrobials, as growth promoters in particular, have caused concern over increased prevalence of resistant bacteria and their dissemination.

It has been shown that prevalence of antimicrobial resistance can be reversed by removing the selective pressure (4, 45, 225). However, in some cases the resistance can

be irreversable or the reversal process can be slow (4, 22, 39, 88). A possible reason for the persistence of resistance might be a co-selection of resistance traits or shared resistance mechanisms, linkage of resistance elements to genes conferring other advantageous traits, dissemination of plasmids containing resistance genes and on account of "plasmid addiction systems" (genes that confer apoptosis of daughter cells that fail to get a copy of the plasmid during division) (1, 4, 55, 132, 144, 154). Not only are resistance genes hard to lose, but with time bacteria aquire new resistance genes and they cluster together, thereby broadening the multiresistant phenotype and further reducing treatment options. Therefore is it essential to implement interventions to slow down the development of resistance early, before the prevalence of resistance rises.

Along with antimicrobial use, globalization has played its part in increased resistance. In the 20th century there were some substantial societal, technological and environmental changes. Urbanisation and increase in international travel and trade, for example with food products, have been a factor in the emergence and dissemination of antimicrobial resistance. Use of antimicrobials, overcrowding and lower hygiene such as at day care centers, intensive care units and animal houses increase the potential for resistance to emerge and disseminate. Soil-dwelling bacteria can be multiresistant and there appears to be a great density of resistance genes in the environment, possibly acting as a reservoir for human pathogens (73, 74). Furthermore, substantial amounts of antimicrobials fed to animals are not absorbed in the animal gut and therefore excreted in urine and feces and ultimately end up in manure, that is applied to agricultural land. These antimicrobials may stay active in the ground for some time and influence the selection of resistant bacteria in the terrestrial environment or be absorbed by plants and later ingested by humans (54, 83, 150). Therefore, antimicrobial resistance must be regarded as a broad ecological problem.

Burden of antimicrobial resistance

The number of infections caused by resistant pathogens continues to increase worldwide (82, 137, 152, 263). The health impact of these infections is diverse: Infection by an antimicrobial resistant strain can lead to a more severe form of the disease, complications and increased likelihood of hospitalization, possibly because of linkage

of resistance genes to virulence genes or other genes that confer further advantages (28, 167, 179, 183, 270, 276); Insufficient response to initial treatment (that is treatment with an inappropriate antimicrobial agent) can lead to longer duration of illness or the need to use less desirable treatment options (28, 71, 186); Antimicrobial use can decrease the gut commensal floras "colonizations resistance" and renders the patient to be increasingly vulnerable to infection by antimicrobial resistant intestinal pathogens unrelated to the original infection causing the antimicrobial use (often called the "attributable fraction"). That is, had the pathogen not been antimicrobial resistant the infection would not have occurred (28, 29, 107, 159). Not only do these infections cause suffering and death, they also entail a financial burden on both healthcare systems and on society in general, because of direct costs due to prolongation of illness and hospital treatment, indirect costs due to loss of productivity, and societal costs due to illness and mortality (35, 47, 61, 62). Likewise, antimicrobial resistance has implications for treatment efficacy in veterinary medicine, for animal welfare and production costs.

The emergence of extensively drug resistant (XDR, resistant to all but one or two antimicrobial classess) and pandrug resistant (PDR, resistant to all available antimicrobial classess) Gram negative bacteria in human infections has been linked to high mortality (231). Infections with these XDR and PDR bacteria has great public health implications as clinicians have no options for antimicrobial treatment. The uninhibited dissemination of these strains could mark the end of the antimicrobial era.

Antimicrobial resistance in production animals in relation to resistance in humans

Although most of the antimicrobial resistance problems in humans stem from overuse in human medicine, there is evidence that antimicrobial resistant enteric bacteria can transfer from animals to humans and thereby establishing a reservoir of resistance genes (13, 95, 181, 239). Transfer of resistant bacteria between animals and humans through food products has been documented and could pose a threat to public health (13, 181, 191). The transfer pathways are diverse and complex, bacteria can transfer from food animals to humans: by direct contact, where farmers, abattoir workers and veterinarians are especially at risk (9, 210, 272) and possibly carrying the bacteria between farms; through the food chain via meat, eggs, water and milk (119, 133, 155); through the

environment as animals produce large amounts of waste that can be used for manure and can contaminate water, soil and crops (211, 224, 287). Furthermore, the situation is made more complex by the transfer of bacteria: from humans to animals and the environment; from pets to humans and vice versa; from feeds to production animals; from environment to wildlife to production animals etc. (Figure 3). The biggest concern is over the rise and dissemination of resistant zoonotic bacteria (e.g. *Salmonella* spp or *Campylobacter* spp) and resistant commensal bacteria (e.g. *E. coli*) which can spread their resistance genes on to the human gut flora and possible human pathogens.

Salmonella

Salmonella spp. are members of the *Enterobacteriaceae*, facultatively anaerobic Gram negative straight rods. They are primarily inhabitants of the gastrointestinal tract and are found in a variety of animal hosts, such as mammals, reptiles and birds, ususally without the display of symptoms. Currently there are around 2400 recognized serotypes (33), but those most often associated with human infections are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (6). Food animals are the main reservoir for human infections, most often originating from meat products (beef, pork and chicken), dairy products and eggs (111). Salmonellas are now recognized as one of the most important causes of foodborne illness worldwide (174, 197), causing mainly gastroenteritis but also more severe extraintestinal diseases such as enteric fever, septicaemia and meningitis (6). *Salmonella* ssp. is the most reported causal agent of foodborne outbreaks in Europe, being responsible for 67.5% of reported outbreaks (268). WHO has estimated that annually there are 1,3 billion cases of acute gastroenteritis due to non-typhoidal salmonellosis in the world and nearly 3 million deaths (197).

The national incidence of human salmonellosis differs between countries, in USA the rate was 13.6 cases per 100,000 inhabitants in 2006 (52), in Denmark it was 31 cases per 100,000 in (69) and in France in 2000 it was 21.9 per 100,000 inhabitants (282). Incidence rates up to 476.2 cases per 100,000 inhabitants have been reported (278). *Salmonella* infections are not common in Iceland. Domestically acquired humans cases are few (16 cases or 5 per 100,000 in 2007) (81) and mainly sporadic, and so are infections in livestock and wild life (7, 122). No S*almonella* has been detected in animal

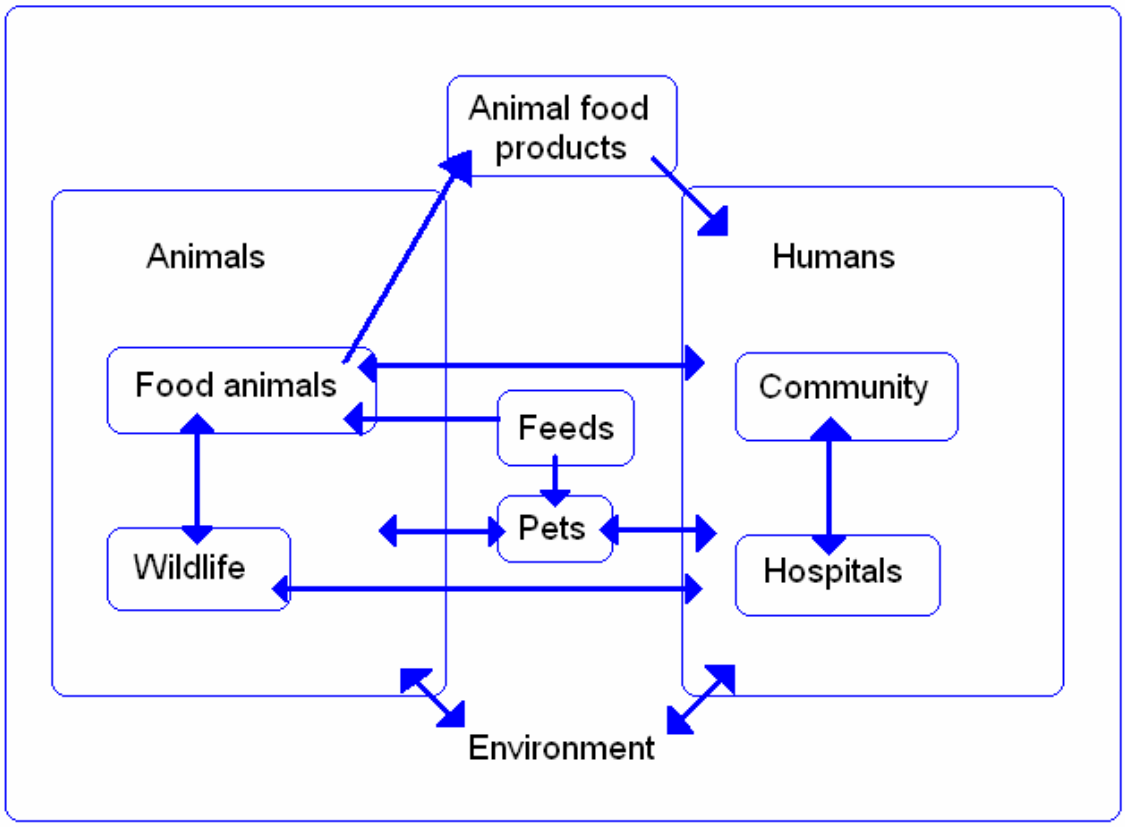

Figure 3. The diverse and complex transfer pathways of antimicrobial resistant bacteria and resistance genes. Modified from (2, 36, 37, 172)

food products in the last few years (261, 262) but there have been sporadic findings of *Salmonella* on imported vegetables and herbs (261). It must be noted that comparison of incidence rates between countries can be problematical as methodology of isolation of infectious agents and registration and reporting of infections varies among countries.

Although salmonellosis is usually self-limiting antimicrobials should be administered to those severely ill or immunocompromized. Ampicillin and trimethoprim-sulfamethoxazole were the drugs of choice but with the introduction of fluoroquinolones and third generation cephalosporins and later increased resistance use of the latter agents is becoming more common (123). Resistance to these newer drugs could have severe implications for human health. In recent years there has been an increasing concern worldwide for the rise of antimicrobial resistance of *Salmonella* spp. Resistant strains are an increasing proportion of all isolates from human infections (53) and many studies have shown that antimicrobial resistant *Salmonella* causing human infection often derive

from production animals (13, 95, 181). Resistance rates vary among countries. The prevalence of antimicrobial resistant nontyphoidal *Salmonella* isolates in domestic human cases in Iceland was around 15% in 2007 to 2008. In the USA in 2005 19% of human nontyphoidal *Salmonella* isolates were resistant to one or more antimicrobial agents (93). In Denmark over 47% of domestic *S*. Typhimurium human cases were resistant in 2005 (68). Between the years 2000 to 2004 resistance rates increased from 57% to 66% in ten European countries, as reported by the Enter-Net surveillance system (175). In all the examples above resistance to ampicillin, tetracyclin and sulfonamides was most common. Resistance to quinolones varies greatly between these countries, resistance to nalidixic acid was 2.4% in the USA (93), 4% in domestic *S*. Typhimurium cases in Denmark (68) and in the Enter-Net study resistance to nalidixic acid had increased from 14% to 21% during the study period (175). Some developing countries show resistance rates among *Salmonella* as high as 70% or more (21, 109). Resistance rates of *Salmonella* of animal origin are similar as in humans, 55.5% in pigs in the USA and 0% resistance to quinolones (93), just over 40% in pigs in Denmark and 1% resistance to quinolones (68), just over 54% in chicken in France and 0% resistance to quinolones (44) and 67% of isolates from poultry and swine in Thailand were reported resistant with around 1% resistance to quinolones (56). The resistance spectrum of *Salmonella* serovars has been expanding in the last decade (95, 104, 120). A plasmid mediated quinolone resistance determinant, *qnrS1*, was found in *S. enterica* strains in the UK (125). The *qnr* genes encode a protein that protects the DNA gyrase from inhibition by the quinolones and confers reduced susceptibility to fluoroquinolones (269). Horizontal gene transfer seems to be most important factor in the evolution of resistance among *Salmonella* as resistance genes are commonly carried on gene cassettes within integrons or on conjugative plasmids, possibly residing and transferring together in clusters (46, 65, 104).

S. Typhimurium DT104 is a known multiresistant phage type that emerged in the 1990s and quickly disseminated worldwide (120, 265). The DT104 clone has caused outbreaks of infection in food animals and humans in a number of countries in Europe and North America (49, 91, 106, 177, 204) and is commonly found in poultry, pigs, cattle and their food products (265). It has a broad host spectrum and a potential of spreading to large

numbers of domestic as well as wild animals and has been shown to spread from food animals to humans (177, 265). This phage type is characterized by resistance to five antimicrobials: ampicillin, chloramphenicol or florfenicol, streptomycin, sulfonamides, and tetracycline (AmpCm/FfSmSuTc) and the majority of DT104 isolates possess an identical chromosomal gene cluster that encodes the entire pentaresistance phenotype, regardless of source or countrie of origin (40, 216). In addition, it has been reported that DT104 isolates have acquired other resistances, such as to trimethoprim, nalidixic acid and ciprofloxacin (120, 177). Reports of quinolone resistant DT104 strains, originating from pigs, causing severe infections in humans resulting in therapeutic failure and deaths (177) are alarming. Fluoroquinolone resistant *Salmonella* strains first emergerd after fluoroquinolones were licensed for use in food animal production (267). The proportion of multiresistant *S.* Typhimurium isolates, such as DT104, can have a strong influence on the total rate of resistance (68) and when the occurrence of these multiresistant isolates declines in the population the overall resistance rates can decline considerably (175, 266).

Campylobacter

Campylobacter spp. are members of the *Campylobacteraceae* family. They are non-sporeforming, oxygen sensitive microaerophilic, Gram negative spirally curved rods. Campylobacters occus primarily in the gastrointestinal tract of humans and animals and are commonly found as commensals of dairy cattle, pigs, rodents, poultry and wild birds. *C. jejuni* and *C. coli* are the most important human pathogens and are a major cause of foodborne diarrhoeal illness worldwide (174, 275). Contaminated food is usually the source of human infections and raw poultry meat appears to be the main risk factor (12, 101, 114, 141).

In some countries, such as Iceland, *Campylobacter* is the most common gastrointestinal pathogen, more common than *Salmonella* (268). In the USA the incidence of *Campylobacter* infections in humans in 2004 was 12.8 cases per 100,000 inhabitants (51). The incidence in Denmark in 2006 was 60 cases per 100,000 inhabitants (69) and has been reported as high as 271.5 cases per 100,000 inhabitants in New Zealand in 2001 (278). Domestically acquired cases of human campylobacteriosis in Iceland are

relatively infrequent although they reached epidemic proportions in 1999, with 362 cases reported or 116 cases per 100,000 (238). With series of interventions in 2000, domestic cases of human campylobacteriosis dropped by 72% to 92 reported cases (33 per 100,000) in 2000 (238) and in 2007 they had dropped to 41 cases (14 per 100,000) (81). Prevalence of *Campylobacter* is relatively low in broiler flocks in Iceland (discussed later) and around 10-13% of broiler meat products are contaminated with *Campylobacter* (260, 262). As noted before comparison of incidence rates between countries can be problematic due to variation in methodology and reporting.

Most human *Campylobacter* infections are self-limiting though some prolonged or severe cases do require treatment with antimicrobial agents. Generally the drugs of choice are erythromycin for microbiologically diagnosed infections and fluoroquinolones for empirical treatment in adults (12, 182). However the increasing resistance in food animal related *Campylobacter* to fluoroquinolones is now considered an emerging public health problem and is thought to cause longer duration of illness and increased risk of adverse events (87, 121, 186). Of special concern is the use of fluoroquinolones in poultry production. It has been shown that fluoroquinolone treatment rapidly selects for high levels of ciprofloxacin resistance in *C. jejuni* (129, 171). Following the introduction of the fluoroquinolone enrofloxacin for veterinary use, especially in poultry, in the Netherlands the prevalence of resistant *Campylobacter* strains isolated from poultry products increased from 0% in 1982 to 14% in 1989 (86). During the same period the prevalence of resistant strains from human infections increased from 0% to 11% (86) and kept on increasing alongside resistance in broilers (256), and in 2004 the resistance rate had increased to 33.4% (274). Generally resistance rates are higher among isolates acquired abroad than in domestic isolates. In the Netherlands 33.4% of domestic *Campylobacter* isolates were resistant to fluoroquinolones while 54% of the travel related isolates were resistant to those agents (274). Similarly in Denmark in 2006, 22.7% of the domestically acquired *Campylobacter jejuni* isolates were resistant to fluoroquinolones while 54.2% of the travel related isolates were resistant to those agents (69). The prevalence of fluoroquinolone resistant *Campylobacter* in domestic infections in Iceland was 8% in 2007-2008 and 65% in infections acquired abroad. In Thailand 100% of human

Campylobacter jejuni isolates were reported resistant to at least one antimicrobial, thereof were 96% resistant to fluoroquinolones (38). In the same Thailand study 84% of poultry isolates were found to be resistant to one or more antimicrobials and of these 77% were resistant to fluoroquinolones. In Denmark resistance rates of *Campylobacter jejuni* isolates from broilers are around 10% and only 6.7% are resistant to fluoroquinolones (69). In the USA 15% of chicken isolates are resistant to fluoroquinolones whereas 21% of the human isolates are fluoroquinolone resistant (93). Quinolone resistance in *Campylobacter* isolates generally arises through chromosomal mutations and resistance to nalidixic acid and fluoroquinolones is often cross-resistant (108). Acquired quinolone resistance in *Campylobacter* is most often linked to a single point mutation in *gyr*A and the function of a constitutively expressed multidrug efflux pump, CmeABC (164, 288). The plasmid-borne *qnr* genes, conferring low level resistance to fluoroquinolones, have not been described in *Campylobacter*.

Escherichia coli

The Gram-negative rod-shaped *Escherichia coli* belongs to the *Enterobacteriaceae* family. *E. coli* is one of the best characterized microorganism. It is facultatively anaerobic and inhabits the gut of humans and other warm-blooded animals as part of the commensal microflora. It can, however, be an opportunistic pathogen causing diarrhea, gastroenteritis, sepsis and urinary tract infections (UTIs) in humans (6), avian colibacillosis, bovine mastitis and swine diarrhea (286). Certain serotypes have in particular been linked to foodborne illnesses, including the enterohaemorrhagic *E. coli* (EHEC) serotype O157:H7 and other Shiga toxin producing *E. coli* (STEC) (174, 245). Human foodborne infections are mainly associated with beef (mainly ground meat and hamburgers) , dairy products and drinking water (6, 111). Although it is considered an important foodborne pathogen the incidence of *E. coli* as such is a good deal lower than for *Salmonella* and *Campylobacter*, usually around 1 case per 100,000 inhabitant or lower for serogroups other than O157 (278). However, there are exceptions as in the Slovak Republic in 1998 where the incidence was 10.4 cases per 100,000 inhabitants (278). The incidence rate of serogroup O157 in the USA in 1999 was 2 cases per 100,000 inhabitants (252). An international outbreak of *E. coli* O157 occurred in

September-October 2007 in Iceland and the Netherlands and shredded, pre-packed lettuce from a Dutch food processing plant was identified as the most probable cause (102). STEC infections are rare in Iceland (<0.66 cases per 100,000 inhabitants) (102) and this was the first outbreak to be reported. The incidence of foodborne non-STEC *E. coli* infections was <0.01 cases per 100,000 inhabitants in Iceland in 2001 (278). In Iceland clinical *E. coli* is most frequently isolated from urine samples (137) and it is the most common cause of UTI (193).

A number of national and multi-national antimicrobial resistance surveillance systems have been initiated in the past years, both in human and veterinary medicine (69, 90, 93). Most of them incorporate *Escherichia coli* as it is a commonly found species in the commensal flora and as a pathogen in animals and humans. It is easy to cultivate and clear guidelines on susceptibility testing are available, making it a suitable bacterial species for determining resistance in commensals. Fecal contamination of carcasses during slaughtering is frequent and makes animal food product an important source of *E. coli*. Indications implicating food-products as a source for resistant *E. coli* infections in humans have been found (103, 133-135) and antimicrobial resistant *E. coli* strains are becoming more frequent among human clinical isolates and can lead to failure in treatment (90, 151, 152). Furthermore, *E. coli* has been shown to be an important source for resistance elements (290) which could spread to other bacteria and possible pathogens (283) .

Antimicrobial agents often used for treatment of human *E. coli* infections are trimethoprim-sulfamethoxazole (TMP-SMZ), quinolones or fluoroquinolones, and extended-spectrum cephalosporins. Increasing resistance to those agents is of concern. In a study in the USA 13.2% of human isolates were antimicrobial resistant, 8.9% were resistant to TMP-SMZ, 5.8% to quinolones/fluoroquinolones and 2.6% to extended-spectrum cephalosporins (136). In the same study 65% of poultry products were antimicrobial resistant, 26.7% to TMP-SMZ, 22.7% to quinolones/fluoroquinolones and 24.9% to extended-spectrum cephalosporins. In a NARMS study in 2005 82.3% of *E. coli* isolates from chickens were resistant to one or more antimicrobials, with only 7.5% resistant to nalidixic acid and 0.4% were resistant to ciprofloxacin (93). In Denmark resistance rates among *E. coli* from healthy humans and broilers were similar, around

21% in humans and around 17% in broilers (69). Much higher resistance rates have been reported, for example in Saudi Arabia where just over 99% of chicken isolates and over 71% of patient and chicken industry worker isolates were resistant to antimicrobials (11). EARSS reported that in 2006 52.3% of invasive *E. coli* isolates in Europe were resistant to one or more antimicrobials, and that resistance to the tested antimicrobials is increasing all over Europe, especially to fluoroquinolones, to which resistance increased significantly in 26 of the 28 participating countries (90). Iceland is one of those countries, with fluoroquinolone resistance among *E. coli* from UTI infections rising from 1% in 1999 to 9% in 2006 (137). Resistance to aminopenicillins was 45%, to aminoglycosides 7% and <1% to third generation cephalosporins (90). An earlier study on *E. coli* causing UTI in Icelandic women reported 35% resistance to ampicillin, 23% to cephalothin and 24% to sulfafurazol (193).

In recent years, the dissemination of fluoroquinolone resistant and extended-spectrum β-lactamase (ESBL) producing *E. coli* isolates has caused much concern. Quinolone resistance in *E. coli* most often arises by stepwise mutations in the DNA gyrase and topoisomerase IV genes and is not as easily induced as in *Campylobacter* (271). In addition, the plasmid mediated quinolone resistance genes (*qnr*) have been detected in *E. coli* isolates of both human and animal origin around the world, often in association with ESBL production (50, 165, 192, 217, 227). ESBLs are enzymes that confer resistance to expanded-spectrum cephalosporins and are plasmid or transposon mediated. ESBLs are classified into groups according to their amino acid sequences, and CTX-M, TEM and SHV are the most prevalent ESBLs (198). β-lactamases hydrolyse the β-lactam ring of β-lactam antibiotics. Many ESBLs can be inhibited by β-lactamase inhibitors such as clavulanic acid. The prevalence of ESBL producing isolates is increasing in Europe and they are spreading both clonally and by horizontal transfer of plasmids (59). ESBL strains also harbouring plasmids encoding other β-lactamases such as metallo-β-lactamases (MBL) and cephamycinases and resistance to fluoroquinolones, rendering them extensively drug resistant (XDR), are emerging and the dissemination of these clones is of high significance to human medicine (59, 149).

Poultry and swine production in Iceland

In 2006 there were 22 swine farms in Iceland. The average size of pig herds in Iceland is 250 piglets and the annual production is around 150 herds. At the time of slaughter pigs are around 22 weeks old. There were 31 broiler farms in Iceland in 2006. The average sizes of broiler flocks are 10,000 broilers and around 600 flocks are slaughtered annually. At the time of slaughter the broilers are about 34 days old. In both swine and broiler farming the "all-in all-out" principle of biosecurity is practiced, where the whole herd/flock is of the same age and is slaughtered at the same time. In between herds/flocks the houses are cleaned and disinfected

The Icelandic *Salmonella* Surveillance programme for pigs and poultry was initiated in 1995. Isolation of *Salmonella* is notifiable and appropriate measures are taken in order to trace the source of the infection and eliminate it. In poultry farming samples are taken both prior to and at slaughter (fecal and cecal samples, respectively), whereas in pig farming samples are taken at slaughter (swaber, meat juice and drainage samples) and at previously positive farms prior to slaughter (fecal). Infected chicken meat is withdrawn from the market and destroyed while infected swine meat can only be put on the market after heat treatment. Slaughterhouses and infected farms are put under restriction, cleaned and disinfected. Efforts to control *Salmonella* in broilers and pig production in Iceland have been very successful, especially in broilers where the prevalence decreased from 15% in 1993 to 0% in 2005 (7). In pigs the prevalence of *Salmonella* decreased from a maximum of 8.4% in 2001 to 1.1% in 2006 (detected by pooled swaber samples at slaughter) (7). Figure 4 shows the prevalence of *Salmonella* and *Campylobacter* in pigs (swaber samples) and broilers (fecal and cecal samples, respectively) in Iceland 2001-2006. These prevalence rates are rather low and comparable to the other Nordic countries, such as Denmark where the prevalence rates in broilers and pigs are 1.5% and 1.3%, respectively (15). Higher prevalence rates are seen elsewhere in Europe (70% in broilers in France (219) and 24.5% in pigs in the Netherlands (273)). No S*almonella* has been detected in poultry or pig related food products in the last few years (261). The most prevalent serotypes are similar as in Denmark (S. Infantis and *S.* Typhimurium), yet there is less serotype diversity in Iceland (15, 264).

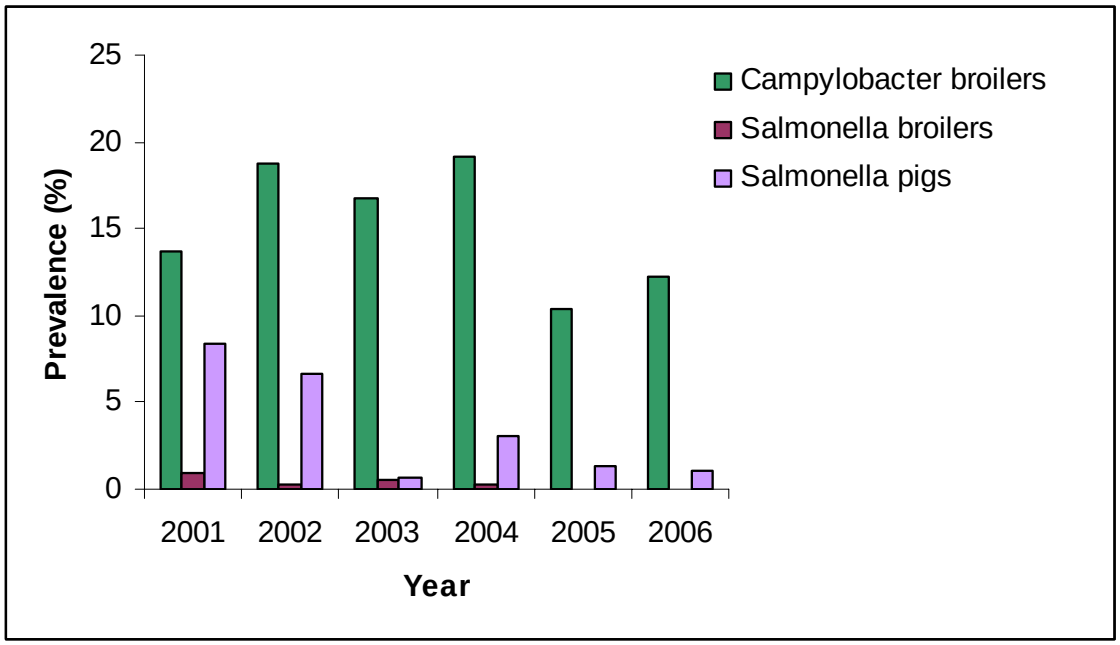

Figure 4: Prevalence of *Salmonella* and *Campylobacter* in pigs and broilers in Iceland 2001-2006 (adapted from (7))

The Icelandic *Campylobacter* Surveillance programme for poultry was initiated in 1995. Series of interventions were introduced in Iceland in 2000, focusing on reducing *Campylobacter* in poultry products and consequent human campylobacteriosis. These interventions included freezing of all *Campylobacter* positive flocks before going to retail and public education to increase consumer awareness. Consequently domestic cases of human campylobacteriosis dropped by 72% (as previously mentioned). Subsequently efforts have been made to control *Campylobacter* in poultry production with considerable success, maintaining the prevalence in broiler chicken at slaughter around 15% during the years 2001-2005 (Figure 4) (7). These prevalence rates are low compared to some European countries (42.7% in France) (213) or the USA (87.5%) (237) but comparable to Sweden (18%) (19) and somewhat higher than in Norway (3.6%) (18). The flock prevalence of *Campylobacter* has a seasonal distribution, peaking in the summer time. The incidence of *Campylobacter* contamination in poultry retail products decreased from 44% in 1999 to 5% in 2000 and has been ranging from 5-23% from 2001-2006 (260-262), which is comparable to the other Nordic countries (12% in Sweden and 19% in Denmark) (16, 19) and much lower than in the USA (70.7%) (289) and United Kingdom (UK) (60.9%) (160).

Antimicrobial susceptibility testing

Shortly after the discovery of the inhibitory affects of penicillin on *Staphylococcus aureus* early in the 20th century the first *in vitro* antimicrobial susceptibiliy tests (ASTs) were developed (99). With increasing demand of reproducibility and comparability of results these ASTs have evolved and methods have been harmonized and standardized (58, 170, 200). Antibiotic susceptibility is defined and usually measured by the concentration of the antimicrobial that it required to have an effect on the specific organism. The bacterium is considered susceptible if the agent restricts bacterial growth, and if it does not the bacteria is considered to be resistant. Minimum inhibitory concentration (MIC) is the lowest drug concentration which completely inhibits growth of the bacteria.

Susceptibility testing of bacterial isolates not only allows for discrimination between isolates, but also for evaluation of developing resistance. There are several methods for *in vitro* AST of bacteria, mostly based on diffusion or dilutions. The agar disk diffusion is the most commonly used method in veterinary medicine (41), mainly because of its fairly low cost and flexibility in the number and types of antimicrobials that can be tested. The test provides qualitative results, that is isolates are reported as susceptible (likelihood of therapeutic success), intermediate (indeterminate or uncertain therapeutic effect) or resistant (likelihood of therapeutic failure). A modification of the diffusion test is the concentration gradient strip (Etest®) that gives quantitative results as MIC values (microgram per milliliter). These tests are rather expensive and therefore not generally used in routine testing. Agar dilution, broth macrodilution and broth microdilution methods all give quantitative results. As both the agar dilution and broth macrodilution methods are generally considered laborious for routine clinical use the broth microdilution is becoming more widely used. Although the broth microdilution method is more expensive and less flexible than the disk diffusion test it gives quantitative results that allow statistical calculations and is therefore preferred in epidemiological and surveillance programs. Microdilution tests are available in a variety of commercial systems, either dehydrated or frozen in microtiter trays.

MIC values of an antimicrobial agent are used to determine breakpoints that predict the probability of clinical success (is the isolate susceptible, intermediate or resistant) or to

detect resistant populations and to follow the evolution of resistance patterns in the population. Breakpoints are defined based on the pharmacokinetic data on the relationship between the drug and specific species, as well as on the basis of a population analysis of the range of drug susceptibility values in the natural bacterial population. Clinical breakpoints are primarily aimed at predicting the outcomes of antimicrobial therapy in the patient, while epidemiological cutoff values (also known as microbiological breakpoints) are aimed at early detection of resistance and are useful in monitoring of the emergence of resistance (140).

Antimicrobial resistance monitoring

Among recommendations for intervention in the *WHO Global Strategy for Containment of Antimicrobial Resistance* is the establishment of national monitoring programmes for antimicrobial use and resistance in both veterinary and human medicine (281). Antimicrobial resistance monitoring programs are important for the control of resistance in humans and animals. Continuous monitoring and surveillance is the only way to efficiently evaluate the resistance problem and detect trends and changes. Monitoring programs identify the prevalence of resistance and resistance trends over time, they can determine the emergence of resistance, help to develop guidelines for the prudent use of antimicrobials and limit the emergence and spread of resistant organisms. If interventions are implemented monitoring programs can help to assess their effectiveness.

Although the importance of resistance monitoring is widely recognized only a few countries have established programs that involve monitoring of resistance among bacteria from animals, foods and humans. Monitoring programs vary in the methodology they use, if isolates come from clinical specimens or healthy individuals or if the program focuses on specific pathogens, infections or antimicrobials. All of the Nordic countries except Iceland have implemented resistance monitoring programs, DANMAP in Denmark, SWEDRES/SWARM in Sweden, NORM/NORM-VET in Norway and FINRES/FINRES-VET in Finland. All these programs include isolates from animals and humans and most also from foods. Other European countries such as Belgium, France, Hungary, Italy (ITAVARM), Spain (VAV), The Netherlands (MARAN) and UK have

active monitoring programs varying in their focus on animal and human pathogens and commensal bacteria. The NARMS program in USA monitor antimicrobial resistance in humans, animals and retail meats, as does the CIPARS program in Canada, while the Japanese program (JVARM) focuses on resistant bacteria from food-producing animals. Many international monitoring programs and studies have been undertaken. The European Antimicrobial Resistance Surveillance System (EARSS) collects information from 31 European countries on antimicrobial susceptibilities of certain human pathogens isolated from invasive infections and analyzes trends over time and between different countries and regions. The SENTRY antimicrobial surveillance program monitors the occurrence and antimicrobial susceptibility of bacterial pathogens causing nosocomial and community acquired infections internationally and furthermore, it explores pathogen resistance mechanisms. Other international programs such as MYSTIC and PROTEKT focus on the susceptibility of certain pathogens to certain antimicrobial agents or classes. Few international monitoring programs focus on resistance in animals and their food products. The WHO Global Salm-Surv (GSS) seeks to strengthen and enhance the ability of national and regional laboratories in the surveillance of *Salmonella*, the other major foodborne pathogens and the antimicrobial resistance in *Salmonella* and *Campylobacter* from humans, food and animals.

Molecular epidemiological analysis

Molecular epidemiology characterises bacterial classification on basis of natural genetic variation. It can provide information regarding the emergence and distribution of specific strains and is important for recognizing outbrakes of infections and determining the source of infection. Determining if phenotypically identical strains are genotypically distinct or clonal can furthermore help in deciding on methods of intervention. Genotyping offers great capacity for differentation of strains and can be helpful in making phylogenetic and epidemiologic inferences. Different genotyping methods have been developed for epidemiological typing of bacteria (DNA “fingerprinting”). All of these methods rely on the generation of a distinct pattern or DNA “fingerprint” that becomes visual by staining with ethidium bromide or by nucleic acid hybridization. When choosing a typing method one must evaluate its discriminatory power, taxanomic

range, its ease of use and interpretation, reproducability and cost. The ideal genotyping method produces results that are constant between laboratories and allows easy comparison.

Among the most commonly used methods are: 1) Pulsed Field Gel Electrophoresis (PFGE): Whole bacterial chromosomes are cleaved with "rare-cutting" restriction endonucleases into large fragments of DNA. The fragments are separated by agarose gel electrophoresis with variations in both the direction and duration of the electric field (194, 199, 257, 277). PFGE is considered by many to be the "gold standard" of genotyping methods (194, 214). It has proven to be highly effective for many different bacterial species, has a high discriminatory power and is easily reproducible. However, comparison of PFGE profiles between laboratories has proved to be difficult. 2) Random Amplified Polymorphic DNA (RAPD) analyses: Arbitrary developed primers are used to amplify random DNA products under low-stringency PCR conditions. The amplification products are separated by agarose gel electrophoresis (194, 199, 257, 277). The RAPD analyses is rapid, cost effective and has great discriminatory power but it has poor reproducability. 3) Amplified Fragment Length Polymorphism (AFLP): Selective primer amplification of a subset of DNA fragments generated by restriction enzyme digestion (194, 277). AFLP is highly reproducible and has good discriminatory powers, yet the method is complex and includes high initial costs 4) Multi Locus Sequence typing (MLST): Determines neutral genetic variation from multiple chromosomal locations by nucleotide sequencing (77). Easily comparable between laboratories. MLST is used for global epidemiology and generational (population) changes rather than short term changes. 5) Restriction Fragment Length Polymorphism (RFLP): Genomic DNA fragments produced by digestion with "frequent-cutting" restriction endonucleases are separeted by gel electrophoresis wich are then "blotted" onto membranes by Southern blotting (194, 199, 257). A moderatly discriminating method.

In the present study, PFGE was used for molecular epidemiological analyses as the method has been shown to be successful in subtyping of *Salmonella, Escherichia coli* and *Campylobacter* isolates (25, 60, 98, 142, 195, 246). RAPD analysis was furthermore used when isolates could not be typed by PFGE.

SUMMARY

Antimicrobial resistance is a growing problem in human as well as veterinary medicine. The use of antimicrobial agents, both in animals and humans, is a major factor in the selection and dissemination of resistant bacteria. Food producing animals can act as a reservoir of resistant bacteria and animal food products are a major source of food-borne infections in humans. Information on the use of antimicrobial agents in animal production in Iceland is limited although there should be no administration of these agents to broiler chicken. Antimicrobial resistance rates of *Campylobacter* spp., *Salmonella* spp. and *E. coli* from humans, production animals and food products are available for many countries. Antimicrobial resistance patterns can vary from one country to another and thus it is important to know the local resistance rates to ensure appropriate and efficient antimicrobial therapy, both in humans and animals. However, until now little has been known about the prevalence of antimicrobial resistant bacteria in animals in Iceland and there has been no research on antimicrobial resistance in production animals or animal food products.

AIMS

The main objective of this study was to determine the prevalence of antimicrobial resistant bacteria in production animals in Iceland and if these resistant bacteria were transferred from animals to humans, possibly through food.

The specific aims were to:

- Determine the overall prevalence of antimicrobial resistant *Salmonella* spp. and *Campylobacter* spp. isolated from broiler chicken and pigs during 2001-2005 in Iceland.

- Analyze the antimicrobial resistance patterns and serotype distribution of *Salmonella* spp. isolated from chickens and pigs.

- Determine the prevalence of antimicrobial resistant *E. coli* isolated from chickens and pigs, their food products, slaughterhouse personnel and outpatients.

- Analyze the genetic relatedness of antimicrobial resistant *Salmonella*, *Campylobacter* and *E. coli* strains.

- Evaluate if there could have been transfer of resistant strains from animals to humans through food.

MATERIALS AND METHODS

Sample collection

Salmonella

One isolate of each serotype from each and every *Salmonella* affected pig herd and broiler flock detected in the national *Salmonella* surveillance program in the years 2001-2005 was chosen for this study. The geographical distribution of the chicken and pig farms that participated in the surveillance programmes in the years 2001-2005 is shown in Figure 5. In 2001-2005 surveillance in pigs was conducted by collecting slaughterhouse drainage samples once a month from every farm slaughtering pigs intended for human consumption. If the drainage samples were positive fecal samples (5 x pooled samples consisting of 5gr) were collected once a month at the farm until the samples had been *Salmonella* negative for two consecutive months. Pigs from *Salmonella* infected farms were slaughtered at specific days of the week and surface swabs were taken from the carcasses (pooled samples consisting of 1-12 swabs). If swab samples were positive carcasses were discarded or put on the market after heat treatment. Appropriate measures were taken at *Salmonella* positive farms to eradicate the infection. For surveillance in broilers in 2001-2005 fecal samples were collected at the farms from all flocks intended for human consumption. Furthermore, neck skin samples were taken from all flocks at slaughter. If samples were *Salmonella* postive the flocks or carcassess were discarded and appropriate measures were taken at the farm and slaughterhouse to eradicate the infection.

Sample examination was carried out at two laboratories, the Institute for Experimental Pathology, University of Iceland, Keldur and the Sýni Laboratory Service, which kindly contributed their *Salmonella* isolates to this study. As samples were taken from each and every pig herd and broiler flock intended for human consumption we find that the sample material is representative for the overall situation in Iceland.

Campylobacter

One isolate from each and every *Campylobacter* affected broiler flock detected in the national *Campylobacter* surveillance programme in the years 2001-2005 was chosen for

this study. The geographical distribution of the chicken farms participating in the surveillance program in the years 2001-2005 is shown in Figure 5. For surveillance in broilers in 2001-2005 fecal samples were collected at the farms from all flocks intended for human consumption. Furthermore, cecal samples (2 x pooled samples consisting of 10 ceca) were taken from all flocks at slaughter. If fecal samples were *Campylobacter* postive the flocks were slaughtered at specific days of the week and the food products were frozen before going to retail. Appropriate measures were taken at the farm and slaughterhouse to eradicate the infection. Furthermore, in 2008 we decided to test 41 *Campylobacter* spp. isolates from broilers, isolated in the National *Campylobacter* surveillance program in the years 2006-2007 for susceptibility to nalidixic acid and ciprofloxacin, to see if resistance prevalence in *Campylobacter* had changed.

As samples were taken from each and every broiler flock intended for human consumption we find that the sample material is representative for the overall situation in Iceland.

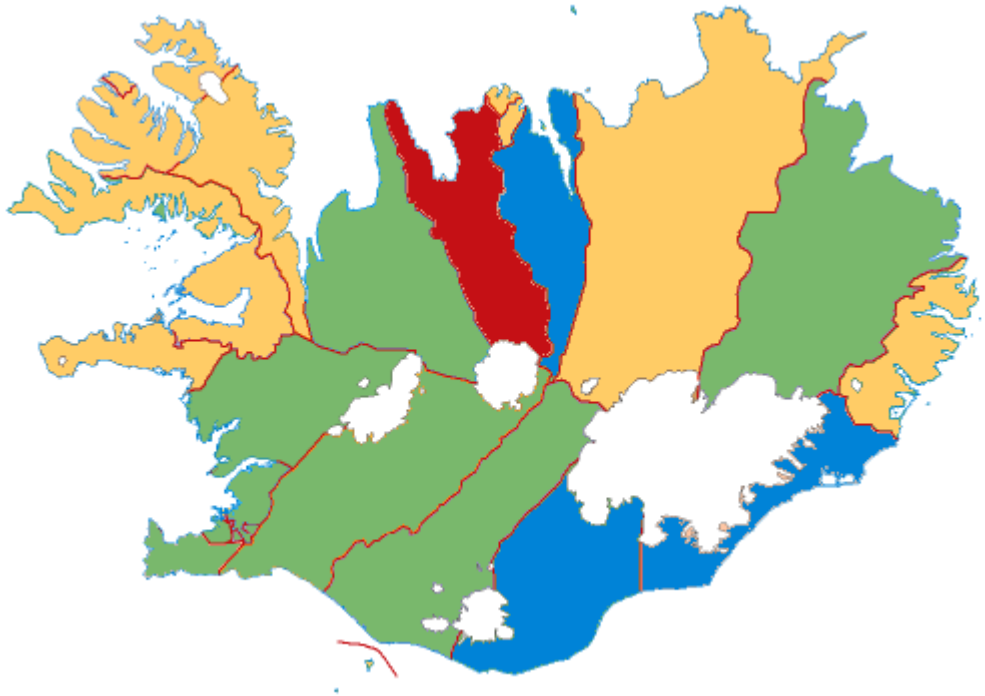

Figure 5: Geographical distribution of chicken and pig farms participating in the Icelandic *Salmonella* and *Campylobacter* surveillance programmes in the years 2001-2005. Blue indicates the counties of participating pig farms, red indicates the counties of participating poultry farms and green indicates counties with both pig and poultry farms participating in the study.

E. coli

Isolates were collected from broilers and pigs in October 2005 to March 2006. To get a representative sample the number of samples collected from each farm was proportional to the number of animals slaughtered per year. Four geographically separated pig slaughterhouses participated in the collection of samples. Samples were taken from 11 pig herds from nine pig farms, accounting for approximately 85% of the total annual volume of pigs slaughtered in Iceland. From each herd, two to ten samples were taken, depending on the size of the herd. Three geographically separated poultry slaughterhouses participated in the collection of samples. Samples were taken from broiler flocks from eight farms producing 20,000 birds or more annually, accounting for approximately 65% of the total annual volume of broilers slaughtered in Iceland. Samples were taken from at least five flocks from the same farm and one sample was taken from each flock. Samples were pooled and consisted of ceca from 20 broilers. Ceca from healthy animals (pigs and broilers) were sampled aseptically at the slaughterhouses by the meat inspection veterinarian.

Upon finding antimicrobial resistant bacteria in animals, further sampling was done from foods and humans. Sampling from these sources was carried out in October 2006 to May 2007. Foods samples were collected from November 2006 to May 2007. Pork and broiler meat was sampled from meat processing plants, prior to packaging, by the meat inspection veterinarian. Pork samples were sampled from two meat processing plants, accounting for approximately 70% of the annual pork production in Iceland. During the sampling period herds from four farms were processed at the two plants. From each herd there were taken five to ten samples, depending on the size of the herd. All three poultry meat processing plants participated in the broiler meat sample collection. During the sampling period flocks from 17 farms were processed at the three plants. From each broiler flock one sample was taken. Samples from humans were collected during October 2006 to January 2007. Isolates were cultured from routine diagnostic fecal specimens of outpatients presented with suspected diarrheal disease submitted to the Department of Clinical Microbiology, Landspitali University Hospital. Specimens from persons taking antimicrobials at the time of collection or recently traveling abroad or from non-Icelandic citizens were excluded. Fecal samples from

healthy slaughterhouse personnel were obtained with a rectal swab and were transmitted to the laboratory in charcoal transport medium. Condition for the participation of slaughterhouse personnel was that they had to have lived in Iceland for at least 3 months and had not taken any antimicrobials for three months prior to sample collection. Slaughterhouse personnel were considered humans with contact to food producing animals and outpatients were considered as humans without any known contact to food producing animals. The study was approved by the National Bioethics Committee.

Moderate to high prevalence of quinolone resistance among *E. coli* isolates from broilers and broiler meat led to further sampling of broilers and broiler feed during the summer of 2008. *E. coli* was isolated from 30 cecal and 20 feed samples. Cecal samples were taken as before from all broiler flocks slaughtered during the sampling period. Feed samples were taken from feed dispensers within the broiler houses at 18 farms (14 from which ceca or meat had previously been sampled) and from the two feed mills operating in Iceland. One sample of a probiotic feed additive was collected from one of the poultry producers.

To evaluate if resistant broiler *E. coli* isolates were genetically related to ciprofloxacin resistant human clinical isolates we selected 34 ciprofloxacin resistant human *E. coli* isolates, isolated from routine clinical specimens at the Landspitali University Hospital during the years 2006-2007 for the study. The human isolates were selected if they had resistance patterns prevalent in the 2005-2007 study (ampicillin, tetracycline, sulfamethoxazole/trimethoprim, ciprofloxacin or ciprofloxacin alone) and only one isolate was chosen from each patient.

Bacterial isolation and identification

Salmonella

Fecal, neck skin and drainage samples are all examined according to the NMKL method No. 71 (5th edition) (187). Swaber samples are examined using TECRA™ Unique test (127) and positive sampels are further examined according to the NMKL method. All *Salmonella* isolates are sent to the Department of Clinical Microbiology, Landspitali University Hospital for confirmation and serotyping and isolates are stored in Brain Heart Infusion (BHI) broth with 20% glycerol at -75°C.

All resistant *Salmonella* Typhimurium strains were sent to the Danish Institute for Food and Veterinary Research, Department of Microbiology and Risk Assessment for definitive phage typing with the Colindale system, M03-03-003.

Campylobacter

Samples for *Campylobacter* identification are examined according to the Campy-Cefex method (235, 236) and isolates are identified to species level on the basis of colony morphology, microscopic appearance (including motility), oxidase, latex agglutination (PanBio, Inc., Baltimore, MD; Latex-Campy), indoxyl acetate hydrolysis and hippurate hydrolysis. Isolates are stored in Brain Heart Infusion (BHI) broth with 20% glycerol at -75°C. All *C. jejuni* and *C. coli* strains were tested for antimicrobial resistance.

E. coli

Pigs and broilers: Ceca (1 g) were suspended in 9 mL Phosphate Buffered Saline (PBS) and thoroughly mixed. Serial dilutions were made and 100 µL of the dilutions were spread on MacConkey agar and MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated overnight at 37°C.

Foods: Meat (25 g) was suspended in 225 mL of Brain Heart Infusion (BHI) broth and thoroughly mixed in a BagMixer laboratory blender (Interscience, St Nom La Breteche, France). Of this suspension 100 mL were incubated overnight at 37°C where after 10 µL were spread on MacConkey agar and 100 µL were spread on MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated again overnight at 37°C.

Humans: Fecal samples from humans were directly spread on MacConkey agar and MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated overnight at 37°C.

Feed: Feed (10 g) was suspended in 90 mL of Buffered Peptone water, thoroughly mixed and incubated overnight at 37°C where after 10 µL were spread on MacConkey agar and 100 µL were spread on MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated again overnight at 37°C.

From all samples of every origin one colony from each agar plate (MacConkey and MacConkey + enrofloxacin) was selected for susceptibility testing. Colonies that were picked had to be lactose fermenting with morphology typical for *E. coli* on sheep-blood

agar (5% v/v), Brilliant Green agar (BG), SSI enteric medium and Triple Sugar Iron agar (TSI) and positive for the production of tryptophanase (indole).

Susceptibility testing

Antimicrobial susceptibility testing was performed using a microbroth dilution method (VetMIC^{TM}, SVA, Sweden) according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) Document M31-A2. Minimum Inhibitory Concentrations (MIC) for the following antimicrobials were determined:

Salmonella; ampicillin (Amp), ceftiofur (Ce), chloramphenicol (Cm), enrofloxacin (Ef), florfenicol (Ff), gentamicin (Gm), nalidixic acid (Nal), neomycin (Nm), streptomycin (Sm), sulphamethoxazole (Su), oxytetracyclin (Te) and trimethoprim (Tm)[1]. For quality control the *E. coli* ATCC 25922 reference strain was tested concurrently.

Campylobacter; ampicillin (Amp), enrofloxacin (Ef), erythromycin (Em), gentamicin (Gm), nalidixic acid (Nal) and oxytetracyclin (Tc). Quinolone susceptibility testing of *Campylobacter* in the summer of 2008 was performed using E-test strips for nalidixic acid and ciprofloxacin, as instructed by the manufacturer (AB Biodisk, Solna, Sweden). For quality control the *C. jejuni* ATCC 33560 reference strain was concurrently tested.

E. coli; ampicillin (Amp), cefotaxime (Ctx), ceftiofur (Ce), chloramphenicol (Cm), ciprofloxacin (Ci), florfenicol (Ff), gentamicin (Gm), nalidixic acid (Nal), kanamycin (Km), streptomycin (Sm), sulphamethoxazole (Su), tetracycline (Tc) and trimethoprim (Tm). Furthermore, MIC for enrofloxacin (Ef) was determined using an agar dilution method according to NCCLS Document M31-A2. For quality control the *E. coli* ATCC 25922 reference strain was tested concurrently.

The cut-off values were the same as those used in the Swedish monitoring program (249, 251). Strains showing resistance to one or more antimicrobial agents were considered resistant and strains resistant to three or more agents were considered multiresistant.

[1] Due to change of VetMIC panel design in the spring 2006 *Salmonella* strains from Sýni ehf were tested for ciprofloxacin (Ci) instead of enrofloxacin, kanamycin (Km) instead of neomycin and tetracyclin (Tc) instead of oxytetracyclin.

Molecular Typing

Pulsed Field Gel Electrophoresis (PFGE)

Resistant *Salmonella* and *E. coli* isolates were analyzed with Pulsed Field Gel Electrophoresis as previously described by Ribot et al. (214) with the following modifications. The DNA-agarose plugs were lysed overnight at 54° C. *Salmonella* plugs were digested with both 25 U/plug *Xba*I and 15 U/plug *Avr*II restriction enzymes overnight at 37° C and *E. coli* plugs with 25 U/plug *Xba*I restriction enzyme overnight at 37°C. Resistant *Campylobacter* isolates were analysed with Pulsed Field Gel Electrophoresis as described by Ribot et al. (215). The DNA-agarose plugs were digested with both *Kpn*I (40U) and *Sma*I (20U) restriction enzymes.

Restricted DNA fragments were separated by PFGE using the CHEF-DR II system (Biorad laboratories, Mississauga, Ontario, Canada). A lambda ladder PFGE marker (New England Biolabs, inc.) was used as a molecular weight standard. The gels were stained with ethidium bromide and photographed under UV transillumination with ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and photos were saved as TIFF files. Analysis of DNA fragmentation profiles was performed by visual inspection and for interpretation the criteria suggested by Tenover *et al*. (258) were used. Macrorestriction patterns were compared with the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and cluster analysis using the Dice coefficient for band matching, with band-position tolerance of 1.5%, were used to generate a dendrogram with the unweighted pair-group method with arithmetic averages (UPGMA). Clusters were defined as ≥3 isolates with ≥ 80% similarity.

E. coli isolates yielding un-interpretable PFGE patterns, due to DNA degradation during the electrophoresis (leaving no bands), were analyzed again, first using *Avr*II restriction enzyme instead of *Xba*I, and if that failed with HEPES buffer as previously described (148).

Randomly amplified polymorphic DNA (RAPD)

E. coli isolates, that did not yield a satisfactory banding pattern by PFGE (using *Xba*I, *Avr*II and HEPES buffer), were genotyped by Randomly amplified polymorphic DNA (RAPD) analysis using two decamer primers, primer 1254 (5´-CCGCAGCCAA-3´) and

primer 1290 (5´-GTGGATGCGA-3´), as previously described (196). Patterns were considered to be different when the profiles differed by at least one band. Reaction products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and gels were photographed under UV light. Similarity among RAPD patterns was compared with the BioNumerics software (Applied Maths) by using Dice similarity coefficient for band matching and band position tolerance of 1.5%, and a dendrogram was constructed by using UPGMA. Clusters were defined as ≥3 isolates with ≥ 80% similarity of composite RAPD profiles.

Antimicrobial residue screening test

Between April and August 2008 slaughterhouse veterinarians sampled broiler chicken at slaughter and farmers collected 5-15 day old broilers at the farm along with feed samples from feed stalls. Samples were taken at all three broiler slaughterhouses in Iceland and from the ten farms with the highest prevalence of resistance in the previous study. One broiler per slaughter flock and three young broilers per flock were sampled for the study. One broiler from slaughter, three young broilers and one feed sample were collected from each farm, in total ten broilers from slaughter, 30 young broilers and ten feed samples. Meat and kidneys from broilers at slaughter, meat from young broilers and feed (slightly condensed with sterile distilled water) were tested for antimicrobial residues with an agar diffusions test (96). Samples giving a questionable reaction were repeated.

Statistical analysis

For *Campylobacter*, frequencies of resistances were tested between isolates from various years using Chi-squared test for trends (R x C contingency table (χ^2 test)), using InStat from GraphPad Software. With *E. coli*, frequencies of resistances from various years were tested using Fisher´s Exact test for 2 x 2 contingency tables using InStat from GraphPad Software.

Ethical considerations

Sampling from slaughterhouse personnel and outpatients was approved by the National Bioethics Committee. No personal data was collected on individuals participating in the study.

RESULTS

Salmonella (Paper I)

A total of 163 isolates of *Salmonella* spp. isolated in the years 2001-2005 were tested for antimicrobial resistance. One hundred and forty one isolates were of pig origin and 22 of chicken origin. Six strains isolated in the national *Salmonella* surveillance programme were omitted as information on the sample origin and confirmation of *Salmonella* spp. and serotyping could not be obtained.

Salmonella serovars

Salmonella enterica serovar Infantis was predominant in both pigs and chickens as 89 pig strains (63%) and 11 chicken strains (50%) were typed as *S.* Infantis. Moreover *Salmonella* serovars Typhimurium (14%), Worthington (23%), Java, Mbandaka and Newport (4.5% each) were isolated in chickens and serovars Typhimurium (36%) and Worthington (1 %) were found in pigs in addition to Infantis.

Antimicrobial susceptibility

Of the 163 isolates tested 21 (12.8%) were resistant. The overall prevalence of resistance was 13.6% in chickens and 12.8% in pigs. All isolates of serovars *S.* Newport and *S.* Java were sensitive to all the antimicrobials tested. Furthermore only one isolate of serovar *S.* Infantis (isolated from pigs) and one of serovar *S.* Worthington (isolated from chickens) were resistant to antimicrobials. On the other hand we found that 19 isolates (35.2%) of serovar *S.* Typhimurium were resistant to antimicrobials, of which 17 were of pig and two of chicken origin, see Table 2. Sixteen (29.6%) of the resistant Typhimurium strains were multiresistant.

PFGE patterns and definitive phage typing of resistant Salmonella

Macrorestriction with *Xba*I and *Avr*II yielded seven different patterns of 11-15 fragments and six patterns of six to ten fragments respectively. Table 3 shows the characteristics of the 19 antimicrobial resistant *S.* Typhimurium strains. No

Table 2: Antimicrobial resistance in *Salmonella* Typhimurium (n=54) isolated from pigs (n=51) and chickens (n=3)in Iceland in the years 2001-2005 (concentration range tested and cut-off values)

Antimicrobial agent	Range tested (mg/L)	Cut-off values[a]	Resistant strains (%)		
			Total	Swine	Chickens
Ampicillin	0.25-32	>8	15 (27.8)	13 (25.5)	2 (66.7)
Ceftiofur	0.12-16	>2	0 (0)	0 (0)	0 (0)
Chloramphenicol	1-128	>16	15 (27.8)	13 (25.5)	2 (66.7)
Enrofloxacin (n=37)	0.03-4	>0.25	1 (2.7)	1 (2.0)	0 (0)
Ciprofloxacin (n=17)	0.008-1	>0.06	0 (0)	0 (0)	0 (0)
Florfenicol	4-32	>16	15 (27.8)	13 (25.5)	2 (66.7)
Gentamicin	0.5-64	>8	0 (0)	0 (0)	0 (0)
Nalidixic acid	1-128	>16	0 (0)	0 (0)	0 (0)
Neomycin/Kanamycin	2-16	>8	0 (0)	0 (0)	0 (0)
Streptomycin	2-256	>32	17 (31.5)	15 (29.4)	2 (66.7)
Sulphamethoxazole	16-2048	>256	16 (29.6)	14 (27.5)	2 (66.7)
Oxytetracyclin/Tetracyclin	0.5-64	>8	16 (29.6)	14 (27.5)	2 (66.7)
Trimethoprim	0.25-32	>8	0 (0)	0 (0)	0 (0)

a: Microbiological cut-off values defining resistance

Table 3: Characteristics of 19 resistant *S.* Typhimurium strains, isolated from chickens and pigs in Iceland 2001-2005, typed by PFGE, bacteriophages (DT) and antimicrobial susceptibilities

Year of isolation	Origin	Farm	Antimicrobial resistance pattern	DT[a]	PFGE profile	
					*Xba*I	*Avr*II
2001	Pig	P1	Sm	3	X1	A4
	Pig	P3	Sm	NT	X7	A1
	Pig	P1	Ef,Te,Su	208	X2	A5
2003	Pig	P1,2,4	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P2	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A2
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Sm	NT	X3	A6
2004	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X4	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X4	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Chicken	C1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Chicken	C1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X6	A3
2005	Pig	P1	Amp,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3

a NT= Non Typeable.

antimicrobial resistant *S.* Typhimurium strains were isolated in the year 2002. Combining the results from both *Xba*I and *Avr*II macrorestrictions gave a dendrogram consisting of eight patterns in three clusters (Fig. 6). The first cluster consisted of three profiles (X1A4, X2A5 and X3A6) from three pig isolates and accounted for 16% of the strains. The second cluster accounted for 79% of the strains and consisted of four profiles (X4A3, X5A3, X6A3 and X5A2, two from chickens and 13 from pigs). X5A3 was the predominant genotype accounting for 58% of all strains. All isolates in this cluster were typed as DT104. One profile (X7A1) comprised the third cluster containing one isolate from a pig. The overall similarity for the three clusters was 82% while the similarity between profiles in cluster two was 94%.

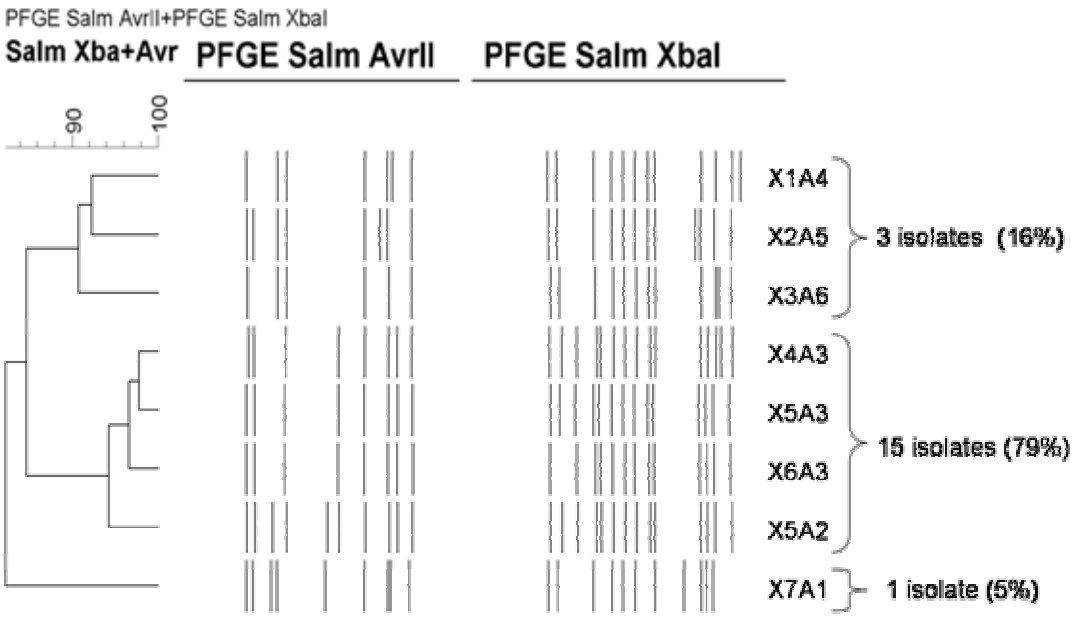

Figure 6: Dendrogram showing the UPGMA cluster analysis of the different PFGE patterns, determined by *Xba*I and *Avr*II digestion, from 19 resistant isolates of *S.* Typhimurium isolated from chickens and pigs in Iceland in 2001-2005.

Campylobacter (Paper II and unpublished)

A total of 390 *Campylobacter* isolates from healthy broiler chickens isolated from slaughter samples in 2001-2005 and 41 isolates from 2006-2007 were included in the study. Fifteen of the 2001-2005 isolates were non-viable after storage at -75° C and 16

of the 2006-2007 isolates. The species distribution of recovered isolates from 2001-2005 was *C. jejuni* 93.0% (349 isolates), *C. coli* 3.5% (13 isolates) and *C. lari* 3.5% (13 isolates). Only *C. jejuni* and *C. coli* isolates were tested for antimicrobial susceptibility. Isolates from 2006-2007 were not indentified to species level as it was assumed that the majority would be *C. jejuni* and *C. coli*.

Antimicrobial susceptibility

Of the 362 *C. jejuni* and *C. coli* isolates tested for antimicrobial susceptibility 337 (93.1%) were susceptible to all the antimicrobials tested and none were multi-resistant. The most commonly observed resistance was to Ampicillin accounting for 3.6% of all isolates or 52% of resistant isolates (Table 4). Resistance patterns differed between *Campylobacter* species. Of 349 *C. jejuni* strains tested 18 (5.2%) were resistant to one or more antibiotics. Resistance to ampicillin was found in 13 (3.7%) strains, one (0.3%) was resistant to oxytetracyclin and four (1.1%) were resistant to enrofloxacin, of which three (0.9%) were also resistant to nalidixic acid. Of the 13 *C. coli* isolates seven (53.8%) were resistant to one or more antibiotics. All seven were resistant to enrofloxacin and four (30.8%) were also resistant to nalidixic acid.

Occurrence of resistance increased from the year 2001 to 2005 (Figure 7) but the difference is not statistically significant, although there is a significant (p=0.0492) linear trend between the years. The 25 viable isolates tested in the 2008 study were fully susceptible to both ciprofloxacin and nalidixic acid.

Table 4: Antimicrobial resistance in thermophilic *Campylobacter* spp. isolated from broilers in Iceland in the years 2001-2005 (n=362) (concentration range tested and cut-off values)

Antimicrobial agent	**Range tested (mg/L)**	**Cut-off values[a]**	**Nr. of resistant strains (%)**		
			All	***C. jejuni***	***C. coli***
Ampicillin	0.5-64	>16	13(3.6)	13(3.7)	0(0)
Erythromycin	0.12-16	>16	0(0)	0(0)	0(0)
Enrofloxacin	0.03-4	>0.5	11(3.0)	4(1.1)	7(53.8)
Gentamicin	0.25-8	>8	0(0)	0(0)	0(0)
Nalidixic acid	1-128	>16	7(1.9)	3(0.9)	4(30.8)
Oxytetracyclin	0.25-32	>8	1(0.3)	1(0.3)	0(0)

a: Microbiological cut-off values defining resistance

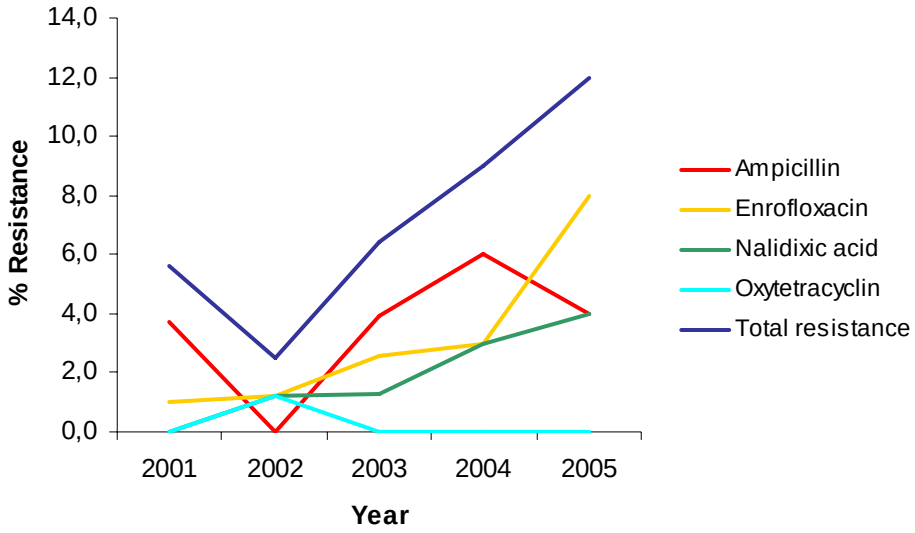

Figure 7: Trends in resistance to the tested antimicrobials among *Campylobacter* isolates isolated from broilers in Iceland 2001-2005.

Pulsed Field Gel Electrophoresis

Macrorestriction of resistant strains with *Sma*I yielded 12 different patterns of four to 14 fragments and with *Kpn*I yielded 13 different patterns of seven to 14 fragments, respectively. With both enzymes 13 pulsed field profiles were obtained from the 25 antibiotic resistant strains, none of wich indicated a predominant genotype (Figure 8). Pulsotypes S11K11 and S12K12 consisted of *C. coli* isolates and the remaining pulsotypes consisted of *C. jejuni* isolates. Although the two *C. coli* PFGE profiles form a cluster with >80% similarity they are only considered possibly related as the patterns differ by four bands after *Kpn*I digestion. Two groups of isolates, PFGE profiles S1K1 and S1K2, with a one band difference between them form a clonal group with >95% similarity and are considered closely related. This clonal group includes four strains, all from the same farm, with strains in the S1K1 profile isolated in 2001 and strains in the S1K2 profile isolated in 2004.

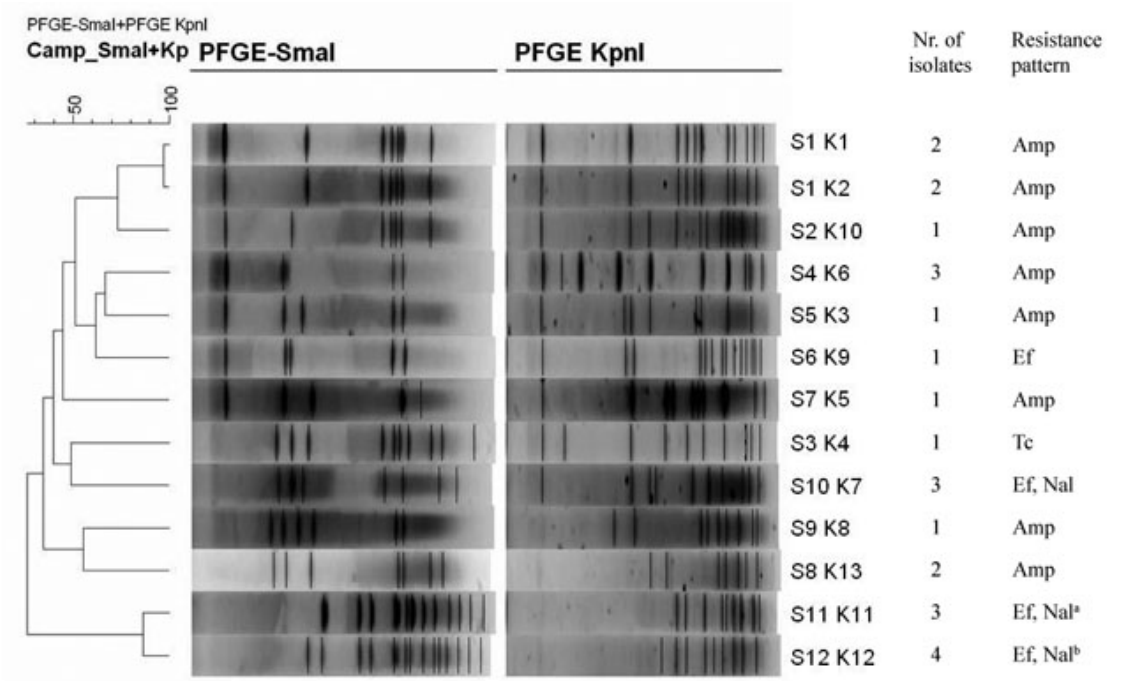

Figure 8: Dendrogram showing the UPGMA cluster analysis of the different PFGE patterns, determined by *Sma*I and *Kpn*I digestion of DNA from *Campylobacter* isolates from broilers in Iceland 2001-2005. [a] = One isolate was resistant only to enrofloxacin. [b] = Two isolates were resistant only to enrofloxacin.

E. coli (Papers III, IV and unpublished)

Sampling

In total 449 samples were taken; 126 broiler samples (96 in 2005-2006 and 30 in 2008), 99 from pigs, 50 broiler meat samples, 60 pork samples, 28 from slaughterhouse personnel, 65 outpatient samples, 20 feed samples and one sample of a probiotic feed additive. Table 5 shows the number of samples taken and the percent of samples positive for *E. coli* (rate of recovery), the number of isolates obtained per origin and the prevalence of resistant isolates and of multiresistant isolates.

Antimicrobial susceptibility

Of the 482 isolates, of all origins, analyzed 197 (41%) were resistant to one or more antimicrobial agents. Prevalence of resistance was highest in pig ceca (54.1%) and lowest in outpatients (23.1%). The percentage of isolates resistant to each antimicrobial agent is reported in Table 6.

Table 5: Numbers of animal, meat, feed and human samples and of *E. coli* isolates available for MIC testing and number of resistant isolates and multiresistant isolates.

Origin		No. of samples (% recovery)	No. of isolates	No. resistant isolates (%)	No. multiresistant isolates (%)
Pig	**Ceca**	99 (97)	109	59 (54.1)	24 (22.0)
	Meat	60 (73)	50	14 (28.0)	3 (6.0)
Broiler	**Ceca 2005-2006**	96 (100)	110	37 (33.6)	16 (14.5)
	Meat	50 (100)	75	39 (52.0)	11 (14.6)
	Ceca 2008	30 (100)	40	20 (50)	1 (2.5)
Feed	**Broiler feed**	20 (75)	22	7 (31.8)	0 (0)
	Probiotic additive	1 (0)	1	0 (0)	0 (0)
Human	**Slaughterhouse personnel**	28 (75)	23	9 (39.1)	4 (17.4)
	Outpatients	65 (80)	52	12 (23.1)	5 (9.6)

Pigs. Of the total 109 pig ceca isolates, 59 (54.1%) were resistant to one or more antimicrobial agents and 24 (22.0%) of them were multiresistant. Resistance patterns were diverse although resistance to tetracycline was the most prevalent pattern (12 isolates). Lower prevalence was noted among pig meat isolates, where 14 (28.0%) isolates were resistant to one or more antimicrobial agents and three (6%) of them were multiresistant. No prevailing resistance pattern was detected among pig meat isolates.

Broilers. Among broiler ceca isolates collected in 2005-2006 a total of 37 (33.6%) were resistant to one or more antimicrobial agents, 16 (14.5%) of them were multiresistant and the most frequent resistance pattern was ampicillin, ciprofloxacin, nalidixic acid, tetracyclin, sulfamethoxazole, trimethoprim and enrofloxacin (nine isolates). Table 7 shows the antimicrobial resistance patterns of broiler, broiler meat and feed isolates. Prevalence of resistance was higher among broiler meat isolates collected in 2006-2007. Thirty-nine (52.0%) isolates were resistant to one or more antimicrobial agents, 11 (14.6%) were multiresistant and the most prevailing resistance pattern was ampicillin, ciprofloxacin, nalidixic acid, tetracyclin, sulfamethoxazole, trimethoprim and enrofloxacin (five isolates). In the 2008 sampling 20 broiler ceca isolates (50%) were resistant to one or more of the antimicrobials tested. Only one isolate (2.5%) was multiresistant, showing resistance to streptomycin, tetracyclin, sulphamethoxazole and trimethoprim. Resistance to ciprofloxacin and nalidixic acid was the most prevalent (42.5%) resistance pattern and these antimicrobial agents always showed cross

Table 6: Antimicrobial resistance of animal, mat, feed and human *E. coli* isolates. [a]

	No of resistant strains (% of total)							
	Pig		Broiler			Feed	Humans	
Antimicrobial	Ceca n=109	Meat n=50	Ceca 2005-2006 n=110	Meat n=75	Ceca 2008 n=40	Feed n=22	Slaughterhouse Personnel n=23	Outpatients n=52
Amp	23 (21.1)	4 (8.0)	20 (18.2)	12 (16.0)	0 (0)	0 (0)	6 (26.1)	8 (15.4)
Ctx	1 (0.9)	1 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ce	1 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cm	6 (5.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.3)	0 (0)
Ci	14 (12.8)	4 (8.0)	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)	3 (13.0)	0 (0)
Ef	13 (11.9)	3 (6.0)	16 (14.5)	25 (33.4)	NT[b]	NT	3 (13.0)	0 (0)
Ff	0 (0)	0 (0)	1 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gm	0 (0)	0 (0)	2 (1.8)	1 (1.3)	0 (0)	0 (0)	0 (0)	0 (0)
Nal	14 (12.8)	4 (8.0)	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)	3 (13.0)	0 (0)
Km	5 (4.6)	0 (0)	1 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sm	25 (23.0)	3 (6.0)	9 (8.2)	7 (9.3)	2 (5.0)	0 (0)	2 (8.7)	5 (9.6)
Su	31 (28.4)	6 (12.0)	21 (19.1)	11 (14.7)	2 (5.0)	0 (0)	3 (13.0)	6 (11.5)
Tc	36 (33.0)	7 (14.0)	15 (13.6)	8 (10.6)	1 (2.5)	0 (0)	5 (21.7)	5 (9.6)
Tm	14 (12.8)	4 (8.0)	16 (14.5)	10 (13.3)	1 (2.5)	0 (0)	3 (13.0)	3 (5.8)

[a] Resistance breakpoints: Ampicillin (Amp) ≥16, Cefotaxime (Ctx) ≥0.5, Ceftiofur (Ce) ≥2, Chloramphenicol (Cm) ≥32, Ciprofloxacin (Ci) ≥0.12, Enrofloxacin (Ef) ≥0.5, Florfenicol (Ff) ≥32, Gentamicin (Gm) ≥8, Nalidixic acid (Nal) ≥32, Kanamycin (Km) >16, Streptomycin (Sm) ≥64, Sulfamethoxazole (Su) ≥512, Tetracycline (Tc) ≥16, Trimethoprim (Tm) ≥4.

[b] NT= Not Tested

Table 7: Antimicrobial resistance patterns of *E. coli* isolates of broiler (cecal and meat), feed and human origin sorted according to their frequencies.

Origin	Resistance patterns[a]	Number of isolates[b]
Broiler ceca 2005-2006	AmpCiNalTcSuTmEf	9
	Sm	6
	Su	5
	Amp ; CiNalEf	4
	AmpCiNalTcSuTm	2
	AmpCiNalGmSmTcKmSuTmEf ; AmpCiNalGmSmTcSuTmEf ; AmpCiNalSuTm ; AmpCiNalTcFfSuTmEf ; AmpTc ; CiNal ; SmSuTm	1
Broiler meat	CiNalEf	16
	Amp ; AmpCiNalTcSuTmEf	5
	CiNal ; CiNalSmTmEf ; Sm	2
	AmpCiNalSmSuTmEf ; AmpSmTcSu ; CiNalTmEf ; GmSmSu ; Su ; TcSu ; TcSuTm	1
Broiler ceca 2008	CiNal	17
	Sm	1
	SmTcSuTm	1
	Su	1
Feed	CiNal	7
Slaughterhouse personnel	AmpCiNalTcEf	2
	Amp ; AmpSmSuTmCm ; AmpSmTcSu ; AmpTc ; CiNalTmEf ; SuTm ; Tc	1
Outpatients	Amp	3
	AmpSmTcSu ; Tc	2
	AmpSmSu ; AmpSmSuTm ; AmpSuTm ; SmSu ; TcTm	1

[a]Resistance patterns are separated by a semicolon. [b]Number of isolates refers to the number of isolates displaying each resistance pattern.

resistance. Prevalence of multiresistant isolates of the total resistant population was 5% compared to 43.2% and 28.2% (ceca and meat respectively) in the 2005-2006 study, which is a significant decrease for the ceca (p=0. 0.0291). Prevalence of resistance to ciprofloxacin and nalidixic acid increased significantly from the previous sampling in 2005-2006 ($p<0.0001$) and decreased significantly for ampicillin (p=0.002) and sulphamethoxazole (p=0.0398) (Figure 9). Of the 22 feed isolates tested, seven (31.8%) were resistant to ciprofloxacin and nalidixic acid. All isolates were susceptible to all other antimicrobials tested. The isolate from the probiotic feed additive was susceptible

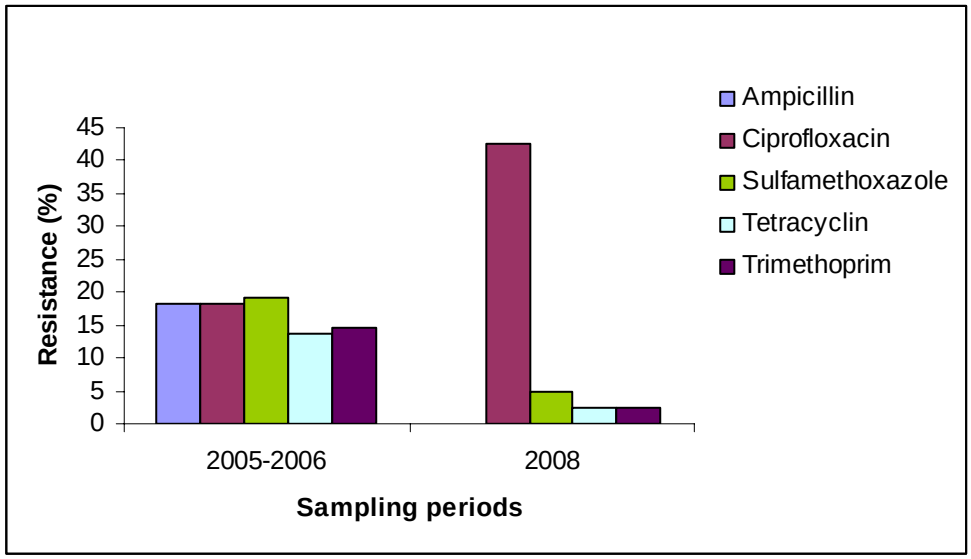

Figure 9: Prevalence of resistance among *E. coli* to five antimicrobials between sampling periods in 2005-2006 and 2008.

to all antimicrobial agents. *E. coli* was not isolated from the feed samples from the two feed mills.

Humans. Nine of the 23 (39.1%) isolates from slaughterhouse personnel were resistant to one or more antimicrobial agents, four (17.4%) were multiresistant and most had unique resistance patterns. From outpatients, 12 (23.1%) isolates were resistant to one or more antimicrobial agents, of which five (9.6%) were multiresistant. Resistance patterns were diverse although most of the resistant strains (66.7%) displayed resistance to ampicillin. Resistance to fluoroquinolones was found among isolates from slaughterhouse personnel but not from outpatients.

PFGE

2005-2007 isolates. Of the 170 resistant isolates subjected to macrorestriction with *Xba*I 159 yielded interpretable PFGE patterns. A total of 123 distinct PFGE profiles were detected and of these, 100 were unique profiles representing a single isolate. Isolates of different origin are considerably intermixed although they rarely cluster together. There were nine clusters of mixed origin. Four clusters of isolates originating from broilers and broiler meat consisting of thirteen, four, three and three isolates each. A cluster of isolates from slaughterhouse personnel and outpatients consisted of four isolates.

Another cluster contained three isolates from swine and broiler meat. There was also a cluster containing four isolates originating from pork and broiler meat. Furthermore there were two clusters, one consisted of two isolates from pork and an outpatient and the other of five isolates from broiler meat and a slaughterhouse personnel. The broilers, of which these isolates originated, were slaughtered at the slaughterhouse where the slaughterhouse employee was working. Different patterns were often seen on the same farm. Furthermore, the same PFGE pattern could occur on several different farms.

2008 isolates. Of the 27 resistant broiler and feed isolates subjected to macrorestriction with *Xba*I 16 yielded interpretable PFGE patterns (data not shown). The other 11 isolates degraded during the electrophoresis using *Xba*I with and without HEPES buffer or *Avr*II, leaving no bands, and underwent RAPD analyses. A total of 16 distinct PFGE profiles were detected, all unique profiles representing a single isolate. At 80% similarity, one cluster containing three broiler isolates was formed. Of the 34 ciprofloxacin resistant human *E. coli* isolates 23 yielded interpretable PFGE patterns (data not shown) with 22 distinct PFGE profiles. There were three clusters with three isolates each, according to 80% similarity. The 2008 strains were compared to broiler and broiler meat isolates collected in 2005-2007 along with the 34 ciprofloxacin resistant human *E. coli* isolates. A total of 137 broiler, broiler meat and human strains were subjected to PFGE analysis, yielding 110 interpretable patterns. There were 92 profiles detected, of which 81 were unique profiles representing a single isolate. Isolates of different origin were intermixed and there was some clustering, with 13 clusters of three or more isolates, of which six were seen in the previous study. Of the seven new clusters four were of mixed origin (Figure 10). Cluster A consisted of eight isolates of broiler (2008) and broiler meat (2006-2007) origin. Cluster B, of five isolates, contained broiler (2005-2006), broiler meat (2006-2007) and a feed (2008) isolate. Cluster C contained broiler meat (2006-2007) isolates and a broiler (2008) isolate. Lastly, cluster D consisted of ciprofloxacin resistant human isolates and feed (2008) isolates. All isolates falling within a cluster were ciprofloxacin resistant. Furthermore, there was close relatedness (≥80%) between human isolates and broiler (2005-2006), broiler meat (2006-2007), broilers (2008) and feed (2008) isolates (Figure 10). As before, different

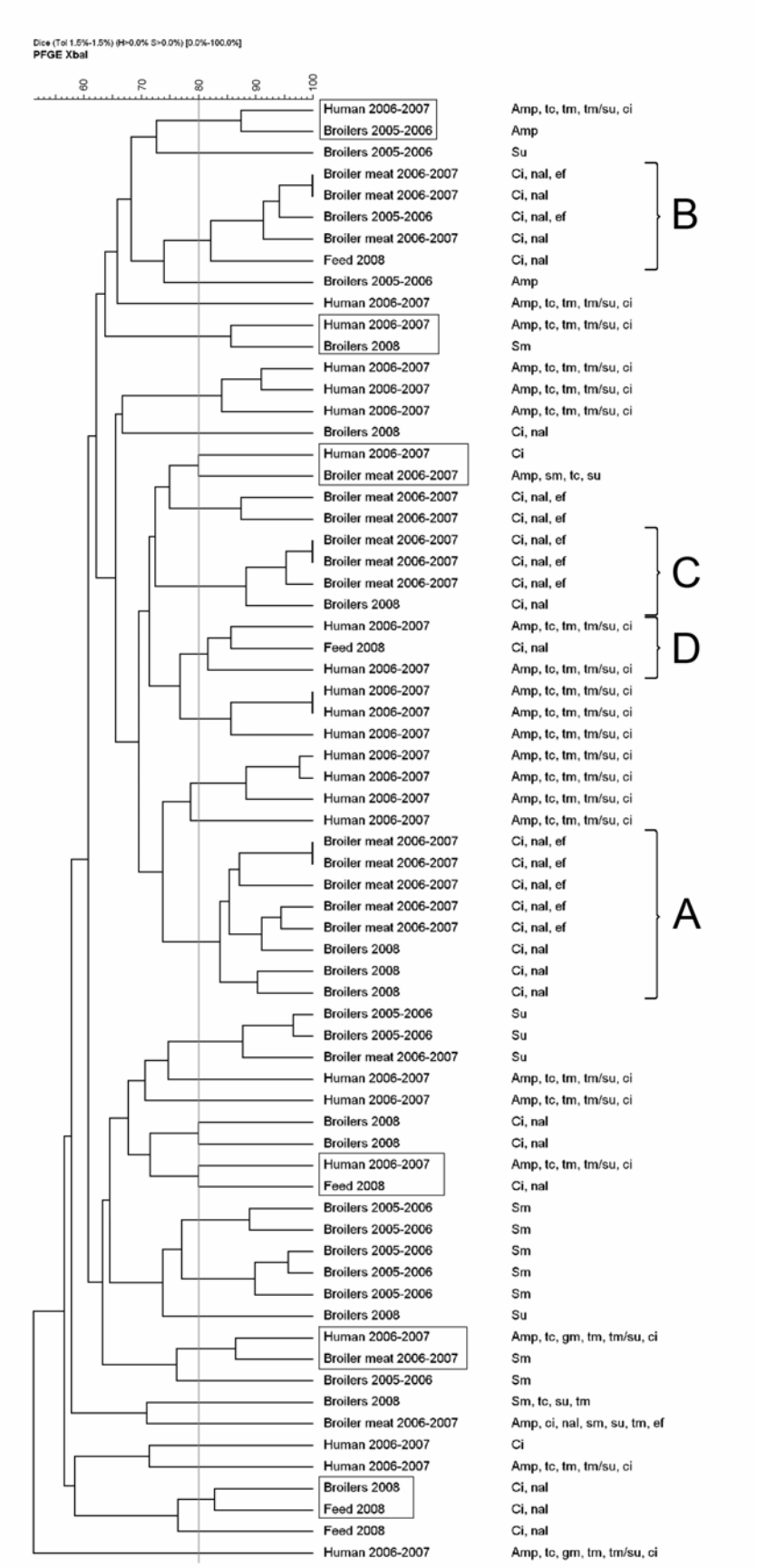

Figure 10: Dendrogram based on UPGMA cluster analysis of PFGE patterns of the broiler (2008), feed (2008) and human ciprofloxacin resistant *E. coli* isolates along with some of the most closely related broiler (2005-2006) and broiler meat (2006-2007) isolates. Brackets denote clusters (isolates with ≥ 80% similarity by Dice coefficient similarity analysis) of mixed origin and isolates within boxes are closely related isolates which do not fall within clusters, who are nevertheless of interest.

PFGE patterns were seen on the same farm and closely related patterns could occur on several different farms.

RAPD

Isolates that did not give interpretable PFGE patterns (one broiler (2005-2006) and four broiler meat (2006-2007) isolates, 11 broiler (2008) and feed (2008) isolates and 11 ciprofloxacin resistant human isolates) were subjected to RAPD analysis. All yielded interpretable patterns displaying 26 distinct profiles, all but one were unique profiles representing a single isolate (Figure 11). There were two clusters seen, thereof one containing six isolates from feed (2008) and broilers (2008) (cluster A). Furthermore, closely related RAPD patterns occurred on several different farms.

Antimicrobial residue screening test

Of the ten broiler meat samples, ten broiler kidney samples, ten young broiler meat samples and ten feed samples only one feed and one young broiler meat sample gave a suspicious reaction in the antimicrobial residue screening test. The test was repeated on these suspicious samples and then gave an unambiguous negative reaction.

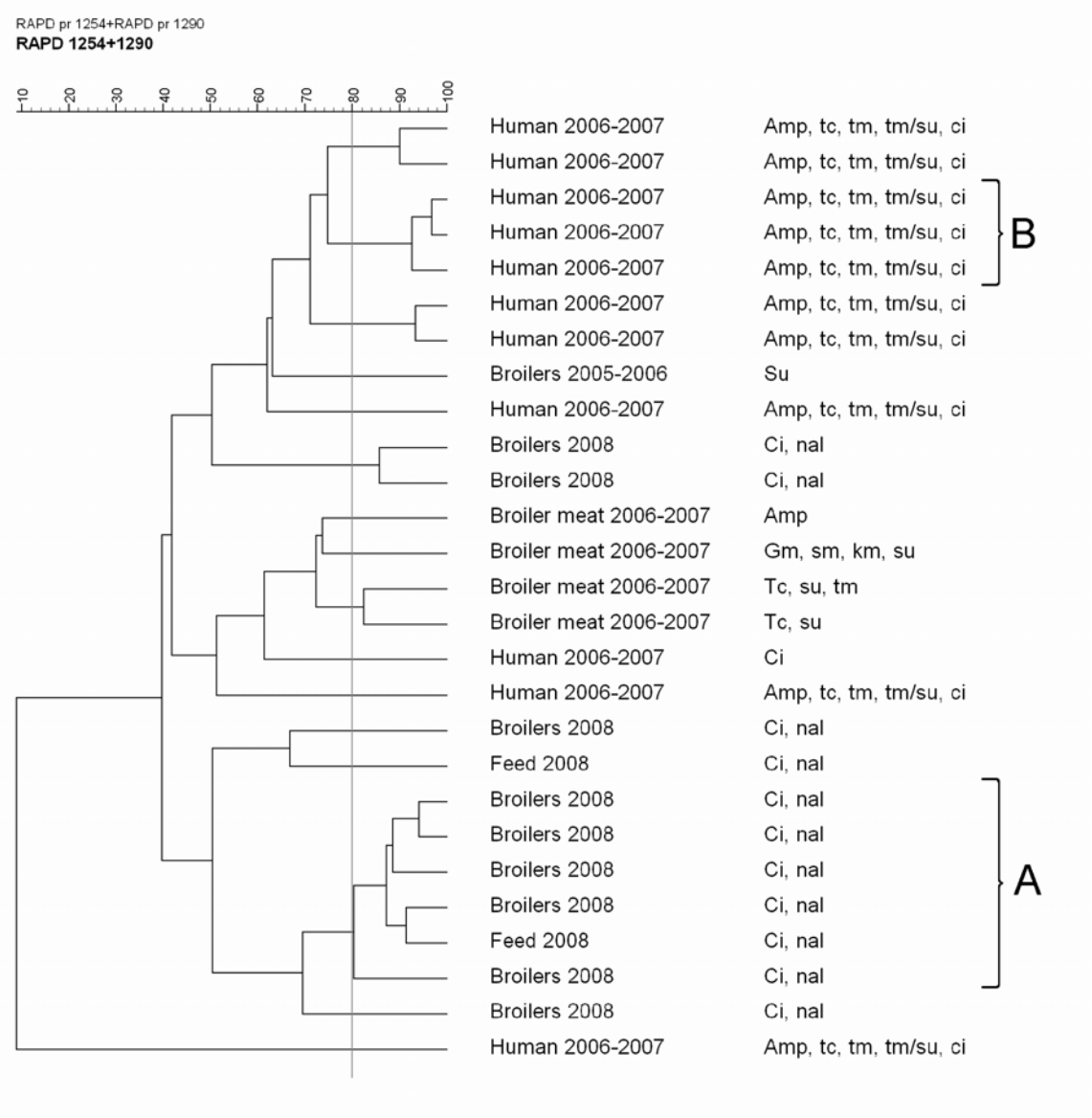

Figure 11: Dendrogram based on UPGMA cluster analysis of RAPD patterns of the broiler (2008), feed (2008) and human ciprofloxacin resistant *E. coli* isolates along with some of the most closely related broiler (2005-2006) and broiler meat (2006-2007) isolates. Brackets denote clusters (isolates with ≥ 80% similarity by Dice coefficient similarity analysis).

DISCUSSION

Salmonella

We found relatively low prevalence of antimicrobial resistant *Salmonella* spp. (13%), which was not surprising. However, finding 16 multi-resistant *S*. Typhimurium strains was unexpected, since the use of antimicrobial agents for growth promotion and as feed additives has never been allowed in Iceland. As antimicrobial agents are not used in chicken production for any purpose one would expect the prevalence of resistance to be low. This appears to be true as only three strains (13.6%) isolated from chickens proved resistant to antimicrobials and these three strains are thought to originate from feed. There are no data available on the therapeutic use of antimicrobials in pig production and therefore it is difficult to estimate its effect on the occurrence of resistance. Results from the present study show that 12.8 % of the pig isolates were resistant to antimicrobials. The prevalence of antimicrobial resistance in Iceland is very similar to that in Denmark (68) while this is a little higher than in Norway and Sweden (0% and 6% resistance of *Salmonella* spp. isolated from food producing animals) (189, 249). However the prevalence in Iceland is still low compared to southern and eastern Europe (over 70% resistance of *S*. Typhimurium isolated from pigs) (8, 89).

Ten of the 16 multiresistant *S.* Typhimurium strains had identical PFGE and resistance profiles (X5A3, Amp,Sm,Te/Tc,Ff,Su,Cm) and were phage typed as DT104, a known multiresistant phage type. All DT104 strains belonged to the same cluster showing 94% overall similarity. All but two of the DT104, X5A3 type isolates came from a single pig farm and the remaining two isolates were from a single chicken farm. Both these farms got their freed from the same feed mill and it is assumed that the feed mill was the source of those strains (Jarle Reiersen, Former Veterinary Officer for poultry diseases, personal communication). However, further investigation on the feed and its possible contamination with DT104 strains was not carried out at that time.

Although previous research has shown that the endemic *S.* Typhimurium strains in Iceland are considerably diverse (113) the DT104 strain appears to be prevailing among the antimicrobial resistant strains, especially in pigs. It is also apparent that the occurrence of these multiple resistant isolates greatly influences the prevalence of

resistance. When the DT104 isolates are excluded the prevalence of resistance among all *Salmonella* isolates is only 4% (6/148). *S.* Typhimurium was not found in pigs or broilers in the years 2006-2008. This disappearance of the DT104 clone further indicates that feed was a vector for the clone into the farms, rather than the clone was persisting on the farms or in their environment.

The single *S.* Typhimurium DT208 strain resistant to enrofloxacin is a cause for concern, although it was not of phage type DT104, since ciprofloxacin is used as the standard empirical treatment for suspected extraintestinal salmonella infections and serious gastroenteritis in humans.

Campylobacter

Low level of antimicrobial resistance (5.2%) was observed among *C. jejuni* isolates from broilers in 2001-2005, with resistance to ampicillin being the most prevalent. Furthermore, isolates from broilers in 2006-2007 were all susceptible to both nalidixic acid and ciprofloxacin. This low level of resistance was to be expected as there is no use of antimicrobials in broiler production in Iceland. However, the prevalence of resistance seems to be increasing, although slowly. These resistance rates are similar and even lower than that seen in the other Nordic countries, where resistance rates among *C. jejuni* isolates from broilers were around 5% to 13% (68, 189, 248). Higher rates are seen in other European countries (>35% in France, Netherlands and the UK) (44). Different national policies in relation to the use of antimicrobials in food animal production might explain the different resistance rates between countries. The low level of resistance, despite the absence of antimicrobial exposure, supports previous findings suggesting that resistant isolates are stable in the broiler house environment and that they are able to transmit to and colonize the flocks without antimicrobial selection pressure (129, 161). It has been implied that antimicrobial resistant *Campylobacter* isolates may arise in the flocks, possibly through intergron mediated gene exchange, after human contamination of the broiler house environment (153).

It has also previously been observed that resistance rates are higher among isolates of *C. coli* than among isolates of *C. jejuni* (3, 129), as is seen in the present study where 53.8% of the *C. coli* strains where resistant to one or more antimicrobial agents while

resistance among *C. jejuni* strains was 5.2%. The difference is even more evident when looking at enrofloxacin resistance, where 1.1% of *C. jejuni* isolates are resistant while 53.8% of *C. coli* isolates are resistant, as has been reported before (3). Moreover the resistance patterns were different between *C. jejuni* and *C. coli* strains, although both species exhibited cross-resistance between enrofloxacin and nalidixc acid. As there were only few *C. coli* isolates available for susceptibility testing it is not possible to conclude if there is a true difference among the species.

Gudmundsdottir et al. (114) found that there was great diversity of *Campylobacter* strains in humans, animals, foods and water in Iceland, where two pulsotypes were dominant and over 60% of the pulsotypes contained only one isolate. Similar results are obtained in the present study where the 25 resistant strains are divided into 13 pulsotypes of wich 6 contain only one strain. On most farms specific clones occurred only once, consistent with the previous report of high diversity, although with three exceptions. On one farm one clone was found in two flocks in two different houses, hatched within one weeks interval. On the same farm, two years later, one clone was found in three flocks in three different houses, hatched within a 12 weeks interval. On a second farm the same clone was found in three flocks from two different houses, hatched within a six weeks interval. As the clones are found in different houses on the same farm and with several weeks interval, persistance within the houses or flock to flock carryover is unlikely. It is most likely that the outdoor environment of the farm is the source of infection in the broilers, as has been reported before (43, 131, 201). Prevoius studies on *Campylobacter* contamination of broiler flocks in Iceland have identified water and vertical ventilation as risk factors for contamination (26, 116), consistent with outdoor environmental source of *Campylobacter* strains. Particular resistance patterns could be associated with different pulsotypes. Specific pulsotypes could arise on different farms located in different parts of the country and slaughtering in separate slaughterhouses, indicating that antimicrobial resistant *Campylobacter* clones can emerge on chicken farms all over Iceland. This is further demonstrated by the fact that antimicrobial resistance patterns were generally uniform within the pulsotypes. Potential dissemination vectors have been identified by other researchers, such as insects, wild birds and water (43, 57, 116, 118). Additionally it has been shown that for

Campylobacter colonization of broiler flocks in Iceland, temperature, age of the birds, flock size and vertical ventilation systems increases the odds of flocks being positive (26, 115-117).

E. coli

In 2005-2007 resistance rates in *E. coli* from pigs and broilers (cecal and meat samples) and slaughterhouse personnel were moderate to high (54.1% and 28% for pigs, 33.6% and 52% for broilers and 39.1% for slaughterhouse personnel, respectively), whereas isolates from outpatients showed more moderate resistance rates (23.1%). This is somewhat similar to what has been reported from the other Nordic countries (pig fecal and meat 22-48% and 23-28%, broilers fecal and meat 15-35% and 16-41%) (67, 69, 97, 189, 249, 251) and quite lower than in Spain (pigs > 75%, broilers 97.5%, foods > 85% and humans > 50%) (220). Of notice is the prevalence of multiresistant isolates as 15% of the *E. coli* isolates had a multiple resistance phenotype. Again, this is somewhat higher than what is seen in the other Nordic countries (3% in broilers in Sweden) (251) although quite lower than in Spain (50% in broilers) (220). This is a cause for concern as studies have shown that the possibility of transferring resistance increases by increased multiresistance (242). Previous studies have shown that *E. coli* isolates from healthy poultry frequently display high prevalence of multiresistance (11, 220). However, high prevalence of resistant and multiresistant isolates is most often seen in areas where the use of antimicrobial agents in poultry production is common (221, 272), which is not the case in Iceland. The use of antimicrobial agents for growth promotion has never been allowed in Iceland and such agents have not been used for any purpose in broiler production for the last decade or so (Jarle Reiersen, former veterinary officer of poultry disease and Gunnar Örn Guðmundsson, District Veterinarian of Gullbringu-and Kjósarsýslu, personal communication). High frequency of multiresistance among the *E. coli* isolates may be due to linkage of resistance genes on plasmids. In the 2005-2007 study certain combinations of resistances are seen more often than others (Table 7), further indicating that the resistance elements are associated and can be mobilized together. Furthermore, resistances to streptomycin and sulphamethoxazole were often associated, as has been documented before in *E. coli* from meat (242). Resistance genes

for streptomycin (*str*A-*str*B) and sulfonamides (*sul*2) are known to co-reside on nonconjugative plasmids (like pBP1) that are widely distributed in *E. coli* and have a broad host range (244).

In 2008 we found that the prevalence of quinolone resistance was still high, and rising, among broilers (42.5%) although prevalence of resistance to other antimicrobial agents seemed to be decreasing considerably along with the prevalence of multiresistant isolates (2.5%). This suggests that quinolone resistance elements are not transferred along with resistance elements of the other antimicrobials and that they are selected for by other forces. We found that isolates from feed had similar resistance patterns and prevalence rates as broiler ceca isolates, both from 2005-2006 and 2008, and broiler meat isolates (2006-2007). High prevalence of resistance to flouroquinolones in animals and their meat products is surprising, especially in broilers where there is no known antimicrobial selection pressure. There clearly is a cross-resistance between ciprofloxacin and nalidixic acid and in most cases also to enrofloxacin, as previously demonstrated(31). Why a full cross-resistance between ciprofloxacin and enrofloxacin was not observed is unclear and will need further studying.

Isolates of *Campylobacter jejuni* are less susceptible to quinolones than are other enteric pathogens such as *E. coli* (42, 108) and fluoroquinolone treatment rapidly selects for high levels of ciprofloxacin resistance in *C. jejuni* (129, 171) while development of resistance in *E. coli* seems to be slower (168). Consistently, it has been shown that broiler flock treatment with enrofloxacin quickly selects for high frequencies of fluoroquinolone resistance among *Campylobacter* while it does not induce resistance among *E. coli* (271). We found moderately high prevalence of fluoroquinolone resistance among *E. coli* whereas we found no resistance among *Campylobacter* to ciprofloxacin and nalidixic acid during the same period and only 3% resistance to enrofloxacin in the previous study in 2001-2005. Therefore it is most plausible that the *E. coli* strains are not acquiring resistance to antimicrobials de novo due to antimicrobial pressure, rather they are acquiring resistance elements from the environment or resistant strains are in some way entering the broiler flocks, e.g. through feed. Although *E. coli* was not isolated from the feed samples from the two feed mills, there was much growth of other *Enterobacteriaceae* on the agar plates which could possibly have overgrown

existing *E. coli* strains, if any, demonstrating that the feed is not sterile. Furthermore, we isolated *E. coli* from the probiotic feed additive (though it was fully susceptible) and from feed samples taken at the broiler houses, demonstrating that the feed and feed additives are contaminated with *E. coli* and other *Enterobacteriaceae* and could be introducing resistant strains into the farm environment.

Khachatryan et al. found that antimicrobial drug selection is not required for short-term maintenance of resistant *E. coli* (146) and that the dietary supplement fed to calves at a dairy farm was probably selecting for strains with a specific resistance pattern (145), concluding that a multifactor selective system might be maintaining a relatively constant level of antimicrobial resistant bacteria. In addition, they concluded that a relatively large genetic element was conferring this specific resistance phenotype and that this element was responsible for maintaining these traits owing to association to genetic traits that give a selective advantage in the calves (144). Genetic elements, such as the transposon *Tn*21, can encode both antimicrobial resistance and heavy-metal resistance to mercuric compounds, and have been found in isolates of human and animal origin (188, 243, 291). Morover, mercury resistance is frequently connected with multiresistance, e.g. resistance to ampicillin, sulfonamides, streptomycin and tetracyclin (184, 284). Mercury can accumulate in fish (76) and the use of fish meal in poultry feed could pose a selection pressure favouring the selection of mercury resistant isolates, along with other resistance elements on the transposon, in poultry, as suggested by Bass et al. (1999) (30). Fish meal is commonly used in broiler feed in Iceland, although during the last few years the feed mills have gradually been replacing it with soy-meal. This could, at least in part, explain the decrease in resistance to antimicrobials other than quinolones and fluoroquinolones. With less fish meal in the feed the selective pressure of mercury decreases and so the prevalence of antimicrobial resistance elements, co-existing with mercury resistance elements on a plasmid or transposon, decreases, while the prevalence of other resistance elements (e.g. fluoroquinolone) is unaffected.

The majority (78.6%) of the resistant *E. coli* isolates from 2005-2007 was genotypically different based on PFGE fingerprint analyses, and clustering was limited. Resistance patterns among *E. coli* of animal origin were diverse and this along with diverse pulsotypes suggests the presence of a large population of resistant *E. coli*. This also

implies de novo acquisition of resistance determinants or mutations. In the 2005-2007 study we found that isolates sharing the same PFGE patterns and the same resistance patterns can arise on different farms. However, it is not easy to point to a single source of those isolates as the farms are geographically separated, they are attended to by different veterinarians and the animals are slaughtered at different slaughterhouses. Furthermore, the broiler production in Iceland adheres to strict biosecurity measures to prevent the transmission of infectious agents or environmental contamination into the farms. We found that feeds contaminated with resistant *E. coli* could be a plausible explanation for similar PFGE and resistance patterns arising on different farms, as there are only two feed mills in Iceland and both produce pig and poultry feed. Consequently, in the 2008 study, we found closely related isolates in feed and broilers (both 2008 and 2005-2006 samples), which is consistent with the findings of da Costa et al. (2007) (64), that antimicrobial resistant *E. coli* could be introduced into the farm environment through feedstuffs and poultry feed. The finding of *E. coli* in feed is of concern as this could indicate that other bacteria and possible pathogens, such as *Salmonella*, could also be found in feed. These results clearly demonstrate a need for further study on bacterial contamination of feed in Iceland.

We found the same resistance pattern and pulsotype among isolates from broiler meat and a slaughterhouse worker at the slaughterhouse where these broilers were slaughtered and their meat processed. This indicates the spread of antimicrobial resistant *E. coli* from animals to humans. Furthermore, it is of interest that resistance to fluoroquinolones was found in isolates from slaughterhouse personnel but not from outpatients. This is in concordance with previous findings, that poultry workers and slaughterers are at a higher risk of colonization of antimicrobial resistant commensal bacteria, such as *E. coli*, because of their work (210, 239, 272). Also, we found considerable similarity (90%) between a pork isolate and an isolate from an outpatient although they did not share a common resistance pattern. Furthermore, in the 2008 study, there was clustering of isolates of different origin and closely related isolates were found in humans, broilers, broiler meat and feed, supporting previous findings of chicken and their products as a possible source of fluoroquinolone resistant *E. coli* in humans (11, 133). With the extensive genomic diversity of *E. coli* and the high discrimination of PFGE as a typing

method for *E. coli*, finding indistinguishable isolates of different origin collected during a large interval of time is unlikely, except within a very large collection (133, 212). Therefore, finding human isolates closely related (⩾80 similarity) to broiler, broiler meat and feed isolates is important and significant. Similarity between isolates from animals, animal food products and humans has been noted before (133, 134, 212). This is however, to our knowledge, the first time that a close relationship has been found between broiler feed and human isolates.

Limitations of the study

In this study we examined antimicrobial susceptibility of *Salmonella* from pigs and broilers, *Campylobacter* from broilers and *E. coli* isolates from pigs, broilers, pork, broiler meat, broiler feed, slaughterhouse personnel and outpatients. As isolates were collected from all major slaughterhouses, pig and broiler farms and the geographical distribution of samples was good we believe this study provides a representative sample of the resistance trends in Iceland. However, this study had some potential limitations. Firstly, the number of isolates in the study is small. Nonetheless, in spite of this low number of isolates, we belief that the sample material is representative, as the swine and broiler production in Iceland is very small. The prevalence of *Salmonella* spp. is low in Iceland so the number of strains available for analyzes were limited. For *Salmonella* and *Campylobacter*, samples were taken from every flock and herd intended for human consumption and all isolates collected for the surveillance programmes were included in the study. Though the same farms are often infected repeatedly as was seen in the present research, where almost 80% of the resistant *Salmonella* strains came from the same pig farm. These persistent infections greatly influence the prevalence of resistance. Only one strain was isolated from each *Campylobacter* positive flock, whereas some flocks may be infected by two or more different strains. We sampled a good proportion of the annual pig and broiler production for the susceptibility testing of *E. coli* and as for *Salmonella* and *Campylobacter* samples were collected from all major slaughterhouses, pig and broiler farms and the geographical distribution of samples was good.

Currently no internationally accepted standards are available for the susceptibility testing of *Campylobacter* spp. However a high degree of agreement between different

methods has been indicated in comparative studies. When studying the broth microdilution test, E-test and the agar dilution method Luber *et al*. produced comparable results and concluded the broth microdilution method to be a reliable method for determination of the MIC´s of antibiotics for *C. jejuni* and *C. coli* (162).

A considerable fraction of the *Campylobacter* isolates from the national surveillance programme in 2006-2007 were lost during freeze storage, prior to antimicrobial susceptibility testing. *Campylobacter* is very sensitive to freezing and there have been reports of reduction in *Campylobacter* counts after freezing (105, 163). Despite these limitations we consider the number of isolates to be sufficient for the evaluation of nalidixic acid and ciprofloxacin susceptibility.

Selection bias might be a cause for concern, regarding the *E. coli* study, as samples of different origin were collected at different time intervals although with some overlap. This is because at first we only intended to collect isolates from animals. However, the resistance prevalence was higher than suspected, which lead to further sampling from foods and humans in 2006-2007 and again in 2008 from broilers and broiler feed. This could explain why little clonal relatedness was seen among cecal and food strains.

By selectively screening for enrofloxacin resistant isolates we increased our chances to find them if present in the sample. However, this makes the comparison of the resistance rates of fluoroquinolones to that of other antimicrobial agents difficult. Nonetheless, we would assume that the changes in the prevalence of different antimicrobials between sampling periods is unaffected by this bias, so that it is safe for us to assume that quinolone resistance is increasing while resistance to other antimicrobials is decreasing.

Data on antimicrobial consumption among individual animal species is not available so evaluation of the effect of consumption on the arising of resistance is not possible. However, there should be no use of antimicrobial agents in broiler production.

GENERAL SUMMARY AND CONCLUSIONS

There is an increasing concern, worldwide, over the development of antimicrobial resistance in pathogenic bacteria. *Salmonella* spp., *Campylobacter* spp. and *E. coli* are known to colonize different animal species and may be transferred from animals to humans, possibly through food. The use of antimicrobial agents in animals and the subsequent emergence of antimicrobial resistant bacteria may therefore have consequences for the treatment of infections in humans. The present study is the first to provide information on the prevalence of antimicrobial resistant *Salmonella*, *Campylobacter* and *E. coli* in pigs, broiler chickens, their food products, broiler feed and slaughterhouse personnel in Iceland.

The prevalence of resistance among *Salmonella* and *Campylobacter* isolated from pigs and broilers is low and comparable to the prevalence in the other Nordic countries. The Nordic countries have similar practices in poultry and swine production and uphold high biosecurity levels, often using the “all-in all-out” principal. The use of antimicrobials in animal production is low and the use of anmticrobial growth promotants has not been allowed for more than a decade or never at all. Given these similarities in the production, we find the Nordic countries useful for comparison and to assess the situation in Iceland. Regarding *Salmonella*, the same farms are often infected repeatedly, as was seen in the present research, where almost 80% of the resistant strains came from the same pig farm. These persistent *S.* Typhimurium DT104 infections greatly influence the prevalence of resistance.

There is moderate to high prevalence of antimicrobial resistance among *E. coli* isolates from production animals and their food products and somewhat lower prevalence among slaughterhouse personnel and outpatients. These prevalence rates are similar, although a bit higher, than what is reported in the other Nordic countries. Of special notice is the high prevalence in broilers and broiler meat where there is no known antimicrobial selection pressure. Furthermore, the high prevalence of resistance to fluoroquinolones in broilers, broiler meat and feed is surprising and the fact that the prevalence of fluoroquinolone resistance remains high, and even increases, while resistance rates for other antimicrobials decrease between sampling periods. We found that slaughterhouse

personnel are at risk of obtaining resistant *E. coli* and that genetically related strains are found in broilers, broiler meat, broiler feed and humans. Our results therefore suggest that broilers can be a source of resistant *E. coli* in humans. These resistant *E. coli* strains can furthermore serve as a reservoir of resistance determinants for other human pathogens.

Feed seems to be a factor in the dissemination of resistant bacteria in animal production in Iceland. The dissemination of resistant *Salmonella* spp. to broiler and pig farms has been connected to feed although it was never studied further. Findings of closely related strains, of *Salmonella*, *Campylobacter* and *E. coli*, on different and geographically distant farms, support the suggestion of feed as a vector for resistant bacteria into the farm environment. In addition, we found closely related *E. coli* strains in broiler feed, broilers and broiler meat and what is more, related strains were found in feed and in humans. Changes in the feed ingredients can possibly explain the changes in resistance patterns among *E. coli* in broilers.

The results presented in this thesis suggest that resistance prevalence among *Salmonella* and *Campylobacter* is low and comparable to countries with similar pig and poultry production and use of antimicrobials. Resistance to fluoroquinolones was detected, although the prevalence was very low. Resistance prevalence among *E. coli* in broilers, however, is surprisingly high in relation to the lack of antimicrobial selection pressure and calls for further research on the origin of these strains. We showed that feed is a plausible vector for the resistant *E. coli* strains into the farm environment, that people working in close contact with animals, such as slaughterhouse personel, are especially at risk of obtaining these resistant strains and that broilers can serve as a source of resistant *E. coli* in humans.

Conversely, these results emphasize the need for more detailed data on the use of antimicrobial agents in production animals in Iceland and the relationship between antimicrobial consumption and resistance in animals needs to be evaluated further. There is a need for continuing surveillance of resistant bacteria in animals and their food products and their possible transfer to humans. Special attention should be put on fluoroquinolone resistance, as it was detected among all the bacterial species studied here and was especially high in *E. coli*, despite the lack of antimicrobial pressure.

Further research is needed and should focus on resistance mechanisms, especially behind fluoroquinolone resistance, on the yet unidentified mechanism selecting for the persistence of fluoroquinolone resistant *E. coli* strains in broilers and on the origin of the resistant strains in the broiler feed, to prevent or minimize the risk of the transfer of these resistant isolates from broilers to humans. Transfer studies might clarify if resistance determinants are passed on together and genotyping of susceptible strains could give further information on strain relatedness and source of resistance. Investigation on the bacterial contamination of feed from the two feed mills in Iceland is of high importance and all suspicion of feed as a vector in bacterial infections must be studied swiftly.

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PAPERS I-IV

PAPER I

I

MICROBIAL DRUG RESISTANCE
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Antimicrobial Resistance and Serotype Distribution among *Salmonella* spp. in Pigs and Poultry in Iceland, 2001–2005

THORUNN R. THORSTEINSDOTTIR,[1] KARL G. KRISTINSSON,[2] and EGGERT GUNNARSSON[1]

ABSTRACT

Little information is available on antimicrobial resistance of bacteria isolated from animals and animal products in Iceland. The objective of this study was to analyze serotype distribution and antimicrobial resistance patterns of *Salmonella* spp. isolated from healthy chickens and pigs in Iceland during 2001–2005. A total of 163 *Salmonella* strains, isolated in the national *Salmonella* surveillance program, were available for study. Isolates were tested for antimicrobial susceptibility using a microbroth dilution method (VetMIC) and resistant strains were compared using pulsed-field gel electrophoresis (PFGE) and phage typing. The most commonly isolated serotypes were *Salmonella* Infantis (61%) and *S.* Typhimurium (33%); other serotypes were less prevalent. The overall prevalence of resistance was 13.6% in chickens and 12.8% in pigs. Twenty one isolates (12.8%) were resistant to one or more antimicrobials, 19 *S.* Typhimurium strains, one *S.* Infantis strain, and one *S.* Worthington strain. Sixteen out of the 19 resistant *S.* Typhimurium strains were multiresistant (to ≥3 antimicrobial agents), and, of these, 15 had identical or closely related PFGE patterns (previously phage typed as DT104). The prevalence of antimicrobial resistance in *Salmonella* spp. in pigs and poultry in Iceland is low; however, we found a multiresistant *S.* Typhimurium clone that causes concern. Continuous resistance surveillance is important, and further research on the source of resistant clones and possible transmission to humans is needed.

INTRODUCTION

In recent years, there has been an increasing concern worldwide for the rise of antimicrobial resistance of *Salmonella* spp. Resistant strains are an increasing proportion of all isolates from human infections,[4] and many studies have shown that antimicrobial-resistant *Salmonella* causing human infection often derive from production animals.[2,10,14]

Salmonella infections have not been common in Iceland in the last few years, with only sporadic cases in humans, livestock, and wildlife.[8,12,13] Efforts to control *Salmonella* in poultry and pig production in Iceland have been very successful, especially in poultry where the prevalence has decreased from 15% to 0% in only 12 years.[16] In recent years, no *Salmonella* has been detected in food products from these animals.[19]

Antimicrobial resistance patterns can vary from one country to another. Little is known about the prevalence of antimicrobial-resistant bacteria in animals in Iceland, and there has been no research on antimicrobial resistance in production animals. The prime objective of this study was to analyze the antibiotic resistance patterns and serotype distribution of *Salmonella* spp. isolated from chickens and pigs intended for human consumption over a 5-year period in Iceland.

MATERIALS AND METHODS

Bacterial isolates

The Icelandic *Salmonella* Surveillance program for pigs and poultry was initiated in 1995. Isolation of *Salmonella* is notifiable and appropriate measures are taken to trace the source of the infection and eliminate it.

Samples are taken both prior to and at slaughter. Infected chicken meat is withdrawn from the market and destroyed, whereas infected swine meat can only be put on the market after heat treatment. Slaughterhouses and infected farms are put under restriction, cleaned, and disinfected.

[1]Institute for Experimental Pathology, University of Iceland, Keldur, Iceland.
[2]Department of Clinical Microbiology, Institute of Laboratory Medicine, Landspitali University Hospital, Reykjavik, Iceland.

Sample examination is carried out at two laboratories—the Institute for Experimental Pathology, University of Iceland, Keldur, and the Sýni Laboratory Service. All *Salmonella* isolates were sent to the Department of Clinical Microbiology, Landspitali University Hospital for confirmation and serotyping. Isolates were stored in brain–heart infusion (BHI) broth with 20% glycerol at −75°C.

In this study, one isolate of each serotype from each *Salmonella*-affected pig herd and chicken flock detected in the national *Salmonella* surveillance program in the years 2001–2005 was tested for antimicrobial resistance. The geographical distribution of the chicken and pig farms that sent in samples for the *Salmonella* surveillance program in the years 2001–2005 is shown in Fig. 1.

Susceptibility testing

Antimicrobial susceptibility testing was performed using a microbroth dilution method (VetMIC™, SVA, Sweden) according to the National Committee for Clinical Laboratory Standards (NCCLS) Document M31-A2. Minimum inhibitory concentrations (MICs) for the following antimicrobials were determined: ampicillin (Amp), ceftiofur (Ce), chloramphenicol (Cm), enrofloxacin (Ef), florfenicol (Ff), gentamicin (Gm), nalidixic acid (Nal), neomycin (Nm), streptomycin (Sm), sulfamethoxazole (Su), oxytetracycline (Te), and trimethoprim (Tm) [Due to change of VetMIC panel design in the spring of 2006, strains from Sýni ehf were tested for ciprofloxacin (Ci) instead of enrofloxacin, kanamycin (Km) instead of neomycin, and tetracycline (Tc) instead of oxytetracycline.] For quality control, the *E. coli* ATCC 25922 reference strain was tested concurrently.

The cut-off values were the same as those used in the Swedish monitoring program.[17] Strains showing resistance to one or more antimicrobial agents were considered resistant, and strains resistant to three or more agents were considered multiresistant.

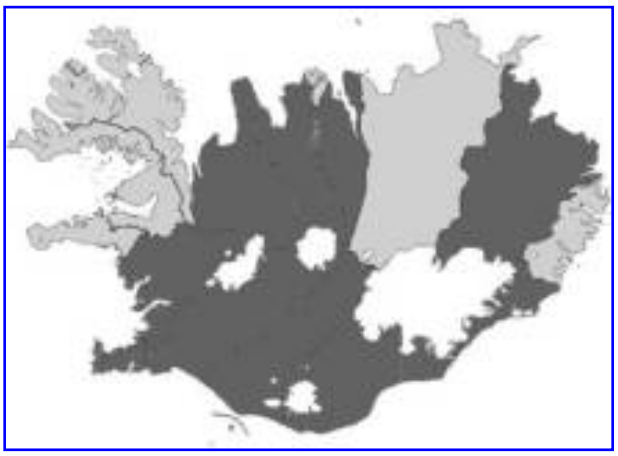

FIG. 1. Geographical distribution of chicken and pig farms participating in the Icelandic *Salmonella* surveillance program in the years 2001–2005. Darker shade denotes the counties of participating farms.

Pulsed-field gel electrophoresis

Resistant *Salmonella* isolates were analyzed with pulsed-field gel electrophoresis (PFGE) as previously described with the following modifications. The DNA-agarose plugs were lysed overnight at 54°C. Plugs were digested with both 25 U/plug *Xba*I and 15 U/plug *Avr*II restriction enzymes overnight at 37°C. Gels were electrophoresed for 22 hr.

Analysis of DNA fragmentation profiles was performed by visual inspection, and, for interpretation, the criteria suggested by Tenover *et al.*[18] were used. Macrorestriction patterns were compared with the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), and cluster analysis using the Dice coefficient for band matching was used to generate a dendrogram with unweighted pair group method with arithmetic mean (UPGMA) clustering.

Definitive phage typing

All resistant *Salmonella* Typhimurium strains were sent to the Danish Institute for Food and Veterinary Research, Department of Microbiology and Risk Assessment for definitive phage typing with the Colindale system, M03-03-003.

RESULTS

A total of 163 isolates of *Salmonella* spp. isolated in the years 2001–2005 were tested for antimicrobial resistance. A total of 141 isolates were of pig origin and 22 of chicken origin. Six strains isolated in the national *Salmonella* surveillance program were omitted because information on the sample origin and confirmation of *Salmonella* spp. and serotyping could not be obtained.

Salmonella serovars

Salmonella enterica serovar Infantis was predominant in both pigs and chickens because 89 pig strains (63%) and 11 chicken strains (50%) were typed as *S*. Infantis. Moreover, *Salmonella* serovars Typhimurium (14%), Worthington (23%), Java, Mbandaka, and Newport (4.5% each) were isolated in chickens and serovars Typhimurium (36%) and Worthington (1%) were found in pigs in addition to Infantis.

Antibiotic susceptibility

Of the 163 isolates tested, 21 (12.8%) were resistant. The overall prevalence of resistance was 13.6% in chickens and 12.8% in pigs. All isolates of serovars *S*. Newport and *S*. Java were sensitive to all the antimicrobials tested. Furthermore, only one isolate of serovar *S*. Infantis (isolated from pigs) and one of serovar *S*. Worthington (isolated from chickens) were resistant to antimicrobials. On the other hand, we found that 19 isolates (35.2%) of serovar *S*. Typhimurium were resistant to antimicrobials, of which 17 were of pig and two of chicken origin (see Table 1).

PFGE patterns and definitive phage typing

Macrorestriction with *Xba*I and *Avr*II yielded seven different patterns of 11–15 fragments and six patterns of 6–10 frag-

TABLE 1. ANTIMICROBIAL RESISTANCE IN *SALMONELLA* TYPHIMURIUM ISOLATED FROM PIGS AND CHICKENS IN ICELAND IN THE YEARS 2001–2005 ($n = 54$) (CONCENTRATION RANGE TESTED AND CUT-OFF VALUES)

Antimicrobial agent	*Range tested (mg/L)*	*Cut-off values*[a]	*Resistant strains (%)*
Ampicillin	0.25–32	>8	15 (27.8)
Ceftiofur	0.12–16	>2	0 (0)
Chloramphenicol	1–128	>16	15 (27.8)
Enrofloxacin ($n = 37$)	0.03–4	>0.25	1 (2.7)
Ciprofloxacin	0.008–1	>0.06	0 (0)
Florfenicol	4–32	>16	15 (27.8)
Gentamicin	0.5–64	>8	0 (0)
Nalidixic acid	1–128	>16	0 (0)
Neomycin/kanamycin	2–16	>8	0 (0)
Streptomycin	2–256	>32	17 (31.5)
Sulphamethoxazole	16–2048	>256	16 (29.6)
Oxytetracycline/tetracycline	0.5–64	>8	16 (29.6)
Trimethoprim	0.25–32	>8	0 (0)

[a]Microbiological cut-off values defining resistance.

ments, respectively. Table 2 shows the characteristics of the 19 antimicrobial-resistant *S.* Typhimurium strains. No antimicrobial-resistant *S.* Typhimurium strains were isolated in the year 2002. Compiling the results from both *Xba*I and *Avr*II macrorestrictions gave a dendrogram consisting of eight patterns in three clusters (Fig. 2). The first cluster consisted of three profiles (X1A4, X2A5, and X3A6) from three pig isolates and accounted for 16% of the strains. The second cluster accounted for 79% of the strains and consisted of four profiles (X4A3, X5A3, X6A3, and X5A2, two from chickens and 13 from pigs). X5A3 was the predominant genotype accounting for 58% of all strains. All isolates in this cluster were typed as DT104. One profile (X7A1) comprised the third cluster containing one isolate from a pig. The overall similarity for the three clusters was 82% whereas the similarity between profiles in cluster two was 94%.

DISCUSSION

The low prevalence of *Salmonella* spp. in chickens (0% in 2005[16]) and in pigs (around 1%[13]) is similar to that in the other Nordic countries,[3,6] and the most prevalent serotypes are similar as in Denmark (S. Infantis and *S.* Typhimurium), yet with

TABLE 2. CHARACTERISTICS OF 19 *S.* TYPHIMURIUM STRAINS, ISOLATED FROM CHICKENS AND PIGS IN ICELAND IN 2001–2005, TYPED BY PFGE, BACTERIOPHAGES (DT), AND ANTIMICROBIAL SUSCEPTIBILITIES

Year of isolation	*Origin*	*Farm*	*Antimicrobial resistance pattern*	*DT*[a]	*PFGE profile*	
					XbaI	*AvrII*
2001	Pig	P1	Sm	3	X1	A4
	Pig	P3	Sm	NT	X7	A1
	Pig	P1	Ef,Te,Su	208	X2	A5
2003	Pig	P1, 2, 4	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P2	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A2
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Sm	NT	X3	A6
2004	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X4	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X4	A3
	Pig	P1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Chicken	C1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Chicken	C1	Amp,Sm,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X6	A3
2005	Pig	P1	Amp,Te,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3
	Pig	P1	Amp,Sm,Tc,Ff,Su,Cm	104	X5	A3

[a]NT, Nontypeable.

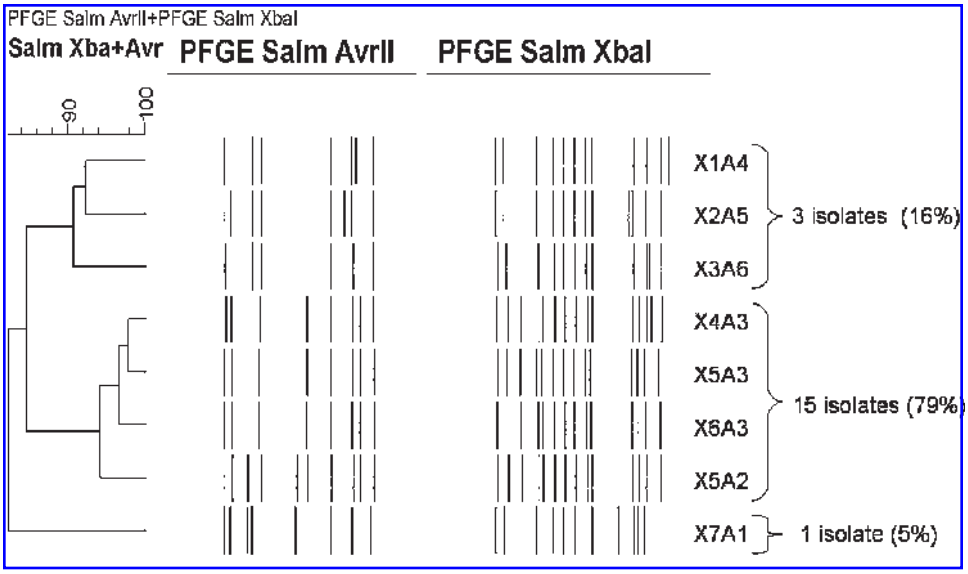

FIG. 2. Dendrogram showing the UPGMA cluster analysis of the different PFGE patterns, determined by *Xba*I and *Avr*II digestion, from 19 isolates of *S.* Typhimurium isolated from chickens and pigs in Iceland in 2001–2005.

less serotype diversity.[3] The relatively low prevalence of antimicrobial-resistant *Salmonella* spp. (13%, 21 of 163) was not surprising, but finding 16 multiresistant *S*. Typhimurium strains was unexpected, because the use of antimicrobial agents for growth promotion and as feed additives has never been allowed in Iceland. Because antimicrobial agents are not used in chicken production for any purpose, one would expect the prevalence of resistance to be low. This appears to be true because only three strains (13.6%) isolated from chickens proved resistant to antimicrobials and these three strains are thought to originate from feed. There are no data available on the therapeutic use of antimicrobials in pig production and therefore it is difficult to estimate its effect on the occurrence of resistance. Results from the present study show that 12.8% of the pig isolates were resistant to antimicrobials. The prevalence of antimicrobial resistance in Iceland is very similar to that in Denmark,[7] whereas this is a little higher than in Norway and Sweden (0% and 6% resistance of *Salmonella* spp. isolated from food producing animals).[15,17] However, the prevalence in Iceland is still low compared to southern and eastern Europe (over 70% resistance of *S*. Typhimurium isolated from pigs).[1,9]

Ten of the 16 multiresistant *S.* Typhimurium strains had identical PFGE and resistance profiles (X5A3, Amp,Sm,Te/Tc,Ff,Su,Cm) and were typed as DT104, a known multiresistant zoonotic pathogen.[20] All DT104 strains belonged to the same cluster, showing 94% overall similarity.

All but two of the DT104, X5A3 type isolates came from a single pig farm, and the remaining two isolates were from a single chicken farm. Both of these farms shared the same feed mill, and it is assumed that the feed mill was the source of those strains (Jarle Reiersen, Veterinary Officer for poultry diseases, personal communication).

Although previous research has shown that the endemic *S.* Typhimurium strains in Iceland are considerably diverse,[11] the DT104 strain appears to be prevailing among the antimicrobial-resistant strains, especially in pigs. It is also apparent that the occurrence of these multiple resistant isolates greatly influences the prevalence of resistance.

Because the prevalence of *Salmonella* spp. is low in Iceland, the number of strains available for analyses were limited. In spite of this low number of isolates, we believe that the results are representative for Iceland. The geographical distribution of samples was good; samples were taken from every flock and herd intended for human consumption and one strain of each serotype from all *Salmonella*-infected flocks or herds was analyzed. However, the same farms are often infected repeatedly, as was seen in the present research, where almost 80% of the resistant strains came from the same pig farm. These persistent infections greatly influence the prevalence of resistance.

The single *S.* Typhimurium strain resistant to enrofloxacin is a cause for concern because ciprofloxacin is used as the standard empirical treatment for suspected extraintestinal salmonella infections and serious gastroenteritis in humans.

These results also emphasize the need for more detailed data on the use of antimicrobial agents in production animals in Iceland. The relationship between antimicrobial consumption and resistance in animals needs to be evaluated further and the transmission of resistant *Salmonella* to humans.

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Address reprint requests to:
Dr. Eggert Gunnarsson
Institute for Experimental Pathology
University of Iceland, Keldur v/Vesturlandsveg
112 Reykjavík, Iceland

E-mail: eggun@hi.is

PAPER II

II

MICROBIAL DRUG RESISTANCE
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Veterinary Microbiology

Antimicrobial Resistance of *Campylobacter* Spp. Isolated from Broiler Flocks in Iceland 2001–2005

Thorunn R. Thorsteinsdottir,[1] Karl G. Kristinsson,[2] Vala Fridriksdottir,[1] and Eggert Gunnarsson[1]

Minimum inhibitory concentrations of six antimicrobial agents were determined for one *Campylobacter* sp. isolate from each of the 362 *Campylobacter*-positive commercial chicken flocks in Iceland in the years 2001–2005. Of all isolates tested, 6.9% were resistant, although none were multiresistant. Resistance to ampicillin was most commonly observed (3.6%) followed by resistance to enrofloxacin (3%), nalidixic acid (1.9%), and oxytetracyclin (0.3%), with cross-resistance between enrofloxacin and nalidixic acid. All isolates were susceptible to erythromycin and gentamicin. Resistance rates among *Campylobacter coli* isolates (7/13 or 53.8%) were much higher than among *Campylobacter jejuni* isolates (18/349 or 5.2%), and resistance patterns differed. Resistant strains were compared using pulsed field gel electrophoresis. Macrorestriction with *Sma*I and *Kpn*I restriction enzymes yielded 13 different pulsotypes, none of which indicated a predominant genotype. Specific pulsotypes with uniform resistance patterns arising on geographically separated farms indicate clonal dissemination. Although resistance levels were low and similar to that seen in the other Nordic countries, further research on this matter is needed as there is no antimicrobial selective pressure in chicken farming in Iceland.

Introduction

Campylobacter spp. have been identified as one of the major causes of human bacterial gastroenteritis worldwide.[2,20,26] Contaminated food is usually the source of human infections, and raw poultry meat appears to be the main risk factor.[2,6,12] Most human *Campylobacter* infections are self-limiting though some prolonged or severe cases do require treatment with antimicrobial agents. Generally, the drugs of choice are erythromycin for microbiologically diagnosed infections and fluoroquinolones for empirical treatment in adults. However, the increasing resistance in food-animal–related *Campylobacter* to fluoroquinolones is now considered an emerging public health problem and is thought to cause longer duration of illness and increased risk of adverse events.[5,9,16]

Series of interventions were introduced in Iceland in 2000, focusing on reducing *Campylobacter* in poultry products and consequent human campylobacteriosis, these included freezing of all *Campylobacter*-positive flocks before going to retail. Consequently, domestic cases of human campylobacteriosis dropped by 72%, from 116 cases per 100,000 in 1999 to 33 cases per 100,000 in 2000.[20] Subsequently efforts have been made to control *Campylobacter* in poultry production with considerable success, maintaining the prevalence in broiler chicken at slaughter around 15% during the years 2001–2005.[11,18] During this same period, the incidence of *Campylobacter* contamination in poultry retail products ranged from 5% to 23%, with the highest prevalence in 2003 and the lowest in 2005.[24,25]

Antimicrobial resistance rates of *Campylobacter* spp. in production animals are available for many countries,[1,3,8,21,27] but little is known about the prevalence of antimicrobial-resistant bacteria in Iceland. The aim of this study was to determine the antibiotic resistance levels of *Campylobacter* spp. isolated from commercial chicken flocks over a 5-year period in Iceland.

Materials and Methods

Bacterial isolates

A total of 390 *Campylobacter* isolates from healthy broiler chickens isolated from slaughter samples were included in the study. The isolates were collected in the Icelandic *Campylobacter* surveillance program at the Institute for Experimental Pathology, University of Iceland, Keldur. For this study, one isolate was selected from each and every *Campylobacter*-affected chicken flock detected in the years 2001–2005 and identified to species level on the basis of colony morphology,

[1]Institute for Experimental Pathology, University of Iceland, Reykjavik, Iceland.
[2]Department of Clinical Microbiology, Institute of Laboratory Medicine, Landspitali University Hospital, Reykjavik, Iceland.

microscopic appearance (including motility), oxidase, latex agglutination (PanBio, Baltimore, MD; Latex-Campy), indoxyl acetate hydrolysis, and hippurate hydrolysis. All *Campylobacter jejuni* and *Campylobacter coli* strains were tested for antimicrobial resistance.

Susceptibility testing

Antimicrobial susceptibility testing was performed using a microbroth dilution method (VetMIC™; SVA, Uppsala, Sweden) according to the National Committee for Clinical Laboratory Standards (NCCLS) Document M31-A2. Minimum inhibitory concentrations (MICs) for the following antimicrobials were determined: ampicillin (Amp), enrofloxacin (Ef), erythromycin (Em), gentamicin (Gm), nalidixic acid (Nal), and oxytetracyclin (Tc). For quality control, the *C. jejuni* ATCC 33560 reference strain was concurrently tested. Microbiological cutoff values were the same as those used in the Swedish monitoring program.[22] Strains showing resistance to one antimicrobial agent were considered resistant, and resistance to three or more agents was considered multiresistant.

Pulsed field gel electrophoresis (PFGE)

Resistant *Campylobacter* isolates were analyzed with PFGE as described by Ribot *et al*.[19] Restricted DNA fragments were separated by PFGE using the CHEF-DR II system (Biorad Laboratories, Mississauga, Ontario, Canada) on 1% SeaKem Gold agarose gels and electrophoresed for 18 hr using an initial switch time of 6.8 sec and a final switch time of 35.4 sec for *Sma*I (20U)–restricted plugs and an initial switch time of 5.2 sec and a final switch time of 42.3 sec for *Kpn*I (40U)–restricted plugs. A lambda ladder PFGE marker (New England Biolabs, Ipswich, MA) was used as a molecular weight standard. The gels were stained with ethidium bromide and photographed under UV transillumination with ChemiImager 5500 (Alpha Innotech, San Leandro, CA), and photos were saved as TIFF files. Analysis of DNA fragmentation profiles was performed by visual inspection, and for interpretation, the criteria suggested by Tenover *et al*.[23] were used. Macrorestriction patterns were compared using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), and cluster analysis using the Dice coefficient for band matching, with band-position tolerance of 1%, was used to generate a dendrogram with unweighted pair group method with arithmetic mean (UPGMA) clustering.

Statistical analysis

Frequencies of resistances were tested between isolates from various years using chi-square test for R×C contingency table, using InStat from GraphPad Software.

Results

Fifteen isolates were nonviable after storage at −75°C. The species distribution of recovered isolates was *C. jejuni* 93.0% (349 isolates), *C. coli* 3.5% (13 isolates), and *Campylobacter lari* 3.5% (13 isolates). Only *C. jejuni* and *C. coli* isolates were tested for antimicrobial susceptibility.

Antibiotic susceptibility

Of the 362 *C. jejuni* and *C. coli* isolates tested for antimicrobial susceptibility, 337 (93.1%) were susceptible to all the antimicrobials tested and none were multiresistant. The most commonly observed resistance was to ampicillin accounting for 3.6% of all isolates or 52% of resistant isolates (Table 1).

Resistance patterns differed between *Campylobacter* species. Of 349 *C. jejuni* strains tested, 18 (5.2%) were resistant to one or more antibiotics. Resistance to ampicillin was found in 13 (3.7%) strains, 1 (0.3%) was resistant to oxytetracyclin, and 4 (1.1%) were resistant to enrofloxacin, of which 3 (0.9%) were also resistant to nalidixic acid. Of the 13 *C. coli* isolates, 7 (53.8%) were resistant to one or more antibiotics. All seven were resistant to enrofloxacin, and four (30.8%) were also resistant to nalidixic acid.

Occurrence of resistance increased from the year 2001 to 2005, but the difference is not statistically significant, although there is a significant ($p = 0.0492$) linear trend between the years.

Pulsed field gel electrophoresis

Macrorestriction of resistant strains with *Sma*I yielded 12 different patterns of 4–14 fragments and with *Kpn*I yielded 13 different patterns of 7–14 fragments, respectively. With both enzymes, 13 pulsed field profiles were obtained from the 25 antibiotic-resistant strains, none of which indicated a predominant genotype (Fig. 1). Pulsotypes S11K11 and S12K12 consisted of *C. coli* isolates, and the remaining pulsotypes consisted of *C. jejuni* isolates. Although the two *C. coli* PFGE profiles form a cluster with >80% similarity, they are only considered possibly related as the patterns differ by four bands after *Kpn*I digestion. Two groups of isolates, PFGE profiles

Table 1. Antimicrobial Resistance in Thermophilic *Campylobacter* Spp. Isolated from Broilers in Iceland in the Years 2001–2005 ($N = 362$) (Concentration Range Tested and Cutoff Values)

			No. of resistant strains (%)		
Antimicrobial agent	*Range tested (mg/L)*	*Cutoff values*[a]	*All*	*Campylobacter jejuni*	*Campylobacter coli*
Ampicillin	0.5–64	>16	13 (3.6)	13 (3.7)	0 (0)
Erythromycin	0.12–16	>16	0 (0)	0 (0)	0 (0)
Enrofloxacin	0.03–4	>0.5	11 (3.0)	4 (1.1)	7 (53.8)
Gentamicin	0.25–8	>8	0 (0)	0 (0)	0 (0)
Nalidixic acid	1–128	>16	7 (1.9)	3 (0.9)	4 (30.8)
Oxytetracyclin	0.25–32	>8	1 (0.3)	1 (0.3)	0 (0)

[a]Microbiological cutoff values defining resistance.

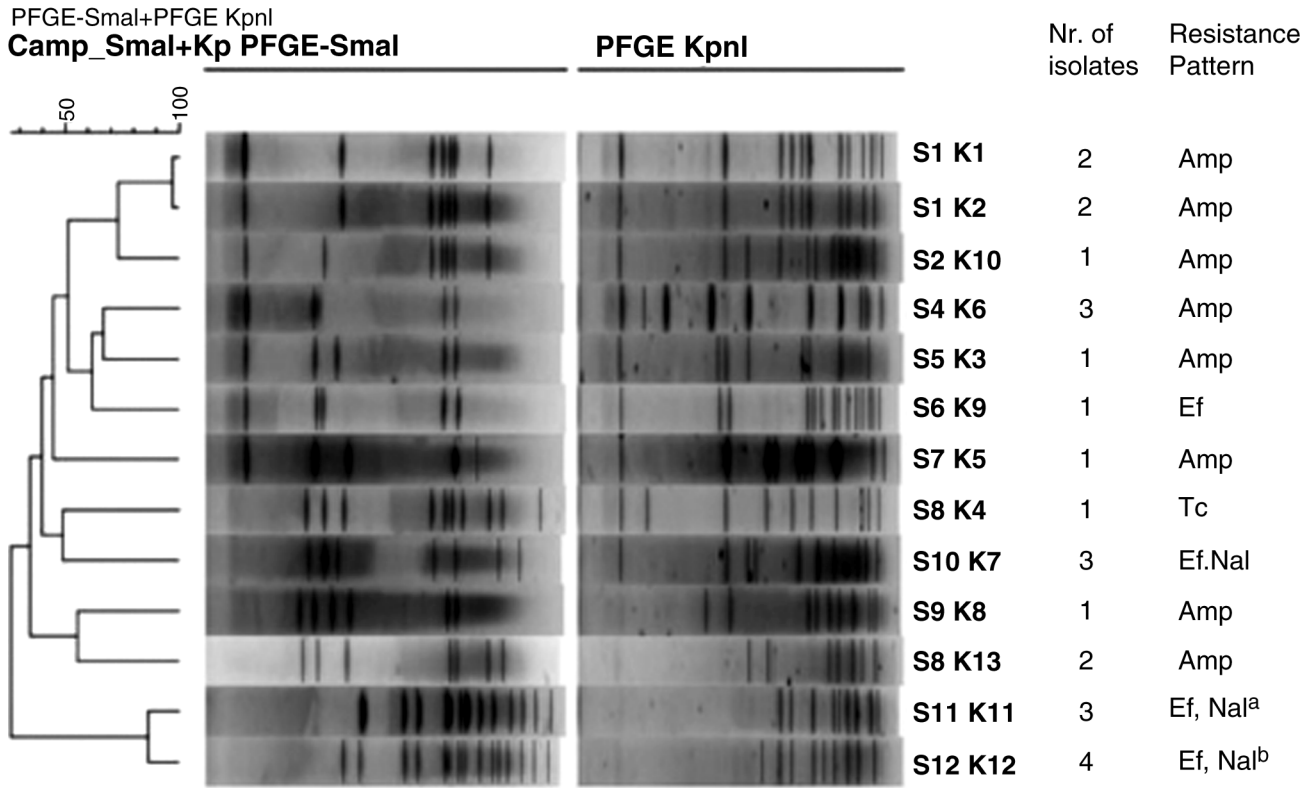

FIG. 1. Dendrogram showing the UPGMA cluster analysis of the different PFGE patterns, determined by *Sma*I and *Kpn*I digestion of DNA from *Campylobacter* isolates from broilers in Iceland 2001–2005. [a] = One isolate was resistant only to enrofloxacin. [b] = Two isolates were resistant only to enrofloxacin.

S1K1 and S1K2, with one-band difference between them form a clonal group with >95% similarity and are considered closely related. This clonal group includes four strains, all from the same farm, with strains in the S1K1 profile isolated in 2001 and strains in the S1K2 profile isolated in 2004.

Discussion

There is an increasing concern on the development of antimicrobial resistance in pathogenic bacteria. Thermophilic *Campylobacter* spp. are known to colonize different animal species and may be transferred from animals to humans. Results from previous studies indicate that human *Campylobacter* infections are most likely derived from poultry and raw milk (cattle).[7] The use of antimicrobial agents in animals and the subsequent development of antimicrobial resistance may therefore have consequences for the treatment of infections in humans.

In the present study, low level of antimicrobial resistance (5.2%) was observed among *C. jejuni* isolates from broilers, with resistance to ampicillin being the most prevalent. This is similar to the situation in other Nordic countries where resistance rates among *C. jejuni* isolates from broilers were around 5% to 13%,[4,17,21] whereas higher rates were seen in other European countries (>35% in France, the Netherlands, and United Kingdom).[3] The use of antimicrobial agents for growth promotion and as feed additives has never been allowed in Iceland, and antimicrobial agents are not used in chicken production for any purpose; therefore, the low level of resistance observed was expected. This low-level resistance, despite the absence of antimicrobial exposure, supports previous findings, suggesting that resistant isolates are stable in the broiler house environment and that they are able to transmit to and colonize the flocks without antimicrobial selection pressure.[10,14] It has been implied that antimicrobial-resistant *Campylobacter* isolates may arise in the flocks, possibly through integron-mediated gene exchange, after human contamination of the broiler house environment.[13]

It has also been previously observed that resistance rates are higher among isolates of *C. coli* than among isolates of *C. jejuni*,[1,10] as is seen in the present study, where 53.8% of the *C. coli* strains were resistant to one or more antimicrobial agents while resistance among *C. jejuni* strains was 5.2%. Moreover the resistance patterns were different between *C. jejuni* and *C. coli* strains, although both species exhibited cross-resistance between enrofloxacin and nalidixic acid. As there were only few *C. coli* isolates available for susceptibility testing, it is not possible to conclude whether there is a true difference among the species.

Gudmundsdottir *et al.*[7] found that there was a great diversity of *Campylobacter* strains in humans, animals, foods, and water in Iceland, where two pulsotypes were dominant and over 60% of the pulsotypes contained only one isolate. Similar results are obtained in the present study, where the 25 resistant strains are divided into 13 pulsotypes, of which 6 contain only 1 strain. On most farms, specific clones occurred only once, with the exceptions of one farm with 10 strains divided into six pulsotypes, a second farm with 4 strains of four different pulsotypes, and the third farm with 3 strains of the same pulsotype. Particular resistance patterns could be associated with different pulsotypes. Specific pulsotypes

could arise on different farms located in different parts of the country and slaughtering in separate slaughterhouses, indicating that antimicrobial-resistant *Campylobacter* clones are disseminated among chicken farms all over Iceland. This is further demonstrated by the fact that antimicrobial resistance patterns were generally uniform within the pulsotypes.

There are limitations to this study as there was only one strain isolated from each *Campylobacter*-positive flock, whereas some flocks may be infected by two or more different strains. Currently no internationally accepted standards are available for the susceptibility testing of *Campylobacter* spp. However, a high degree of agreement between different methods has been indicated in comparative studies. When studying the broth microdilution test, E-test, and the agar dilution method, Luber *et al.* produced comparable results and concluded the broth microdilution method to be a reliable method for determination of the MICs of antibiotics for *C. jejuni* and *C. coli*.[15]

In conclusion, this study is the first to provide information on the prevalence of antimicrobial-resistant thermophilic campylobacters in broiler chickens in Iceland and shows that the prevalence is low and similar to that seen in the other Nordic countries. Nevertheless, there is a need for continuing surveillance as resistance to fluoroquinolones was detected despite the lack of antimicrobial pressure, and further research is needed on resistance mechanisms and the vehicles of clonal dissemination.

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Address reprint requests to:
Thorunn R. Thorsteinsdottir
Institute for Experimental Pathology
University of Iceland
Keldur v/Vesturlandsveg
112 Reykjavík
Iceland

E-mail: thoruth@hi.is

PAPER III

III

Prevalence and genetic relatedness of antimicrobial resistant *Escherichia coli* isolated from animals, foods and humans in Iceland

Thorunn R. Thorsteinsdottir[1,2], Gunnsteinn Haraldsson[2,3], Vala Fridriksdottir[1], Karl G. Kristinsson[2,3] and Eggert Gunnarsson[1]

Institute for Experimental Pathology, University of Iceland, Keldur[1], Faculty of Medicine, University of Iceland[2], Department of Clinical Microbiology, Landspitali University Hospital[3], Reykjavik, Iceland.

Corresponding author:

Thorunn R. Thorsteinsdottir,

Institute for Experimental Pathology, University of Iceland, Keldur v/Vesturlandsveg, 112 Reykjavík, Iceland.

Tel: +354-585 5100.

Fax: +354-567 3979

E-mail: thoruth@hi.is

Keywords: Antimicrobial resistance, *Escherichia coli*, human, animal, food, Pulsed Field Gel Electrophoresis.

Bullet points:

- Antimicrobial resistance rates among *Escherichia coli* isolates in animals, foods and humans in Iceland are moderate and quite similar to the other Nordic countries. However, quinolone resistance in broiler chicken is of concern as there is no known antimicrobial selection pressure in chicken farming in Iceland.
- The same resistance pattern and DNA fingerprint pattern (PFGE) was found among isolates from broiler meat and a slaughterhouse worker at the slaughterhouse where these broilers were slaughtered and their meat processed. This indicates the spread of antimicrobial resistant *E. coli* from animals to humans.
- These findings emphasize the need for further study on the origin of the resistant strains and for continuing surveillance of antimicrobial resistance among bacteria from production animals and their food products in Iceland.

Summary

The prevalence of resistant bacteria in food products in Iceland is unknown and little is known of the prevalence in production animals. The aim of this study was to investigate the prevalence and genetic relatedness of antimicrobial resistant *Escherichia coli* from healthy pigs and broiler chicken, pork, broiler meat, slaughterhouse personnel and outpatients in Iceland.

A total of 419 *E. coli* isolates were tested for antimicrobial susceptibility using a microbroth dilution method (VetMIC) and resistant strains were compared using Pulsed Field Gel Electrophoresis (PFGE). All samples were screened for enrofloxacin resistant strains with selective agar plates.

The resistance rates among *E. coli* isolates were moderate to high from cecal and meat samples of pigs (54.1% and 28%), broilers (33.6% and 52%) and slaughterhouse personnel (39.1%), whereas isolates from outpatients showed moderate resistance rates (23.1%). Of notice was resistance to quinolones (MIC´s: nalidixic acid ≥32, ciprofloxacin ≥0.12 and enrofloxacin ≥0.5), particularly among broiler and broiler meat isolates (18.2% and 36%) as there is no known antimicrobial selection pressure in the broiler production in Iceland. The majority (78.6%) of the resistant *E. coli* isolates was genotypically different based on PFGE fingerprint analyses and clustering was limited. However, the same resistance pattern and pulsotype was found among isolates from broiler meat and a slaughterhouse worker indicating spread of antimicrobial resistant *E. coli* from animals to humans. Diverse resistance patterns and pulsotypes suggest the presence of a large population of resistant *E. coli* in production animals in Iceland.

This study gives baseline information on the prevalence of antimicrobial resistant *E. coli* from production animals and their food products in Iceland and the moderate to high resistance rates emphasize the need for continuing surveillance. Further studies on the origin of the resistant strains and the genetic relatedness of strains of different origin are needed.

Introduction

Antimicrobial resistance is a growing problem in human as well as veterinary medicine. The use of antimicrobial agents, both in animals and humans, is a major factor in the selection and dissemination of resistant bacteria. Food producing animals can act as a reservoir of resistant bacteria and animal food products are a major source of food-borne infections in humans. Transfer of resistant bacteria between animals and humans through food products has been documented and could pose a threat to public health (Angulo, et al., 2004; Mølbak, 2004; O'Brien, 2002).

A number of national and multi-national antimicrobial resistance surveillance systems have been initiated in the past years, both in human and veterinary medicine. Most of them incorporate *Escherichia coli* as it is a commonly found species in the commensal flora and as a pathogen in animals and humans. It is easy to cultivate and clear guidelines on susceptibility testing are available, making it a suitable bacterial species for determining resistance in commensals. Fecal contamination of carcasses during slaughtering is frequent and makes animal food product an important source of *E. coli*. Indications implicating food-products as a source for resistant *E. coli* infections in humans have been found (Garau, et al., 1999; Johnson, et al., 2006; Johnson, et al., 2005; Johnson, et al., 2003) and antimicrobial resistant *E. coli* strains are becoming more frequent among human clinical isolates and can lead to failure in treatment (European Antimicrobial Resistance Surveillance System, ; Lautenbach, et al., 2005; Lautenbach, et al., 2004). Furthermore, *E. coli* has been shown to be an important source for resistance elements (Zhao, et al., 2001) which could spread to other bacteria and possible pathogens (Winokur, et al., 2001).

Information on the use of antimicrobial agents in animal production in Iceland is limited although there should be no administration of these agents to broiler chicken. The prevalence of resistant bacteria in food products in Iceland is unknown and little is known of the prevalence in production animals. The aim of this study was to determine the prevalence and genetic relatedness of antimicrobial resistant *E. coli* strains isolated from healthy pigs and broiler chicken, pork, broiler meat, slaughterhouse personnel and outpatients.

Materials and methods

Sample collection

Pigs and broilers. Samples were collected from October 2005 to March 2006. Ceca from healthy animals were sampled aseptically at the slaughterhouses by the meat inspection veterinarian. The number of samples collected from each farm was proportional to the number of animals slaughtered per year. At the time of slaughter pigs were around 22 weeks old and broilers were about 34 days old. The average size of pig herds in Iceland is 250 piglets and the annual production is around 150 herds, The average size of broiler flocks is 10,000 broilers and annually are slaughtered around 600 flocks. Four geographically separated pig slaughterhouses participated in the collection of samples. Samples were taken from 11 pig herds from nine pig farms, accounting for approximately 85% of the total annual volume of pigs slaughtered in Iceland. From each herd, two to ten samples were taken, depending on the size of the herd. Three geographically separated poultry slaughterhouses participated in the collection of samples. Samples were taken from broiler flocks from eight farms, accounting for approximately 65% of the total annual volume of broilers slaughtered in Iceland. From each broiler flock one sample was taken, consisting of ceca from 20 broilers.

After finding higher prevalence of resistance in animals than expected we decided to further sample foods and humans.

Foods. Samples were collected from November 2006 to May 2007. Pork and broiler meat was sampled from meat processing plants, prior to packaging, by the meat inspection veterinarian. Pork samples were sampled from two meat processing plants, accounting for approximately 70% of the annual pork production in Iceland. During the sampling period herds from four farms were processed at the two plants. From each herd there were taken five to ten samples, depending on the size of the herd. All three poultry meat processing plants participated in the broiler meat sample collection. During the sampling period flocks from 17 farms were processed at the three plants. From each broiler flock one sample was taken.

Humans. Samples were collected during October 2006 to January 2007.

Isolates were cultured from routine diagnostic fecal specimens of outpatients presented with suspected diarrheal disease submitted to the Department of Clinical Microbiology, Landspitali University Hospital. Specimens from persons taking antimicrobials at the time of collection or recently traveling abroad or from non-Icelandic citizens were excluded. Fecal samples from healthy slaughterhouse personnel were obtained with a rectal swab and were transmitted to the laboratory in charcoal transport medium. Condition for the participation of slaughterhouse personnel was that they had to have lived in Iceland for at least 3 months and had not taken any antimicrobials for three months prior to sample collection. The study was approved by the National Bioethics Committee.

Bacterial isolation and identification

Pigs and broilers. Ceca (1 g) were suspended in 9 mL Phosphate Buffered Saline (PBS) and thoroughly mixed. Serial dilutions were made and 100 µL of the dilutions were spread on MacConkey agar and MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated overnight at 37°C.

Foods. Meat (25 g) was suspended in 225 mL of Brain Heart Infusion (BHI) broth and thoroughly mixed in a BagMixer laboratory blender (Interscience, St Nom La Breteche, France). Of this suspension 100 mL were incubated overnight at 37°C where after 10 µL were spread on MacConkey agar and 100 µL were spread on MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated again overnight at 37°C.

Humans. Fecal samples from humans were directly spread on MacConkey agar and MacConkey agar containing enrofloxacin (0.25 mg/L) and incubated overnight at 37°C.

From all samples of every origin one colony from each agar plate was selected for susceptibility testing. Colonies that were picked had to be lactose fermenting with morphology typical for *E. coli* on sheep-blood agar (5% v/v), Brilliant Green agar (BG), SSI enteric medium and Triple Sugar Iron agar (TSI) and positive for the production of tryptophanase (indole).

Susceptibility testing

Antimicrobial susceptibility testing was performed using a microbroth dilution method (VetMIC^{TM}, SVA, Sweden) according to the National Committee for Clinical Laboratory Standards (NCCLS) Document M31-A2. Minimum Inhibitory Concentrations (MIC) for the following antimicrobials were determined: ampicillin (Amp), cefotaxime (Ctx), ceftiofur (Ce), chloramphenicol (Cm), ciprofloxacin (Ci), florfenicol (Ff), gentamicin (Gm), nalidixic acid (Nal), kanamycin (Km), streptomycin (Sm), sulphamethoxazole (Su), tetracycline (Tc) and trimethoprim (Tm). Furthermore, MIC for enrofloxacin (Ef) was determined using an agar dilution method according to NCCLS Document M31-A2. For quality control the *E. coli* ATCC 25922 reference strain was tested concurrently.

The cut-off values were the same as those used in the Swedish monitoring program (SWARM 2004, 2005; SWARM 2006, 2007). Strains showing resistance to one or more antimicrobial agents were considered resistant and strains resistant to three or more agents were considered multiresistant.

Pulsed Field Gel Electrophoresis (PFGE)

Resistant *E. coli* isolates were analyzed with PFGE as previously described by Ribot et al (2006)(Ribot E.M., et al., 2006) with the following modifications. The DNA-agarose plugs were lysed overnight at 54°C. Plugs were then incubated with 25 U/plug *Xba*I restriction enzyme overnight at 37°C. A lambda ladder PFGE marker (New England Biolabs, Inc., Ipswich, MA) was used as a molecular weight standard. Analysis of DNA fragmentation profiles was performed by visual inspection and for interpretation the criteria suggested by Tenover *et al.* (1995) were used. Macrorestriction patterns were compared using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). For cluster analysis the Dice coefficient and band-position tolerance of 1.5% were used to generate an unweighted pair-group method with arithmetic averages (UPGMA) dendrogram.

Results

Prevalence

A total of 419 isolates of *E. coli* from pigs, broilers (ceca and meat) and humans were analyzed. Table 1 shows the number of samples taken and the percent of samples positive for *E. coli* (rate of recovery), the number of isolates obtained per origin and the prevalence of resistant isolates and of multiresistant isolates.

Antibiotic susceptibility

Of the 419 isolates analyzed 170 (40%) were resistant to one or more antimicrobial agents. Prevalence of resistance was highest in pig ceca (54.1%) and lowest in outpatients (23.1%). The percentage of isolates resistant to each antimicrobial agent is reported in Table 2. Resistance patterns of isolates of animal origin (ceca and meat) and human origin along with the number of isolates displaying each pattern are shown in Table 3.

Pigs. Of the total 109 pig ceca isolates, 59 (54.1%) were resistant to one or more antimicrobial agents and 24 (22.0%) of them were multiresistant. Resistance patterns were diverse although resistance to tetracycline was the most prevalent pattern (12 isolates). Lower prevalence was noted among pig meat isolates, where 14 (28.0%) isolates were resistant to one or more antimicrobial agents and three (6%) of them were multiresistant. No prevailing resistance pattern was detected.

Broilers. A total of 37 (33.6%) broiler ceca isolates were resistant to one or more antimicrobial agents, 16 (14.5%) of them were multiresistant and the most frequent resistance pattern was AmpCiNalTcSuTmEf (9 isolates). Prevalence of resistance was higher among broiler meat isolates, 39 (52.0%) isolates were resistant to one or more antimicrobial agents, 11 (14.6%) were multiresistant and the most prevailing resistance pattern was Amp,Ci,Nal,Tc,Su,Tm,Ef (5 isolates).

Humans. Nine of the 23 (39.1%) isolates from slaughterhouse personnel were resistant to one or more antimicrobial agents, five (21.7%) were multiresistant and most had unique resistance patterns. From outpatients, 12 (23.1%) isolates were resistant to one or more antimicrobial agents, of which five (9.6%) were multiresistant. Resistance patterns

were diverse although most of the resistant strains (66.7%) displayed resistance to ampicillin.

Pulsed Field Gel Electrophoresis (PFGE)

Of the 170 resistant isolates subjected to macrorestriction with *Xba*I 159 yielded interpretable PFGE patterns (data not shown). A total of 123 distinct PFGE profiles were detected and of these, 100 were unique profiles representing a single isolate. Isolates of different origin are considerably intermixed although they rarely cluster together. There were 12 clusters of mixed origin. Five clusters of isolates originating from broilers and broiler meat consisting of 13, four, three, three and two isolates each. Two clusters of isolates from slaughterhouse personnel and outpatients consisted of two and four isolates each. One cluster consisted of one swine isolate and one pork isolate. Another cluster contained three isolates from swine and broiler meat. There was also a cluster containing four isolates originating from pork and broiler meat. Furthermore there were two clusters, one consisted of two isolates from pork and an outpatient and the other of five isolates from broiler meat and a slaughterhouse personnel. The broilers, of which these isolates originated, were slaughtered at the slaughterhouse where the slaughterhouse employee was working. Different patterns were often seen on the same farm. Furthermore, the same PFGE pattern could occur on several different farms.

Discussion

The results of this study shows that resistance rates in *E. coli* from pigs and broilers (cecal and meat samples) and slaughterhouse personnel were moderate to high (54.1% and 28% for pigs, 33.6% and 52% for broilers and 39.1% for slaughterhouse personnel, respectively), whereas isolates from outpatients showed more moderate resistance rates (23.1%). This is somewhat similar to what has been reported from the other Nordic countries (pig fecal and meat 40% and 22.7%, broilers fecal and meat 34.9% and 41.4%) (NORM/NORM-VET 2004, 2005) and quite lower than in Spain (pigs > 75%, broilers 97.5%, foods > 85% and humans > 50%) (Saenz, et al., 2001).

High prevalence of resistance to flouroquinolones in animals and their meat products is surprising, especially in broilers where there is no known antimicrobial selection pressure. There clearly is a cross-resistance between ciprofloxacin and nalidixic acid and in most cases also to enrofloxacin, as previously demonstrated (Bazile-Pham-Khac, et al., 1996). Why a full cross-resistance between ciprofloxacin and enrofloxacin was not observed is unclear and will need further studying. Of notice is the prevalence of multiresistant isolates as 15% of the *E. coli* isolates had a multiple resistance phenotype. Again, this is somewhat higher than what is seen in the other Nordic countries (3% in broilers in Sweden) (SWARM 2007, 2008) although quite lower than in Spain (50% in broilers) (Saenz, et al., 2001). This is a cause for concern as studies have shown that the possibility of transferring resistance increases by increased multiresistance (Sunde and Norstrom, 2006). Previous studies have shown that *E. coli* isolates from healthy poultry frequently display high prevalence of multiresistance (Al-Ghamdi, et al., 1999; Saenz, et al., 2001). However, high prevalence of resistant and multiresistant isolates is most often seen in areas where the use of antimicrobial agents in poultry production is common (Schroeder, et al., 2004; van den Bogaard, et al., 2001), which is not the case in Iceland. The use of antimicrobial agents for growth promotion has never been allowed in Iceland and such agents have not been used for any purpose in broiler production for the last decade or so (Jarle Reiersen, former veterinary officer of poultry disease and Gunnar Örn Guðmundsson, District Veterinarian of Gullbringu-and Kjósarsýslu, personal communication).

High frequency of multiresistance among the *E. coli* isolates may be due to linkage of resistance genes on plasmids. In the present study certain combinations of resistances are seen more often than others (Table 3), further indicating that the resistance elements are associated and can be mobilized together. Furthermore, resistances to streptomycin and sulphamethoxazole were often associated, as has been documented before in *E. coli* from meat (Sunde and Norstrom, 2006). Resistance genes for streptomycin (*str*A-*str*B) and sulphonamides (*sul*2) are known to co-reside on nonconjugative plasmids (like pBP1) that are widely distributed in *E. coli* and have a broad host range (Sundin and Bender, 1996).

The majority (78.6%) of the resistant *E. coli* isolates was genotypically different based on PFGE fingerprint analyses and clustering was limited. Resistance patterns among *E. coli* of animal origin were diverse and this along with diverse pulsotypes suggests the presence of a large population of resistant *E. coli*. This also implies de novo acquisition of resistance determinants or mutations.

Isolates sharing the same PFGE patterns and the same resistance patterns can arise on different farms. However, it is not easy to point to a single source of those isolates as the farms are geographically separated, they are attended to by different veterinarians and the animals are slaughtered at different slaughterhouses. Furthermore, the broiler production in Iceland adheres to strict biosecurity measures to prevent the transmission of infectious agents or environmental contamination into the farms. Studies have shown that feeds can be contaminated by resistant *E. coli* (da Costa, et al., 2007), which could be a plausible explanation as many of the poultry farms and some of the pig farms in this study share a common source of feed. Additional genotyping and research on resistance elements could further elucidate the strain relatedness and origin.

We found the same resistance pattern and pulsotype among isolates from broiler meat and a slaughterhouse worker at the slaughterhouse where these broilers were slaughtered and their meat processed. This indicates the spread of antimicrobial resistant *E. coli* from animals to humans. This is in concordance with previous findings, that poultry workers and slaughterers are at a higher risk of colonization of antimicrobial resistant commensal bacteria, such as *E. coli*, because of their work (Price, et al., 2007; Stobberingh, et al., 1999; van den Bogaard, et al., 2001). Furthermore, we found considerable similarity (90%) between a pork isolate and an isolate from an outpatient although they did not share a common resistance pattern. Similarity between isolates from animals, animal food products and humans has been noted before (Johnson, et al., 2006; Johnson, et al., 2005; Ramchandani, et al., 2005).

In this study we examined antimicrobial susceptibility of *E. coli* isolates from pigs, broilers, pork, broiler meat, slaughterhouse personnel and outpatients. As isolates were collected from all major slaughterhouses and pig and broiler farms we believe this study provides a representative sample of the resistance trends in Iceland. However, this study had some potential limitations. Firstly, the number of isolates in the study is too small to

draw concrete conclusions. However, we do believe that the sample material is representative as the swine and broiler production in Iceland is very small and we sampled a good proportion of the annual production Selection bias might be a cause for concern as samples of different origin were collected at different time intervals although with some overlap because at first we only intended to collect isolates from animals. However, the higher than suspected prevalence lead to further sampling from foods and humans. This could explain why little clonal relatedness was seen among cecal and food strains. Genotyping of susceptible strains would also have given further information on strain relatedness and source of resistance. By selectively screening for enrofloxacin resistant isolates we were certain to find them if present in the sample. However, this makes the comparison of the resistance rates of fluoroquinolones to that of other antimicrobial agents difficult. Further study on the genetic determinants of resistance would be beneficial and transfer studies might clarify if these determinants are passed on together with others. Data on antimicrobial consumption among individual animal species is not available so evaluation of the effect of consumption on the arising of resistance is not possible. However, there should be no use of antimicrobial agents in broiler production.

In conclusion, the results of this study show a moderate to high prevalence of antimicrobial resistance among *E. coli* isolates from production animals and their food products and somewhat lower prevalence among slaughterhouse personnel and outpatients. Of special notice is the high prevalence in broilers and broiler meat where there is no known antimicrobial selection pressure. These findings emphasize the need for further study on antimicrobial consumption in animal husbandry, the origin of the resistant strains and for continuing surveillance of antimicrobial resistance among bacteria from production animals and their food products in Iceland.

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Table 1: Numbers of animal, meat and human samples and of *E. coli* isolates available for MIC testing and number of resistant isolates and multiresistant isolates.

Origin		No. of samples (% recovery)	No. of isolates	No. resistant isolates (%)	No. multiresistant isolates (%)
Pig	**Ceca**	99 (97)	109	59 (54.1)	24 (22.0)
	Meat	60 (73)	50	14 (28.0)	3 (6.0)
Broiler	**Ceca**	96 (100)	110	37 (33.6)	16 (14.5)
	Meat	50 (100)	75	39 (52.0)	11 (14.6)
Human	**Slaughterhouse personnel**	28 (75)	23	9 (39.1)	4 (17.4)
	Outpatients	65 (80)	52	12 (23.1)	5 (9.6)

Table 2: Antibiotic resistance of animal, meat and human *E. coli* isolates. [a]

	No of resistant strains (% of total)					
	Pig		Broiler		Humans	
Antibiotics	**Ceca n=109**	**Meat n=50**	**Ceca n=110**	**Meat n=75**	**Slaughterhouse Personnel n=23**	**Outpatients n=52**
Amp	23 (21.1)	4 (8.0)	20 (18.2)	12 (16.0)	6 (26.1)	8 (15.4)
Ctx	1 (0.9)	1 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)
Ce	1 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cm	6 (5.5)	0 (0)	0 (0)	0 (0)	1 (4.3)	0 (0)
Ci	14 (12.8)	4 (8.0)	20 (18.2)	27 (36.0)	3 (13.0)	0 (0)
Ef	13 (11.9)	3 (6.0)	16 (14.5)	25 (33.4)	3 (13.0)	0 (0)
Ff	0 (0)	0 (0)	1 (0.9)	0 (0)	0 (0)	0 (0)
Gm	0 (0)	0 (0)	2 (1.8)	1 (1.3)	0 (0)	0 (0)
Nal	14 (12.8)	4 (8.0)	20 (18.2)	27 (36.0)	3 (13.0)	0 (0)
Km	5 (4.6)	0 (0)	1 (0.9)	0 (0)	0 (0)	0 (0)
Sm	25 (23.0)	3 (6.0)	9 (8.2)	7 (9.3)	2 (8.7)	5 (9.6)
Su	31 (28.4)	6 (12.0)	21 (19.1)	11 (14.7)	3 (13.0)	6 (11.5)
Tc	36 (33.0)	7 (14.0)	15 (13.6)	8 (10.6)	5 (21.7)	5 (9.6)
Tm	14 (12.8)	4 (8.0)	16 (14.5)	10 (13.3)	3 (13.0)	3 (5.8)

[a] Resistance breakpoints: Ampicilin (Amp) ≥16, Cefotaxime (Ctx) ≥0.5, Ceftiofur (Ce) ≥2, Chloramphenicol (Cm) ≥32, Ciprofloxacin (Ci) ≥0.12, Enrofloxacin (Ef) ≥0.5, Florfenicol (Ff) ≥32, Gentamicin (Gm) ≥8, Nalidixic acid (Nal) ≥32, Kanamycin (Km) >16, Streptomycin (Sm) ≥64, Sulphamethoxazole (Su) ≥512, Tetracycline (Tc) ≥16, Trimethoprim (Tm) ≥4.

Table 3: Antibiotic resistance patterns of *E. coli* isolates of pig and broiler origin (cecal and meat samples) and human origin sorted according to their frequencies.

Origin	Resistance patterns[a]	Number of isolates[b]
Pig	Tc	14
	AmpCiNalTcSuTmEf	8
	Sm ; Su	5
	AmpSmSu	4
	AmpTc ; CiNalEf ; SmSu	3
	AmpCiNalSmTcSuTmCmEf ; AmpSmSuTm ; AmpSmTcSu ; CiNalTcEf ; SmTc ; SmTcSuTmCm	2
	Amp ; AmpSmKmSu ; AmpSmTcKmSu ; AmpSmTcKmSuTm ; AmpTcSuTm ; AmpTm ; CiNal ; CiNalTc ; CiNalCeSmTcKmCtxEf ; Ctx ; SmSuCm ; SmTcSu ; SuCm ; SuTm ; TcKm ; TcSu	1
Broiler	CiNalEf	20
	AmpCiNalTcSuTmEf	14
	Amp	9
	Sm	8
	Su	6
	CiNal	3
	AmpCiNalTcSuTm ; CiNalSmTmEf	2
	AmpCiNalGmSmTcKmSuTmEf ; AmpCiNalGmSmTcSuTmEf ; AmpCiNalSmSuTmEf ; AmpCiNalSuTm ; AmpCiNalTcFfSuTmEf ; AmpSmTcSu ; AmpTc ; CiNalTmEf ; GmSmSu ; SmSuTm ; TcSu ; TcSuTm	1
Human	Amp	4
	AmpSmTcSu ; Tc	3
	AmpCiNalTcEf	2
	AmpSmSu ; AmpSmSuTm ; AmpSmSuTmCm ; AmpSuTm ; AmpTc ; CiNalTmEf ; SmSu ; SuTm ; TcTm	1

[a]Resistance patterns are separated by a semicolon. [b]Number of isolates refers to the number of isolates displaying each resistance pattern.

PAPER IV

IV

Probable broiler-feed origin of fluoroquinolone resistant *Escherichia coli* isolated from broilers, broiler meat and humans in Iceland

Thorunn R. Thorsteinsdottir[1,2], Gunnsteinn Haraldsson[2,3], Vala Fridriksdottir[1], Karl G. Kristinsson[2,3], and Eggert Gunnarsson[1]

Institute for Experimental Pathology, University of Iceland, Keldur[1], Faculty of Medicine, University of Iceland[2], Department of Clinical Microbiology, Landspitali University Hospital[3], Reykjavik, Iceland.

Abstract

Investigating possible feed source of quinolone resistant *E. coli* in broilers we compared resistant broiler, broiler feed and meat isolates to ciprofloxacin resistant human clinical isolates by PFGE. Our results implicate feed as a source of ciprofloxacin resistance in broilers and chickens as a source of ciprofloxacin resistance in humans.

Previously we have found a relatively high prevalence of antimicrobial and quinolone resistance among *Escherichia coli* isolates from broilers and broiler meat (*1*), despite no known antimicrobial selection pressure in chicken farming in Iceland and biosecurity measures to prevent the transmission of infectious agents into farms. Therefore, it is unlikely that resistant bacteria persist in the broiler houses. However, animal feed can be contaminated with antimicrobial resistant *E. coli* (*2*).

This high prevalence of quinolone resistant *E. coli* isolates obtained from broilers and broiler meat coincides with an increasing prevalence of fluoroquinolone resistance among human clinical *E. coli* isolates in Iceland. This was correlated with increased use of fluoroquinolones in the clinical settings (*3*).

We examined changes in the prevalence of resistance between years and whether broiler feed could be a source for resistant *E. coli* strains in broilers. Furthermore, we compared the genotypes of ciprofloxacin resistant broiler, broiler meat and broiler feed *E. coli* isolates with ciprofloxacin resistant human clinical *E. coli* isolates.

The study

The sampling period was May to November 2008. Pooled cecal samples (20 ceca from each flock) were taken from all flocks slaughtered at all three broiler slaughterhouses in Iceland. Ceca were stomachered in Phosphate Buffered Saline, spread on MacConkey agar with and without enrofloxacin (0.25 mg/L) and incubated overnight. Feed was sampled from feed stalls at 18 farms (of which 14 participated in the previous study) and from two feed mills, suspended in Buffered Peptone water, mixed, incubated overnight and spread on MacConkey agar as described above. One colony from each agar plate was

selected for susceptibility testing as described previously (*1*). As isolates were collected from all major broiler farms, all the broiler slaughterhouses and the only two feed mills operating in Iceland we believe this study provides a representative sample.

From all human *E. coli* isolates, isolated from routine clinical specimens (mostly urine and blood) at the main clinical microbiology/reference laboratory in Iceland (Landspitali University Hospital) 2006-2007, we selected all available isolates (n=34) which had similar susceptibility patterns as the strains previously isolated from broilers (ampicillin – tetracycline – sulphamethoxazole/trimethoprim - ciprofloxacin or ciprofloxacin alone). Only one isolate was chosen from each patient.

Susceptibility testing was performed with a microbroth dilution method (VetMIC^{TM}, SVA, Sweden). Minimum inhibitory concentrations were determined for ampicillin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, nalidixic acid, kanamycin, streptomycin, sulphamethoxazole, tetracycline and, trimethoprim. The cut-off values were the same as those used in the Swedish and Norwegian monitoring programs (*4, 5*). Strains resistant to three or more antimicrobial classes were considered multiresistant.

All *E. coli* broiler ceca and feed isolates, resistant to one or more antimicrobials, and the 34 ciprofloxacin resistant human *E. coli* isolates, were compared to resistant *E. coli* isolates from the previous study (2005-2007) (*1*) using Pulsed Field Gel Electrophoresis (PFGE) using a slightly modified method of Ribot et al (2006) (*6*).

Comparison of PFGE patterns was by visual inspection and by the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). For cluster analysis, the Dice coefficient for band matching (band-position tolerance 1.5%) was used to generate a UPGMA

dendrogram. Isolates from both the previous and present study, not yielding a satisfactory banding pattern by PFGE, were genotyped by Randomly Amplified Polymorphic DNA (RAPD) analysis as previously described (*7*). Reaction products were analyzed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide. Patterns were considered to be different when the profiles differed by at least one band. Similarity among RAPD patterns was compared as described for PFGE. Clusters, for both PFGE and composite RAPD profiles, were defined as ≥2 isolates with ≥ 80% similarity. Resistance rates were compared with the Fisher´s Exact test.

Of the 40 broiler isolates 20 were resistant to one or more of the antimicrobials tested (Table 1), of which only one was multiresistant (resistant to streptomycin, tetracyclin, sulphamethoxazole and trimethoprim). Ciprofloxacin and nalidixic acid were always cross resistant. Compared with the previous sampling, the prevalence of resistance had increased significantly for ciprofloxacin and nalidixic acid (18.2% to 42.5%, $p<0.0001$), but had decreased significantly for ampicillin (18.2% to 0.0%, $p=0.002$) and sulphamethoxazole (19.1% to 5.0%, $p=0.0398$). This suggests that quinolone resistance was not transferred with resistance to the other antimicrobials and that it was selected for by other factors. Of the 22 *E. coli* isolates obtained from feed, seven (31.8%) were resistant to ciprofloxacin and nalidixic acid and all were susceptible to the other antimicrobials tested. Although no *E. coli* was isolated from the feed samples taken at the two feed mills, there was much growth of other Enterobacteriaceae on the agar plates which could possibly have overgrown existing *E. coli* strains, if any, demonstrating that the feed is not sterile.

The 27 resistant broiler and feed isolates were compared to 76 resistant isolates analyzed in the previous study (*1*) along with the 34 ciprofloxacin resistant human *E. coli* isolates. Out of 137 broiler, broiler meat, feed and human isolates, 110 yielded interpretable, reproducible PFGE patterns. There were 92 profiles detected, of which 81 were unique profiles represented by a single isolate. Isolates of different origin were intermixed forming 26 clusters, of which 12 were seen in the previous study. Of the 14 new clusters ten were of mixed origin (Figure 1). Human isolates clustered with broiler (2005-2006), broiler meat, broiler (2008) and feed isolates in six clusters (Figure 1). This supports previous findings of chicken and their products as a possible source of fluoroquinolone resistant *E. coli* in humans (*8, 9*). With the extensive genomic diversity of *E. coli* and the discriminative power of PFGE typing, finding indistinguishable isolates of different origin collected over several years is unlikely, except from a very large collection (*8, 10*). Therefore, finding human isolates closely related (≥80 similarity) to broiler, broiler meat and feed isolates is important and significant. To our knowledge, this is the first time that a close relationship has been found between broiler feed and human isolates. Additionally, we found closely related isolates from feed and broilers (both 2008 and 2005-2006 samples), supporting prevous findings, that antimicrobial resistant *E. coli* could be introduced into the farm environment through feedstuffs and poultry feed (*2*).

The isolates that did not give interpretable PFGE patterns (one broiler (2005-2006) and four broiler meat (2006-2007) isolates, 11 broiler (2008) and feed (2008) isolates and 11 ciprofloxacin resistant human isolates) were subjected to RAPD analysis. All yielded interpretable patterns displaying 26 distinct profiles, all but one unique profiles

representing a single isolate. At 80% similarity, one cluster was of mixed origin, containing six isolates from feed and broilers (2008).

Conclusions

Prevalence of fluoroquinolone resistant *E. coli* remains moderately high in broilers but resistance to other antimicrobials decreases. Fluoroquinolone resistant *E. coli* isolated from broiler feed, implicates feed as the source of resistant strains into farms. Resistant isolates from feed, broilers, broiler meat and humans were found to be closely related, demonstrating that poultry and their food products can be a source of resistant *E. coli* in humans.

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Ms Thorsteinsdottir is a biologist and a PhD fellow at the Institute for Experimental Pathology, University of Iceland, Keldur. Her primary research interest is antimicrobial resistance of bacteria, particularly in relation to zoonoses.

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Correspondence:

Thorunn R. Thorsteinsdottir,

Institute for Experimental Pathology, University of Iceland,

Keldur v/Vesturlandsveg,

112 Reykjavík, Iceland.

Tel: +354-585 5100.

Fax: +354-567 3979

E-mail: thoruth@hi.is

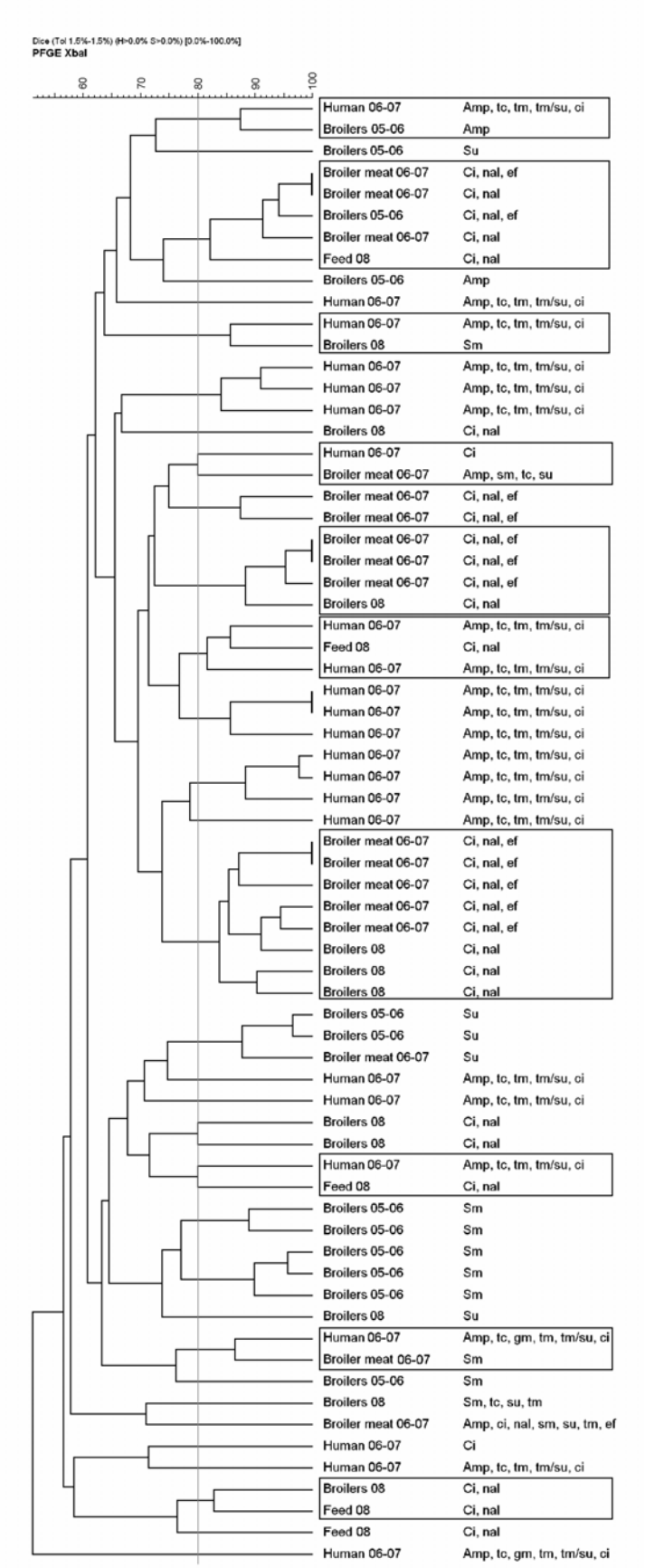
Table 1: Antimicrobial resistance of broiler and feed *E. coli* isolates compared to broiler ceca and meat isolates from a previous study in 2005-2007 (*1*) *

	No resistant strains (% total)			
	2005-2007		2008	
Antimicrobials	Ceca n=110	Meat n=75	Ceca n=40	Feed n=22
Ampicillin	20 (18.2)	12 (16.0)	0 (0)	0 (0)
Cefotaxime	0 (0)	0 (0)	0 (0)	0 (0)
Ceftiofur	0 (0)	0 (0)	0 (0)	0 (0)
Chloramphenicol	0 (0)	0 (0)	0 (0)	0 (0)
Ciprofloxacin	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)
Enrofloxacin	16 (14.5)	25 (33.4)	NT†	NT†
Florfenicol	1 (0.9)	0 (0)	0 (0)	0 (0)
Gentamicin	2 (1.8)	1 (1.3)	0 (0)	0 (0)
Nalidixic acid	20 (18.2)	27 (36.0)	17 (42.5)	7 (31.8)
Kanamycin	1 (0.9)	0 (0)	0 (0)	0 (0)
Streptomycin	9 (8.2)	7 (9.3)	2 (5.0)	0 (0)
Sulphamethoxazole	21 (19.1)	11 (14.7)	2 (5.0)	0 (0)
Tetracycline	15 (13.6)	8 (10.6)	1 (2.5)	0 (0)
Trimethoprim	16 (14.5)	10 (13.3)	1 (2.5)	0 (0)

* Resistance breakpoints: Ampicillin ≥16, Cefotaxime ≥0.5, Ceftiofur ≥2, Chloramphenicol ≥32, Ciprofloxacin ≥0.12, Enrofloxacin ≥0.5, Florfenicol ≥32, Gentamicin ≥8, Nalidixic acid ≥32, Kanamycin ≥16, Streptomycin ≥64, Sulphamethoxazole ≥512, Tetracycline ≥16, Trimethoprim ≥4.

† NT= Not Tested

Figure 1: Dendrogram based on UPGMA cluster analysis of PFGE patterns of the 20 broiler (2008), seven feed (2008) and 34 human ciprofloxacin resistant *E. coli* isolates along with 29 of the most closely related broiler (2005-2006) and broiler meat (2006-2007) isolates from the previous study. Boxes denote clusters (isolates with ≥ 80% similarity by Dice coefficient similarity analysis) of mixed origin. Antimicrobial abbreviations: amp=ampicillin, ci=ciprofloxacin, gm=gentamicin, nal=nalidixic acid, sm=streptomycin, su=sulphamethoxazole, tc=tetracycline, tm=trimethoprim and, tm/su=trimethoprim-sulfamethoxazole.


Dice (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE XbaI
60
70
80
90
100
Human 06-07 Amp, tc, tm, tm/su, ci
Broilers 05-06 Amp
Broilers 05-06 Su
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal
Broilers 05-06 Ci, nal, ef
Broiler meat 06-07 Ci, nal
Feed 08 Ci, nal
Broilers 05-06 Amp
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Broilers 08 Sm
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Broilers 08 Ci, nal
Human 06-07 Ci
Broiler meat 06-07 Amp, sm, tc, su
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broilers 08 Ci, nal
Human 06-07 Amp, tc, tm, tm/su, ci
Feed 08 Ci, nal
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broiler meat 06-07 Ci, nal, ef
Broilers 08 Ci, nal
Broilers 08 Ci, nal
Broilers 08 Ci, nal
Broilers 05-06 Su
Broilers 05-06 Su
Broiler meat 06-07 Su
Human 06-07 Amp, tc, tm, tm/su, ci
Human 06-07 Amp, tc, tm, tm/su, ci
Broilers 08 Ci, nal
Broilers 08 Ci, nal
Human 06-07 Amp, tc, tm, tm/su, ci
Feed 08 Ci, nal
Broilers 05-06 Sm
Broilers 05-06 Sm
Broilers 05-06 Sm
Broilers 05-06 Sm
Broilers 05-06 Sm
Broilers 08 Su
Human 06-07 Amp, tc, gm, tm, tm/su, ci
Broiler meat 06-07 Sm
Broilers 05-06 Sm
Broilers 08 Sm, tc, su, tm
Broiler meat 06-07 Amp, ci, nal, sm, su, tm, ef
Human 06-07 Ci
Human 06-07 Amp, tc, tm, tm/su, ci
Broilers 08 Ci, nal
Feed 08 Ci, nal
Feed 08 Ci, nal
Human 06-07 Amp, tc, gm, tm, tm/su, ci