Faculty of Sciences

Department of Biochemistry, Physiology and Microbiology

Laboratory for Microbiology

Antimicrobial resistance in human and broiler chicken *Escherichia coli* isolates

Jonas Ghyselinck

Promotor: Dr. Geert Huys
Co-Promotor: Drs. Davy Persoons, DVM

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Jonas Ghyselinck

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Abstract

Antimicrobial use in broiler chickens may select for antimicrobial resistant *Escherichia coli* that can be transmitted to humans. Two slaughter plants were sampled and *Escherichia coli* isolates were obtained from broiler chicken neck skins and intestines.

For every isolate, resistance was tested against amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur, chloramphicol, enrofloxacin, flumequin, florfenicol, gentamicin, nalidixic acid, neomycin, streptomycin, tetracycline and trimethoprim-sulfonamide. The *Escherichia coli* isolates were also screened for presence of ESBL genes CTX-M, TEM, SHV and OXA. Screening was performed using a PCR with specific primers, followed by gel electrophoresis. The obtained amplicons were sequenced to provide information about the ESBL subtype.

Two groups of human *Escherichia coli* isolates (hospital and community) were tested for their resistance against the before mentioned antimicrobial agents. The isolates were also screened for presence of the before mentioned ESBL genes. Obtained data from the veterinary and human *Escherichia coli* isolates were compared.

Finally, REP-PCR was performed for typing of the veterinary *Escherichia coli* isolates and comparing of resistance profiles.

Contents table

Abbreviations

ABC	ATP binding cassette
AD	Distilled water
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
BLAST	Basic local alignment and search tool
CAT	Chloramphenicol acetyltransferase
D	Dapsone
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMACA	Dimethylaminocinnamaldehyde
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
ERIC	Enterobacterial Repetitive Intragenic Consesus
ESBL	Extended Spectrum Beta-Lactamase
HIV	Human Immunodeficiency Virus
IRT	Inhibitor resistant TEM
IS	Insertion sequence
MATE	Multidrug and toxin extrusion
MFP	Membrane Fusion Protein
MFS	Major facilitator super
MIC	Minimal inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mRNA	Messenger ribonucleic acid
OMF	Outer Membrane Factor
PABA	para-Aminobenzoate
PBP	Penicillin Binding Protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM	Pyrimethamine
RAPD	Randomly amplified polymorphic DNA

REP	Repetitive extragenic palindromic
Rep-PCR	Repetitive PCR
RND	Resistance-nodulation-division
rRNA	Ribosomal ribonucleic acid
SD	Sulfadoxine
SMR	Small multidrug resistance
SMZ	Sulfamethoxazole
Taq	Thermus aquaticus
TBE	Tris boric acid
TMP	Trimethoprim
tRNA	Transfer ribonucleic acid
US	United States

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Introduction

The discovery of antimicrobials meant an evolution in the field of curing bacterial and fungal infections; substances, produced by micro-organisms to eliminate their competitors, could be applied for elimination of human and animal pathogens. This soon led to an overall use of these substances in medical applications.
Due to the overall use, and abuse, a selection pressure for organisms that own antimicrobial resistance genes occurred. Resistance genes are genes encoding resistance mechanisms that enable the organism to neutralise antimicrobial substances so that they cannot damage the cell. The origin of these genes can be found in the antibiotic producing organisms; they are not susceptible to the agent they produce.
Resistant micro-organisms are able to pass their resistance genes on to other micro-organisms, with the result that these will also become resistant against the agent. This is a major problem that we are facing today; the use of antimicrobials both in human and veterinary medicine, has led to a selection pressure inside the host resulting in survival of only resistant organisms.

To maintain their eliminating properties, the agents need to be modified, or new agents ought to be developed that are insensible to the organisms resistance mechanisms.

It is interesting to research the antimicrobial resistance in *Escherichia coli* because this organism is part of the intestinal flora in humans and animals. Results learn that these organisms are resistant to a large number of antimicrobial agents, which is of course a major problem. Moreover, a large number of isolates seem to be multiresistant; making antimicrobial treatments very difficult.

Part I

Literature

1. Antimicrobials

Antimicrobials are substances that are used to treat bacterial or fungal infections in people and animals. These components either kill micro-organisms (bactericidal) or stop them from reproducing (bacteriostatic), allowing the body's natural defence mechanisms to eliminate the invading organism. A differentiation has to be made between antibiotics and antimicrobial chemotherapeutics. Antibiotics are substances produced by micro-organisms, whilst chemotherapeutics are semi-synthetic (derived from antibiotics) or synthetic drugs.

Before the discovery of antibiotics, treatments often contained chemical compounds with also a high toxicity for the subject in therapy, whilst antibiotics usually have a high specificity for the target organism 'without' causing damage to the host. Absences of or differences in cell components between prokaryotic and eukaryotic cells, largely explain this latter thesis.
"Without causing damage to the host" should of course not be interpreted as such; there are numerous side effects that can occur during therapeutic treatment. Besides the respective interactions between antibiotics and bacteria and between the immune system and bacteria, antibiotics also directly interact with the immune system. Immunomodulatory effects of antibiotics include alteration of phagocytosis, chemotaxis, endotoxin release, cytokine production, and tumoricidal effects of certain cells. Moreover, some antibiotic agents can affect the life span of cells through inducing or inhibiting apoptosis (Jun *et al.*, 2003).

1.1 Classes of antimicrobials and their mode of action

There are several classification schemes for antimicrobials, based on bacterial spectrum, route of administration (injectable, oral, local, topical), or type of activity. The most useful however is based on chemical structure. In this section we describe the mode of action of these antibiotics for which the resistance profile for *Escherichia coli* was determined.
Different antibiotics will have different spectra. An overview is given in figure 1.

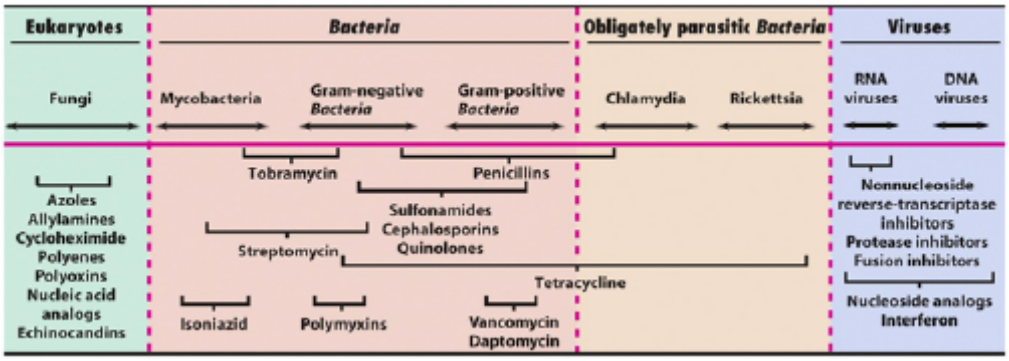

Fig. 1: Overview of the spectra of different classes of antibiotics (Madigan & Martinko, 2006).

1.1.1 Antibiotics

1.1.1.1 Aminoglycosides

Aminoglycoside antibiotics exhibit in vitro activity against a wide variety of clinically important gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., etc (Vakulenko & Mobashery, 2003). They lack activity against anaerobic micro-organisms. They are derived from bacteria belonging to the genus *Streptomyces* or *Micromonospora*.

Despite their nephrotoxicity (poisonous effect on the kidney), ototoxicity (damage to the ear related nervous system) and interference with Ca^{++} metabolism in the nervous system, these antibiotics remain valuable and sometimes indispensable for treatment of various infections (serious, life-threatening gram-negative infections, complicated skin, bone or soft tissue infections, complicated urinary tract infection, septicaemia). Aminoglycosides are effective even when the bacterial inoculum is large, and resistance rarely develops during the course of treatment. These potent antimicrobials are used as prophylaxis and treatment in a variety of clinical situations. Aminoglycosides exhibit several characteristics that make their use interesting, such as plasmaconcentration-dependent bactericidal activity, postantibiotic effect (period of time after seizing therapy during which there is no growth of the target organism) and synergism with other antibiotics. The bactericidal activity of aminoglycosides depends more on their concentration than on duration of bacterial exposure to inhibitory concentrations of the antibiotic (Vakulenko & Mobashery, 2003). The killing potential of aminoglycosides increases with increasing plasmaconcentrations of the antibiotic.

It has been proposed that penetration of aminoglycoside antibiotics into aerobically growing bacterial cells occurs in three steps. The first step is the energy independent binding of the positively charged aminoglycosides to the negatively charged parts of phospholipids, lipopolysaccharides and outer membrane proteins in gram-negative bacteria, and to phospholipids and teichoic acids in gram-positive bacteria. This binding results in displacement of Mg^{2+} and Ca^{2+} ions, that link adjacent lipopolysaccharides, resulting in damage of the outer membrane and enhancement of its permeability.
The energy independent first step is followed by a second one of actual uptake of the aminoglycoside, during which a transmembrane potential generated by a membrane-bound respiratory chains is required. Micro-organisms with deficient electron transport systems, such as anaerobes, can for this reason not be penetrated and are thus resistant to aminoglycosides.
It is thought that during this latter phase, only a small quantity of antibiotic molecules penetrate the cytoplasmic membrane, resulting in the binding of the antibiotic to the ribosome. This results in misreading of mRNA and production of inactive proteins. Some of these proteins are incorporated in the cytoplasmic membrane, resulting in loss of membrane integrity and leading to a cascade of events with increased uptake of aminoglycosides. During this last phase (also energy-dependent), additional quantities of aminoglycosides are transported through the damaged membrane. As a result, antibiotics accumulate rapidly in the cytoplasm and irreversibly saturate all ribosomes leading to inevitable cell death.
The higher the concentration of the aminoglycoside, the more rapid is the onset of the latter energy-dependent phase and subsequent bacterial death.

During protein synthesis, the ribosome decodes information from the mRNA and catalyzes incorporation of amino acids into a growing polypeptide chain. High accuracy during this process is achieved by the ability to discriminate between conformational changes in the ribosome, induced by binding of correct and incorrect tRNAs at the A site of the ribosome.
The kind of interaction with the ribosome depends on the type of aminoglycoside. Paromomycin, for example, increases the error rate of the ribosome by allowing incorporation of incorrect tRNAs. The antibiotic does not only inhibit protein synthesis, it also interferes with the assembly of the 30S ribosomal subunit. Streptomycin induces misreading of the genetic code, but the underlying mechanism is different.
Other aminoglycosides are neomycin, gentamicin, amikacin, netilmicin and tobramycin.

1.1.1.2 Penicillins

The class of the penicillins contains natural as well as synthetic agents. The antibiotics are derived from fungi (*Penicillium*). The penicillin family of antibiotics is divided into five categories (Miller, 2002): (1) natural penicillins, (2) penicillinase resistant penicillins, (3) aminopenicillins, (4) extended spectrum penicillins and (5) aminopenicillin/beta-lactamase inhibitor combinations.
The natural penicillins have the narrowest spectrum of activity: aerobic, gram-positive organisms.
Penicillinase resistant penicillins are synthetically composed penicillins. This group achieves their effectiveness by the addition of a large side chain to the penicillin molecule which prevents penicillinase (beta-lactamase produced by *Staphylococcus* spp.) from entering the penicillin molecule and cleaving the beta-lactam ring.
Aminopenicillins and extended spectrum penicillins are effective against a broader range of bacteria, including some gram-negative organisms such as *H. influenza*, *N. gonorrhoeae* and *E. coli* but ineffective against beta-lactamase producing organisms. Addition of beta-lactamase inhibitors, which brings us to the fifth group of penicillins, improves the spectrum of their activity. These inhibitors have no intrinsic antimicrobial activity and can work in two ways: (1) binding to the active site of the beta-lactamase enzyme, thereby preventing their attack on the beta-lactam ring and (2) enhancing the affinity of penicillin-binding proteins in bacteria, thereby facilitating breakdown of the bacterial cell wall.

The incidence of adverse response to penicillin ranges from 0,7 to 10 percent and may manifest in the immune, nervous, renal, gastrointestinal, integumentary (concerning the external covering of the body) and vascular system.

Penicillin and other beta-lactam antibiotics inhibit the growth of peptidoglycan-containing bacteria by inhibiting penicillin-binding proteins (PBPs). PBPs have transpeptidase and carboxypeptidase functions, and are involved in the late stages of peptidoglycan synthesis, the latter being an important cell wall polymer. Interference with its synthesis or structure leads to loss of cell shape and integrity.
Peptidoglycan cross-linking extends from the carboxy-terminal D-alanine residue at position 4 of a stem tetrapeptide to the lateral amino group at position 3 of another, unbranched or branched, stem peptide. The interpeptide linkages or cross-bridges are made by specialized acetyltransferases which are immobilized by penicillin (Goffin & Ghuysen, 2002). Initially, it was assumed that inhibition of these cross-linking reactions led to the existence of a

mechanically weakened cell wall, which would eventually burst due to increasing osmotic pressure. However, timely addition of penicillinase to a penicillin-inhibited culture could reinitiate culture growth; leading to the conclusion that the latter assumption was wrong; cell death is not due to rupture of the cell wall by osmotic pressure.

The model had to be revised and this led to the insight that killing of the bacterial cells by penicillin is due to autolysis (Novak *et al.*, 2000). Maintenance of the covalently closed peptidoglycan network requires enzymes capable of cleaving the cell wall during bacterial growth and cell separation. The roles of autolysins in the growth of *Bacillus subtilis* are now clear (Koch, 2001); they function by cleaving the outermost layer of the cell wall.

New layers of peptidoglycan are added just outside the cytoplasmic membrane and inside the existing layer of peptidoglycan. As additional layers are added, a given layer moves outward and is stretched as the cell grows. This stretching is of importance for the cleaving by autolysins: the cell's autolysins dissolve the outermost peptidoglycan most effectively when the peptidoglycan is stretched as far as its elastic limit will permit. These hydrolases can also act as suicidal enzymes, although this function seems strange. Nevertheless, when we place this in another context it seems more acceptable: prokaryotic cell death might be the single-celled organism's analogue that corresponds to the phenomena of apoptosis and altruism considered for the cells of multicellular organisms under the heading of programmed cell death.

This emphasizes the need for efficient and strict regulation of hydrolytic activity! Antibiotics like penicillin deregulate autolysin control, resulting in autolysis of the cell.

Remark: amoxicillin is often used in combination with clavulanic acid. The combination of amoxicillin and clavulanic acid specifically addresses the problem with beta-lactamase enzymes and penicillinases that destroy penicillin antibiotics. Clavulanate protects the amoxicillin by binding to these bacterial enzymes so they cannot destroy the beta-lactam ring structure that makes the penicillin molecule so effective (Brooks, 2001).

1.1.1.3 Cephalosporins

Cephalosporins belong, together with the penicillins, to the group of the beta-lactam antibiotics and are produced by *Cephalosporium* spp.. They differ from the penicillins in that way that penicillins have a beta-lactam ring attached to a thialazolidine ring with one side chain, while cephalosporins have a beta-lactam ring attached to a dihydrothiazine ring with two side chains (figure 2).

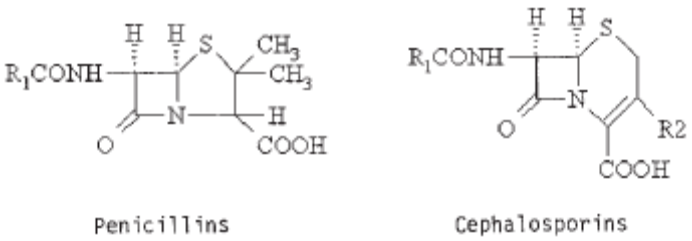

Fig. 2: Comparison of chemical structures of penicillins and cephalosporins (Hameed & Robinson, 2002).

Because of their similar structure, it is possible that a penicillin allergic patient will also react to cephalosporins. Typical allergic reactions are an abnormally low blood pressure, urticaria (lesions of the skin), dyspnea (difficult breathing), nausea (dizziness) and severe headaches.

There are different generations of cephalosporins:

- First generation cephalosporins possess excellent coverage against most gram-positive pathogens and variable to poor coverage against most gram-negative pathogens.
- Second generation cephalosporins show an extended gram-negative spectrum.
- Some members of the third generation cephalosporins have decreased activity against gram-positive organisms, but their gram-negative activity is expanded.
- Fourth generation cephalosporins eventually are extended-spectrum agents with similar activity against gram-positive organisms as first generation agents. They also have a greater resistance to beta-lactamases than the third generation cephalosporins.

As mentioned for the penicillins, these antibiotics also affect bacteria by two mechanisms targeting the inhibition of cell wall synthesis. Firstly, they are incorporated in the bacterial cell wall and inhibit the action of transpeptidase enzymes responsible for completion of the cell wall. Secondly they attach to the PBPs whose function amongst others is to suppress cell wall hydrolases, which in turn act to lyse the bacterial cell wall (Samaha-Kfoury & Araj, 2003).

In this work the resistance of *Escherichia coli* against ceftiofur is especially monitored. Ceftiofur is a third generation cephalosporin (extended-spectrum cephalosporin). These cephalosporins have been developed in response to the increased prevalence of β-lactamases in certain organisms and the spread of these enzymes into new hosts (Paterson & Bonomo, 2005).

A problem that occurred after introduction of these drugs was the introduction of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins. These lactamases were referred to as Extended Spectrum Beta Lactamases (ESBLs).

1.1.1.4 Tetracyclines

Tetracyclines are a group of antibiotics produced by *Streptomyces* spp. and active against a broad range of gram-positive and gram-negative bacteria; they inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (A-site). The wide spectrum, together with the fact that they don't cause major side effects, has led to their extensive use in the therapy of human and animal infections (Chopra & Roberts, 2001).
In some countries, tetracyclines are added to animal feeds, acting as growth promoters. The mechanisms responsible for growth promotion appear to include enhancement of vitamin production by gastrointestinal micro-organisms, elimination of subclinical populations of pathogenic organisms, and increased intestinal absorption of nutrients.
The result of this intensive use of tetracyclines has led to an increase in microbial resistance against this agent. Therefore the use of tetracyclines and other antibiotics as animal growth promoters is becoming increasingly controversial because of concerns that this practice leads to the emergence of resistance in human pathogens. In Europe, all use of antimicrobial feed additives has been banned since 2005.

To interact with their targets these molecules need to traverse one or more membrane systems, depending on the bacteria being gram-positive or -negative.
Tetracyclines traverse the outer membrane of gram-negative bacteria through specific channels as positively charged cation complexes (probably magnesiumtetracycline). This complex is attracted by the Donnan potential across the outer membrane. The Donnan potential is the result of solutions separated by a semi-permeable membrane: the smallest ions are able to pass through the semi-permeable membrane while the larger ones are retained, causing a charge imbalance between the two solutions. Eventually the energy required to bring about further separation of charges becomes too large to allow any further net diffusion to take place, and the system settles into an equilibrium state in which a constant potential difference is maintained (the Donnan potential).
The complex will accumulate in the periplasm, where the metal ion-tetracycline complex probably dissociates to liberate uncharged tetracycline, a lipophilic molecule able to diffuse through the lipid bilayer of the (inner) cytoplasmic membrane (Chopra & Roberts, 2001).

Within the cytoplasm, the drug binds reversibly to the ribosome, providing an explanation of the bacteriostatic effects of these antibiotics.

Remark: It has been established that the thia-tetracyclines and a number of other tetracycline analogs, collectively referred to as *"atypical tetracyclines",* exhibit a different activity from the majority of the tetracyclines. These molecules directly perturb the bacterial cytoplasmic membrane, leading to a bactericidal response. This differs from the typical tetracyclines, which interact with the ribosome and display a reversible bacteriostatic effect.
The atypical tetracyclines are trapped in the hydrophobic cytoplasmic membrane, disrupting its function. These molecules are therefore of no interest for therapeutic use; they show no selectivity for prokaryotic cell membranes and thus cause adverse side effects in human cells.

1.1.1.5 Chloramphenicol

Chloramphenicol, a broad-spectrum bacteriostatic antibiotic originally derived from *Streptomyces venezuelae*, has been used to treat severe infections for several decades. Its use in contemporary medical practice has fallen out of favour due to the adverse effects this agent causes. An important one is its bone marrow toxicity, but it can also inhibit mitochondrial protein synthesis in mammalian cells. Relatively uncommon, but possible, are skin rashes which occur as a result of hypersensitivity. Fever may appear simultaneously.
Angioedema, a rapid swelling of the skin, mucosa and submucosal tissues, can occur but this is very rare. Other adverse effects are nausea, vomiting, unpleasant taste, diarrhea and perineal irritation.
Due to these adverse reactions, therapy with chloramphenicol must be limited to infections for which the benefits of the drug outweigh the risks of the potential toxicities. When other antimicrobial drugs are available that are equally effective and potentially less toxic, these should be used.
The use of chloramphenicol in veterinary medicine has been banned since 1995, mostly because of increasing microbial resistance and possible impact on human health.

Chloramphenicol interferes with protein synthesis by binding reversibly to the 50 S ribosomal subunit; it blocks peptidyltranferase activity, binding and movement of ribosomal substrates through the peptidyltransferase center and translation termination (Xaplanteri *et al*., 2003). Peptidyltransferase is the enzyme that catalyzes the formation of a peptide bond between the α amino group of the second amino acid (which is present at the A-site of the ribosome)

and the first amino acid (present at the P-site of the ribosome). It thus covalently links amino acids during protein synthesis.
Two binding sites for this antibiotic have been reported in structures of antibiotic-ribosomal subunit complexes solved through X-ray crystallography (Long & Porse, 2003). In one complex, chloramphenicol binds to the A site. The position of the bound drug suggests that it hinders substrate binding directly by interfering with the positioning of the aminoacyl moiety in the A site. In the other complex , chloramphenicol binds at the entrance to the peptide exit tunnel. This binding site suggests that chloramphenicol inhibits protein synthesis.

1.1.2 Anti-infectious chemotherapeutics

1.1.2.1 Sulfa drugs

Severe allergic reactions to sulfa drugs are known. In some cases, e.g. when using drugs like sulfadoxine, such reactions can be life-threatening. The sulfa drugs are usually not allergenic by themselves, but when a sulfonamide molecule is metabolized in the body, it is capable of attaching to proteins, forming a larger complex that could serve as an allergen. Thus, the allergy is not due to the original drug, but to a drug-protein complex. It is estimated that a skin rash occurs in about 3.5% of hospitalized patients receiving sulfonamides, but people with HIV infection seem to have a considerably higher sensitivity (Dharmananda, 2005).
Stevens-Johnson syndrome is a severe hypersensitivity reaction that can be caused by sulfa drugs. This leads to epidermal blistering, necrosis (death of cells and living tissue) and sloughing (the act of casting off the skin, as do insects). Prognosis depends on how early the syndromes are diagnosed and treated. Mortality may reach 40%. The disorder affects between 1 and 5 people/million (Stevens-Johnson Syndrome (SJS), 2005).

Sulfa drugs are synthetic drugs that interfere with the de novo biosynthesis of folic acid by competing with *p*-aminobenzoate (PABA), the cosubstrate of dihydropteroate synthase (DHPS), to which they are structurally related. DHPS catalyzes the condensation of PABA and hydroxymethyldihydropterin-pyrophosphate to produce dihydropteroate, which is subsequently converted into dihydrofolate by dihydrofolate synthetase. Dihydrofolate is then reduced by dihydrofolate reductase into tetrahydrofolate, a cofactor essential for various biochemical pathways (Meneau *et al.*, 2004). This process is presented in figure 3.
The sulfa drugs sulfamethoxazole (SMZ), sulfadoxine (SD), and (D) inhibit the dihydropteroate synthetase (DHPS), whereas the diaminopyrimidines, trimethoprim (TMP)

and pyrimethamine (PM) are inhibitors of the dihydrofolate reductase (DHFR) (Nahimana *et al.*, 2004).

Remark: Sulfa drugs such as sulfamethoxazole are competitive inhibitors of DHPS and work synergistically with trimethoprim, which inhibits microbial DHFR.

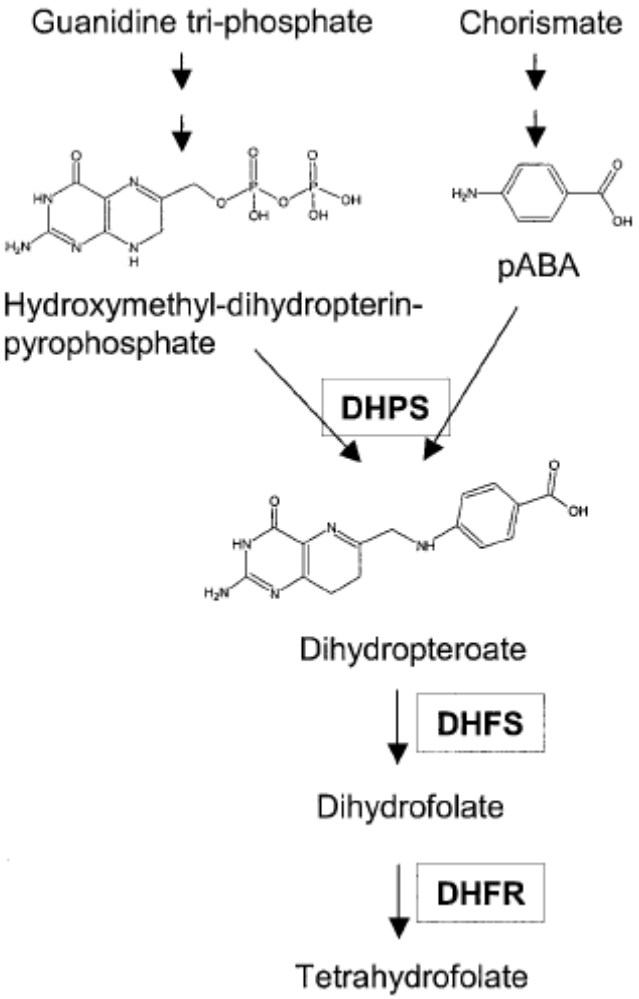

Fig. 3: Schematic representation of de novo folate biosynthesis (Meneau *et al.*, 2004).

Folate compounds are essential cofactors for the formation of purines and thymidine nucleotides and important precursors for DNA synthesis. Mammalian cells do not perform this de novo biosynthesis of folate; they possess a carrier-mediated active transport system for the uptake of performed folates. Thus, the de novo folate pathway is unique to non-mammalian cells, and as such provides a target for drug therapy.

1.1.2.2 Quinolones

Quinolone drugs are a widely used class of synthetic antibacterial agents. First generation quinolones include nalidixic acid and oxolinic acid. Subsequent generations have been modified to increase spectrum and potency. Fluoroquinolones have a fluoro group attached to the central ring system.

Quinolones used to be considered as being relatively safe, but several side effects have surfaced with extensifying use of quinolones. Examples of occurring effects are spontaneous tendon damage or ruptures and nerve damage. Nerve damage can result in paresthesia (sensation of pricking, numbness or tingling of a person's skin), hypoaesthesia (condition where the body is much less sensitive than normal to stimulation from such things as light, touch, or pain), dysesthesia (tactile hallucination; it signals that damage is being done to tissue when none is occurring) and weakness.
It has to be noticed that occurrence of these effects fortunately is quite rare!

DNA gyrase is a type II topoisomerase, and is the only topoisomerase that is able to introduce negative supercoils into DNA. Because this enzyme is absent in humans, gyrase is a successful target for antibacterial drugs. It acts by creating transient DNA breaks and facilitates DNA replication and other key DNA transactions (Aubry *et al.*, 2004).
DNA gyrase is a tetrameric A_2B_2 protein. The A subunit carries the 'breakage-reunion' active site, the B subunit promotes ATP hydrolysis needed for energy transduction. Quinolone drugs bind strongly to gyrase-DNA complexes, but only very little to either gyrase or DNA (Heddle & Maxwell, 2002). The exact interaction of quinolones with the gyrase-DNA complex still remains unclear. A proposition is that it results in additional stabilization of the quinolone-gyrase-DNA complex in the DNA-cleaved state (Heddle & Maxwell, 2002).

1.1.2.3 Florfenicol

Florfenicol is a synthetic, broad-spectrum fluorinated analogue of thiamphenicol. Like chloramphenicol and thiamphenicol, it shows activity against many gram-positive and gram-negative bacteria. Bacterial resistance to chloramphenicol and thiamphenicol is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase (CAT) enzymes. Due to the replacement of the hydroxyl group at position C-3 with a fluorine residue, the acceptor site for acetyl groups was structurally altered in florfenicol. This

modification rendered florfenicol resistant to inactivation by CAT enzymes, and consequently, chloramphenicol-resistant strains, in which resistance is solely based on CAT activity, are susceptible to florfenicol (Kehrenberg & Schwarz, 2006).

Florfenicol is a bacteriostatic antibiotic which interferes with protein synthesis. It binds to the 50S ribosomal subunit, inhibiting peptidyl transferase and thereby preventing the transfer of amino acids to growing peptide chains. The site of action of florfenicol is considered to be the same as that of chloramphenicol.

2. Antimicrobial resistance

The overall use and abuse of antimicrobials has created a rise in the number of resistant micro-organisms. Antimicrobials are sometimes used there where they do not have any curative potential; these agents are active against bacteria and some against fungi and parasites, but not against viruses or non-infected inflammation. Prescribing antimicrobials in case of a solely viral infection is futile. Correct use of antimicrobials in medical applications is thus important. Misuse can also be found in the feed industry, where antimicrobials such as tetracyclines were once used as growth promoting feed additives.

It is important, however, to remark that the evolution of a resistance mechanism must have involved very difficult step-by-step processes and long times because a series of mutations and very complex evolutionary pathways are generally required to create a totally new protein structure (Koch, 2003). However, it is widely accepted that antibiotic resistance genes may have originated in antibiotic-producing organisms in order to avoid the deleterious effect of the antibiotic on them. These genes could have further evolved in organisms in an ecological consortium with antibiotic producers. This way, the resistance genes were able to evolve further and eventually be transferred to other bacterial species.

The use of antimicrobials both in human and veterinary medicine, has led to a selection pressure inside the host resulting in survival of only resistant organisms. Resistant organisms are able to pass their resistance genes on to other organisms, which makes it possible for a resistant organism like *Escherichia coli*, which is part of the normal gut flora, to pass its resistance on to (facultative) pathogenic species like e.g. *Salmonella*

2.1 Important factors in antimicrobial resistance

2.1.1 Insertion sequences

Insertion sequences (IS) have two major characteristics: they are small compared to other transposable elements (generally around 0,7 to 2,5 kb in length) and many carry a single open reading frame encoding a transposase which catalyses the enzymatic reaction allowing the IS to move. Others carry several open reading frames, encoding products that may act as regulators in the transposition process. IS are thus different from transposons, which also carry accessory genes such as antimicrobial resistance genes. The coding region in an insertion sequence is usually flanked by inverted repeats.

IS may be present in one or several copies and can be localised on the chromosome, on plasmids or on both and are dependant of conjugative elements for intercellular transfer. IS elements may contain partial or complete promoters, and are capable of activating the expression of neighbouring genes. In this sense, IS have an effect on antimicrobial resistance genes. In contrast, insertion inactivation is the predominant effect of IS elements on genes involved in the modulation of resistance levels (Depardieu *et al.*, 2007).

As mentioned above, IS elements are capable of activating the expression of resistance genes. Transcriptional activation may result from IS insertion into a region carrying a weak, an incomplete or no promoter. The other effect that IS can cause is a disruption of resistance-modulating genes; IS elements may inactivate genes encoding proteins that modulate the efficiency of a given resistance mechanism. These proteins include multidrug efflux pumps, pores that condition antibiotic influx across the outer membrane in gram-negative bacteria, and others. IS-mediated gene disruption leading to pyrazinamide resistance in *Mycobacterium tuberculosis* has been reported (Depardieu *et al*., 2007). The susceptibility of this species to pyrazinamide is due to the production of the enzyme pyrazinamidase, which transforms the drug into a bactericidal derivative. Analysis of pyrazinamide-resistant organisms has shown that resistance is due to insertion of an IS into the gene encoding pyrazinamidase, leading to an inactivation.

2.1.2 Integrons

Integrons are genetic elements that are able to capture genes on small mobile elements (gene cassettes) in a process of site-specific recombination. They contain a recombinase gene (integrase) (*intI*), a recombination site (*attI*) and a promoter region that drives the expression of the cassette-associated genes (C1 & C2, figure 4).

The *attI* site is recognised by the integrase, and the incoming genes are incorporated at this site. To be inserted, incoming genes must be associated with a recombination site that is recognised by the integrase. Different 59-base elements function as recombination sites and can participate in recombination events involving either *attI* or a second 59-base element.

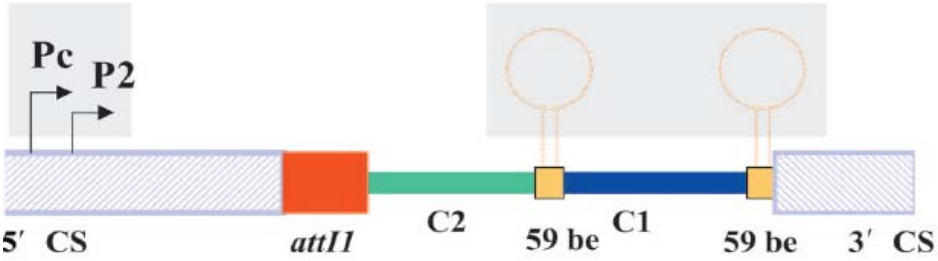

Fig. 4: Schematic representation of an integron structure (Depardieu *et al.*, 2007).

Integrons can be subdivided into two categories; the mobilised integrons and the chromosomal integrons. Cassettes that encode antimicrobial resistance are typically found in mobilized integrons.

Integrons are grouped in different classes according to their *intI* sequences. The class 1 integrons are the most abundant. The major part resides on transposons and conjugative plasmids, which is responsible for their wide distribution. The cassettes in this class of integrons encode a variety of enzymes, aminoglycoside-modifying enzymes, DHFRs, β-lactamases and chloramphenicol acyltransferases. More recently identified cassettes have shown to encode resistance to rifampin, quinolones and ESBLs (Depardieu *et al*., 2007).

2.1.3 Plasmid transfer

Bacterial conjugation is a highly specific process in which DNA is transferred from donor to recipient bacteria by a specialized multiprotein complex, referred to as the conjugation apparatus (Grohmann *et al*, 2003). Important for conjugative transfer is an intimate association between the cell surfaces of both cells. In gram-negative bacteria, this is established by sex pili; complex extracellular filaments. For the majority of gram-positive bacteria, the means to achieve this close cell-cell contact have not been achieved yet (Grohmann *et al.*, 2003). Gram-negative bacteria possess two very efficient barriers which have to be traversed by macromolecules during export from and import into the cell: the outer membrane and the inner membrane, which are separated by a cellular compartment, the periplasm. A transport channel is needed to cross the two membranes and the periplasmic space.

2.1.4 Conjugative transposons

Conjugative transposons are integrated DNA elements that excise themselves to form a covalently closed circular intermediate. This circular intermediate can either reintegrate in the same cell (intracellular transposition) or transfer by conjugation to a recipient and integrate into the recipient's genome (intercellular transposition).

Conjugative transposons were first found in gram- positive cocci but are now known to be present in a variety of gram-positive and gram-negative bacteria also. These elements have a surprisingly broad host range, and they probably contribute as much as plasmids to the spread of antibiotic resistance genes in some genera of disease-causing bacteria (Salyers *et*

al., 1995). Resistance genes need not be carried on the conjugative transposon to be transferred.

2.1.5 Regulation of resistance expression

It is essential for an organism, to be able to adapt to changing conditions in the environment. Signaling proteins, that promote information transfer within and between proteins, are important in this field. One such system, the 'two-component regulatory system', comprises two proteins: a sensor, usually located in the membrane, that detects certain environmental signals, and a cytoplasmic response regulator that mediates a response; usually a change in gene expression (Depardieu *et al.*, 2007). Communication between the two proteins occurs by the transfer of a phosphate group from a histidine residue of the sensor to an aspartate residue in the receiver domain of the regulator. Response regulators consist of a conserved domain of approximately 125 amino acids, attached by a linker sequence to a domain with an effector function. The effector domain generally has DNA binding activity and response regulator phosphorylation results in the activation of transcription. Response regulators thus act as transcriptional activators or repressors!

2.2 Mode of action

Resistance can be caused by different mechanisms (Fluit, Visser & Schmitz, 2001):

- presence of an enzyme that inactivates the antimicrobial agent,
- presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent,
- mutation in the target of the antimicrobial agent, which reduces the binding of the antimicrobial agent,
- posttranslational or posttranscriptional modification of the antimicrobial's target, which reduces the binding of the agent,
- reduced uptake of the antimicrobial agent,
- efflux pumps, actively pumping the antimicrobial agent out of the cell,
- overproduction of target of the antimicrobial agent.

Efflux pumps are described in more detail below.

2.2.1 Efflux pumps

The function of these efflux pumps is to pump out the antimicrobial agent, and thus limiting the intracellular accumulation of antimicrobial agents. The pumping out is energized by ATP hydrolysis or by an ion antiport mechanism. This mechanism confers, by a single mechanism, resistance to various drug classes.

The envelope of gram-negative bacteria consists of two membranes, separated by a periplasmic space, the gram-positive bacterial envelope consists of a single membrane (figure 5).

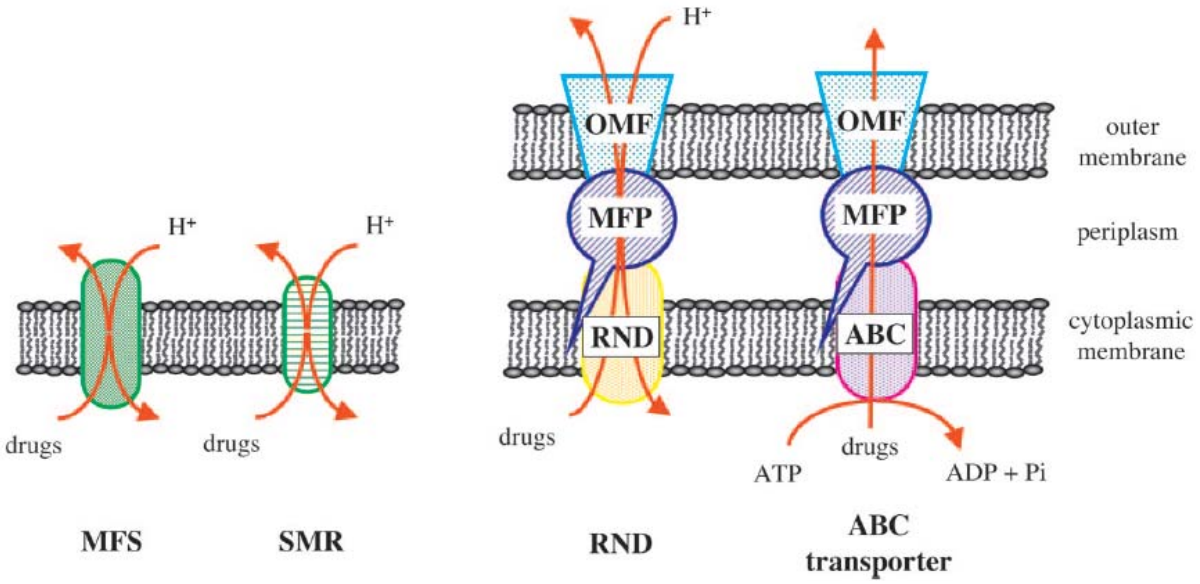

Fig. 5: Schematic representation of the cell membranes with examples of multidrug efflux systems (Depardieu *et al.*, 2007).

The membrane located transporters can be grouped into five categories, based on homology, mechanisms and molecular characteristics (Depardieu *et al.*, 2007): the ATP binding cassette (ABC) family, the major facilitator super family (MFS), the multidrug and toxin extrusion family (MATE), the resistance-nodulation-division (RND) family, and the small multidrug resistance (SMR) family.

OMF stands for Outer Membrane Factor and MFP is Membrane Fusion Protein. The illustration thus shows that in gram-negative bacteria, the efflux machinery is complex; comprising a cytoplasmic membrane-located transporter, a periplasmic membrane adaptor protein and an outer membrane channel protein.

Generally, drug-specific efflux pumps tend to be encoded by plasmids and are thus transmissible, whilst multi-drug resistance efflux pumps are usually encoded on the chromosome.

2.3 Mechanisms for the different classes of antibiotics

2.3.1 Aminoglycosides

Resistance to these agents is caused by aminoglycoside-modifying enzymes and is widespread. Most of the genes coding for these enzymes are associated with gram-negative bacteria. Depending on the modification they cause, these enzymes are classified as aminoglycoside acetyltransferases, aminoglycoside adenyltransferases and aminoglycoside phosphotransferases. Aminoglycosides modified at amino groups by the first group of enzymes or at hydroxyl groups by the latter two enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis.
Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described.

2.3.2 β-lactam antibiotics

Resistance is most often caused by the presence of β-lactamases, but mutations in PBP's, resulting in reduced affinity for β-lactam antibiotics, are also observed. Resistance is less frequently caused by reduced uptake due to changes in the cell wall or active efflux.
Genes encoding β-lactamases can be located either on plasmids or the bacterial chromosome and are found among both gram-positive and gram-negative organisms. Plasmids play a major role in bacterial resistance spreading. Their transferability is responsible for many outbreaks of resistance (Samaha-Kfoury & Araj, 2003).
In gram-positive bacteria, β-lactamases are secreted to the outside membrane environment as exoenzymes. In gram-negative bacteria, they remain in the periplasmic space where they attack the antibiotic before it can reach its receptor site.
β-lactamases destroy the β-lactam ring by two mechanisms of action. Most common β-lactamases have a serine based mechanism of action. These enzymes contain an active site consisting of a narrow longitudinal groove with a cavity which is loosely constructed in order to have conformational flexibility in terms of substrate binding. Close to this lies the serine

residue that irreversibly reacts with the carbonyl carbon of the β-lactam ring, finally resulting in an open ring and regenerating the β-lactamase (Samaha-Kfoury & Araj, 2003).
A less common group of β-lactamases are the metallo-β-lactamases. These use a divalent ion linked to a histidine or cysteine residue or both to react with the carbonyl group.

Because of the existence of these β-lactamases, and the rising resistance of organisms against the β-lactam agents, alternative antimicrobials had to be developed and different generations of β-lactam antibiotics arose. However, the activity of the β-lactamases expanded, even against the third and fourth generation cephalosporins. These new β-lactamases are called extended spectrum β-lactamases (ESBLs) and they have evolved from point mutations altering the configuration of the active site of the original β-lactamases (designated TEM-1, TEM-2 and SHV-1) (chapter 5, part I: Literature). The ESBL producing bacteria are typically associated with multidrug resistance, because genes coding for resistance against other agents often reside on the same plasmid as the ESBL gene. Consequence of this is that some ESBL producing organisms are also resistant to quinolones and aminoglycosides.

2.3.3 Tetracyclines

There are two important tetracycline resistance mechanisms which do not destroy the compound: efflux and ribosomal protection. Efflux is mediated by energy-dependent efflux-pumps, the other mechanism involves a protein that confers ribosome protection.
Oxidative destruction of tetracyclines has been found in a few species (Fluit, Visser & Schmitz, 2001).

Twenty-nine different tetracycline resistance (tet) genes and three oxytetracycline resistance (otr) genes have been characterised (Chopra & Roberts, 2001). The genes involved in the efflux resistance mechanism code for membrane-associated proteins which export tetracycline from the cell. Export of the agent reduces the intracellular drug concentration and thus protects the ribosomes.
The ribosome protection genes code for a protein that interacts with the ribosome in a way that protein synthesis is unaffected by the presence of the antibiotic. Ribosome protection proteins confer a wider spectrum of resistance to tetracyclines than is seen with bacteria that carry tetracycline efflux proteins, with the exception of Tet(B) (Chopra & Roberts, 2001).

There are six groups of membrane-bound efflux proteins, based on amino acid sequences.

The group one gram-negative efflux genes are widely distributed and normally associated with large plasmids. These plasmids often carry other antimicrobial resistance genes, heavy metal resistance genes and/or pathogenic factors such as toxins. Thus, selection for any of these factors selects for the plasmid. This phenomenon of cross-selection has contributed to the increase in the number of multiple-drug-resistant bacteria.

The gram-negative efflux system consists of two genes, one coding for an efflux protein and one coding for a repressor protein. Both genes are regulated by tetracycline. In the absence of tetracycline, the repressor protein blocks transcription of the structural genes for both the repressor and the efflux protein. Induction in the system occurs when a tetracycline-Mg^{2+} complex enters the cell and binds to the repressor protein. Drug binding changes the conformation of the repressor so that it can no longer bind the operator region, with transcription of the efflux gene and repressor gene as a consequence. Production of the repressor will result in rebinding of this protein when tetracycline concentrations in the cell are low.
No repressor proteins have been found in genes of gram-positive bacteria. These genes are regulated by translational attenuation.

2.3.4 Sulfa drugs

Resistance to sulfa drugs occurs through mutations in the gene encoding DHPS, leading to an amino acid change in the enzyme. These mutations are located in the sulfa binding site of DHPS, leading to reduced binding of the drugs to the enzyme and reduced susceptibility to the antimicrobial (Nahimana *et al.*, 2004).

Alteration of DHFR enzyme is a common resistance machanismin clinically important microbial pathogens such as *Plasmodium falciparum* and *Streptococcus pneumoniae* (Nahimana *et al*., 2004).

2.3.5 Fluoroquinolones

Resistance mechanisms to these antibiotics fall into two categories: alterations in drug target enzymes and alterations that limit the permeation of the drug to the target (Fluit, Visser & Schmitz, 2001). Alterations of target enzymes appear to be the most dominant factors in expression of resistance.

Resistance mechanisms affecting the DNA gyrase enzyme involve changes in amino acid composition in regions of the enzyme that are involved in its transient covalent binding to the DNA phosphate groups during the enzyme's DNA strand-passing reactions. The amino acid substitutions responsible for the antimicrobial resistance consist of the replacement of a hydroxyl group with a hydrophobic group; a replacement that may be important for quinolone-DNA gyrase interaction.

DNA gyrase and type II topoisomerase are located in the cytoplasm of the bacterial cell. Thus, to reach their target, fluoroquinolones have to traverse the cell envelope. Decreased uptake due to changes in the cell envelope (particularly in the outer membrane) has been demonstrated with gram-negative bacteria. This mechanism of resistance has not yet been found in gram-positive bacteria (Fluit, Visser & Schmitz, 2001).

2.3.6 Chloramphenicol

Resistance to chloramphenicol is generally due to inactivation of the antibiotic by a chloramphenicol acetyltransferase (CAT). The gene encoding this enzyme is most commonly found on plasmids. CAT catalyses transfer of the acetyl moiety from acetyl coenzyme A to a chloramphenicol molecule. This modified chloramphenicol no longer binds to the ribosomes and protein synthesis is no longer inhibited!
Regulation of the gene encoding chloramphenicol acetyltransferase occurs at posttranscriptional level. An inverted repeat structure preceding the CAT-coding region plays an important role in this mechanism; mRNA transcribed from this inverted repeat could form a stable stem-loop in which the ribosome binding site of the cat gene is present. As a consequence, the mRNA cannot be translated because no base pairing can occur between the cat Shine-Dalgarno and the 16S rRNA. Induction is accomplished by opening this stem-loop or hindering its formation. This conformational change is mediated by ribosomes modified by the inducing antimicrobial agent (Brückner & Matzura, 1985).
Another mechanism of resistance in both gram-negative and gram-positive bacteria is the presence of efflux pumps. This mechanism however, can only provide low-level resistance to the organism.

Sometimes decreased outer membrane permeability or active efflux is observed in gram-negative bacteria (Fluit, Visser & Schmitz, 2001). Kehrenberg & Schwarz (2005) mentioned an rRNA methylase which methylates 23S rRNA.

2.3.7 Florfenicol

Bacterial resistance to chloramphenicol is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase (CAT) enzymes. As mentioned earlier, the replacement of the hydroxyl group at position C-3 with a fluorine residue alters the acceptor site for acetyl groups in florfenicol. Due to this modification florfenicol becomes resistant to inactivation by CAT enzymes.

The use of florfenicol has been restricted to veterinary purposes only, and monitoring studies have indicated that virtually all target bacteria isolated from respiratory tract infections of cattle and pigs were susceptible to florfenicol. However, a first florfenicol resistant *Pasteurella multocida* isolate that carried a plasmid-borne *floR* gene, coding for a chloramphenicol/florfenicol exporter, has been detected (Kehrenberg & Schwarz, 2005).

Other resistance mechanisms have also been discovered. The *cfr* gene, coding for an rRNA methylase, which mediates resistance to chloramphenicol and florfenicol by methylation of the 23S rRNA, and *fexA*, encoding a protein which represents a novel type of efflux protein. Its substrate spectrum contains only florfenicol and chloramphenicol. Both genes are plasmid-encoded.

3. Antimicrobial resistance in poultry flocks and risk of transfer to humans

3.1 Illustrating the problem

Over the past half century, food-animal production has changed from small-scale, individual farms to large-scale industries; a mode of production in which a small number of companies control all aspects of production, from breeding and feeding to slaughter and distribution of consumer products. High numbers of animals are grouped together in one house, providing the possibility for micro-organisms to easily 'travel' from one host to another and infect all animals within the same flock.
The use of antimicrobials in food production became controversial because of data suggesting that usage may lead to an increase in drug resistant human pathogens. Long term use of antimicrobials in animal production industries for therapeutic and growth promotion purposes, created a selective pressure; an environment in which only resistant organisms can survive. Since elements such as plasmids and transposons are common vectors for the spread of antimicrobial resistance genes, bacteria can acquire resistance genes through horizontal gene transfer. Commensal and environmental bacteria, in environments where antimicrobial usage occurs, might thus form a reservoir for the transfer of antimicrobial resistance genes to pathogenic bacteria. Different resistance elements are often clustered on plasmids or on the chromosome. Selection of one resistance gene may therefore lead to selection of other resistance genes, not under direct selection pressure. The phenomenon of clustering of resistance genes also ensures the inheritance of all resistance elements.

Research in this field has brought a number of insights. A few topics are described below, to picture the problem of rising antimicrobial resistance.

Smith *et al.* (2007) found a high prevalence of resistance to tetracycline, sulfonamides and streptomycin in flocks of chickens, although these drugs were not used in most cases. This means that even in controlled settings with clean pens and fresh bedding, there was a high prevalence of resistance to antimicrobials not commonly used in broiler chicken industry. This is in accordance to other studies, implying that antimicrobial resistance may not correlate with antimicrobial usage. Miles, McLaughlin and Brown (2006) reported that bacteria in the soil could acquire resistance to tetracycline from environmental exposure,

creating a reservoir of resistance factors generated outside host animals. Environmental exposure can be due to contact with animal wastes, animal bedding, air both inside and downwind of animal feeding operations, in groundwater contaminated with resistant organisms, use of litter as fertiliser, etc. Certain organisms are able to survive in this litter. Floors of chicken houses are covered with a bedding material of softwood shavings that, during maturation of each flock, becomes mixed with chicken faeces, urine, skin and feathers. The resulting mixture is called litter. Some companies remove this litter from the house prior to every new flock, others place fresh bedding on top of used litter and replace the litter a few times a year. The co-evolution of *E. coli* populations and the antimicrobial resistance gene load in litter may have a greater influence on prevalence of antimicrobial resistance than antimicrobial usage alone has. According to Smith *et al.* (2007), previous studies have shown that the litter contained the same antimicrobial resistance genes that were detected in the commensal *E. coli* strains. The litter environment can thus serve as a reservoir for antimicrobial resistance gene carriage and genetic exchange among abundant members of the litter bacterial community.

Miles, McLaughlin and Brown (2006) concluded from their research that there was significant antimicrobial resistance of *E. coli* isolates from broiler chickens raised on farms without recorded antimicrobial use. However, Bazile-Pham-Khac *et al.* (1996) investigated the resistance to fluoroquinolones in *E. coli* isolates from poultry and concluded that the introduction of the antibiotic in veterinary medicine in Saudi Arabia meant an increase in fluoroquinolone resistance. In the year following the introduction, the proportion of quinolone-resistant strains isolated by diagnostic laboratories increased with more than fifty percent.

Kariuki *et al.* (1999) researched resistance patterns in *E. coli* strains isolated from children living in close contact with chickens. The majority of the isolates from children were multidrug resistant, while the majority of the isolates from chickens were either fully susceptible or resistant only to tetracycline. Further they also learned that the isolates in children were different from the isolates in chickens; meaning that periods of feeding and collecting eggs were not sufficient to allow colonization of the children with *E. coli* from chickens. However, Linton *et al.* (1977) reported that colonization of the intestinal tract with resistant *E. coli* from chickens had been shown in human volunteers.

A study performed by Price *et al.* (2007), to assess the risk for colonization with antimicrobial-resistant *E. coli* from occupational exposure to live chickens in the broiler chicken industry, showed that these workers have a great risk in getting colonized by antimicrobial resistant *E. coli.* Evidence was provided by colonization with gentamicin-resistant *E. coli*. Gentamicin cannot be administered orally and is therefore minimally used in medical applications. This means that there is a minimal selection of gentamicin resistant *E.*

coli in the community. Nevertheless, fifty percent of the poultry workers were colonized with gentamicin resistant *E. coli.* Knowing that gentamicin has been reported to be the most commonly used antibiotic in broiler production in the US, the results of this study are beyond doubt. Moreover, the results became more clear when comparing these results with the proportion of community referents colonized with gentamicin resistant strains (3%) and hospital isolates (6,3%). This study thus shows the possibility of transfer of resistant strains from animals to humans during exposure.

3.2 Spread of antimicrobial resistance

Poppe *et al.* (2005) studied the possibility of gene transfer between micro-organisms. Turkeys were dosed with *Escherichia coli* harbouring a plasmid encoding the CMY-2 β-lactamase and other drug resistance determinants. Their study showed that 25,3% of *Salmonella enterica* subsp. *Enterica* serovar Newport acquired the plasmid and other drug resistance genes. The plasmid containing the *cmy-2* gene was transferred not only from the donor *E. coli* to *Salmonella*, but also to another *E. coli* serotype present in the intestinal tract. According to the authors, this is a demonstration of the ease with which transfer of resistance genes can occur in the absence of antimicrobial selection! Transfer of the gene occurred predominantly inside the intestinal tract and much less frequently in the environment.

A study performed by van den Bogaard *et al.* (2001) indicated that transmission of resistant clones and resistance plasmids of *E. coli* from poultry to humans commonly occurs. In this study the prevalence of resistance in faecal *E. coli* in broilers and turkeys was analysed, both with relatively high antimicrobial use, and laying hens with relatively low use. The faecal *E. coli* from the farmers, who had daily contact with the animals, were also studied. Of the three poultry populations, the highest prevalence of resistance was detected in turkey samples, closely followed by those from broilers. The laying-hen population showed remarkably lower resistance. In the human populations, turkey farmers showed the highest percentage of resistance, the lowest resistance rates were observed in the laying-hen farmers. The results from this study strongly suggest a spread of antimicrobial resistant *E. coli* from animals to people – not only to farmers but also at lower level to the consumers of poultry meats.

Lietzau *et al.* (2006) examined the spread of resistant bacteria between healthy individuals in the community. They noted that family members of colonized children had significantly more resistant isolates than those of non-colonized children. They suggested that within family transmission is likely to play a major role in the spread of antimicrobial resistance in the community.

Antimicrobial resistant bacteria from food animals may colonize the human population via the food chain, contact through occupational exposure or waste runoff from animal production facilities. Evidence for the possible transmission from food animals to humans is given by tetracycline-resistant isolates that have been found in human isolates. Tetracycline is an antibiotic that is infrequently used to treat human enteric infections, yet a substantial number of human *E. coli* isolates were tetracycline resistant (Schroeder *et al.*, 2002).

3.3 Conclusion

This chapter clearly illustrates the problems associated with the use of antimicrobials. The creation of resistant micro-organisms as a result of antimicrobial usage, the possible transfer of resistance genes to other micro-organisms by horizontal gene transfer, the transmission of these resistant organisms to humans and finally the transmission of resistant organisms between humans in a community has clearly been illustrated, using only results from previously performed studies. It is thus clear that the problem is of present interest and measures should be taken!

In humans, the control of resistance is based on hygienic measures: prevention of cross contamination and a decrease in the usage of antimicrobial agents. In food animals, held closely together, hygienic measures such as prevention of oral-faecal contact are not feasible. Therefore a reduction in antimicrobial use is the only possible way of controlling resistance in large groups of animals (van den Bogaard & Stobberingh, 1999). This can be achieved by improvement of animal husbandry systems, feed composition and eradication of or vaccination against infectious diseases. Van den Bogaard & Stobberingh stated in 1999 that abolishing the use of antimicrobial agents as feed additives for growth promotion in animals that are to be a food source for humans, on a worldwide scale, would decrease the use of antimicrobial drugs in animals by nearly 50%.

4. New lights on antimicrobial resistance

In biology, any limiting condition for the majority is a golden opportunity for the minority. Bacteria that are capable of surviving and multiplying under these conditions will gain access to organic spaces in which competition with other micro-organisms is avoided (Martinez & Baquero, 2002). Thus, purely theoretic, antimicrobial use should mean a decrease in the size of pathogenic populations and an increase in the number of antimicrobial resistant micro-organisms. The consequence would then be that less use of antibiotics is required, resulting in a restoration of antibiotic susceptibility. Unfortunately, this seems not to be true.

In this chapter, uncommon problems associated with antimicrobial resistance will be highlighted. Martinez and Baquero (2002) performed research in this field and went beyond known issues; resistance was related to virulence and epidemiology.

4.1 Resistance versus virulence

Most virulence determinants are either located in chromosomal gene clusters or in transmissible elements such as plasmids and phages. At first sight, pathogenicity and resistance should be unlinked phenomena. However, several examples indicate that this is not the case for several bacterial pathogens:

- Some bacteria are able to travel from cell to cell without any significant contact with the extracellular environment. This way these organisms are able to avoid the immune system and the presence of antimicrobial agents (which are unable to enter mammalian cells).
- Biofilm-associated organisms are insensitive to antimicrobials. Antibiotic use might thus select for biofilm-forming bacteria, thereby increasing the prevalence of chronic infections.
- Formation of abscesses by certain bacteria leads to a reduced susceptibility due to the fact that these agents are inactivated or altered as a consequence of localized pH changes or free proteins.
- *Bordetella pertussis* is a pathogen responsible for whooping cough. The cell wall of the virulent strains is infrequently susceptible to autolysis triggered by β-lactams, only avirulent *B. pertussis* strains are known to be lysed. The lifestyle of an organism will thus influence its resistance profile!

In the first three cases, the mechanism of pathogenicity serves as a mechanism for antibiotic resistance.

Could antimicrobial resistance determinants also have its effect on virulence? Multi drug resistance mechanisms such as efflux pumps are able to extrude not only a broad range of antimicrobial agents, but also solvents, dyes and quorum-sensing signals. These multi drug resistance efflux pumps will also influence the virulence of an organism. A prerequisite for any pathogen to colonize the intestinal tract is the ability to grow in the presence of bile salts. It has been reported that *Escherichia coli* and *Salmonella enterica* extrude bile salts through these efflux pumps. This means that these pumps are involved in both resistance and virulence, and confirms the latter question that antimicrobial resistance mechanisms can have their effects on virulence properties. Not only do these pumps have the possibility to extrude certain components which makes their colonization in certain niches possible, these pumps also provide the possibility to actively extrude defensins (family of potent antibiotics made within the body that play an important role against invading microbes).
Selection for antimicrobial resistance might thus simultaneously select for more virulent organisms. However, the opposite situation has also been found: antimicrobial resistance may also result in a decrease of virulence. E.g., the KatG catalase-peroxidase activity is important for the survival of *Mycobacterium tuberculosis* in the host. Mutations that eliminate this activity prevent the activation of isoniazid and are the major cause of resistance to this drug in this particular organism. Isoniazid-resistant organisms might thus be less virulent than wild type strains.

It is assumed that acquisition of novel genetic determinants may have a cost for the bacterial host. This may happen because of partial incompatibility of previous and acquired lifestyles, or because of the extra energy required to maintain the genetic vectors carrying the new genes. It might thus have an effect on bacterial fitness, making the organism less virulent. However, the cost in bacterial fitness is rapidly compensated for due to the possibility of bacterial genomes to adapt to unfortunate situations.

Martinez & Baquero (2002) stated that the effect of antimicrobials in inducing the transfer of plasmids and transposons has been demonstrated in vitro. Results in their laboratory suggested that bacterial expression of factors in cell-to-cell DNA transfer in some organisms may be triggered by inflammatory products (as a result of infection by a virulent organism). It can then be expected that bacteria evolve more rapidly inside the host and under selective pressure, so that an infected patient under antimicrobial therapy may act as an evolutionary accelerator!

As mentioned before, plasmids are major vectors for the dissemination of resistance genes, but also for virulence determinants. Co-selection is a problem that occurs when virulence genes and antimicrobial resistance genes are located on the same plasmid. This means that when there is a selection for one of the properties, this will lead to the selection of the other property. This applies as well for genes present in transposons, phages and integrons. An example of a virulence gene found on a transposon is the *E. coli* enterotoxin STII. The presence of virulence genes together with resistance genes in the same phage, however, has not been reported. An explanation for this phenomenon is the limited amount of genetic material that can be encapsulated by a phage particle.

Elements with a role in virulence may be involved in expression of resistance genes. An example: expression of multi drug resistance efflux pumps can be induced by salicylate. Salicylate is also a virulence factor in *Pseudomonas* spp. that is produced during infection. Salicylate production by *Pseudomonas* species may thus induce a phenotype of antimicrobial resistance (Martinez & Baquero, 2002).
A linkage between resistance and virulence gene regulation might thus result in situations of in host resistance at the site of infection that is impossible to predict by routine laboratory susceptibility testing!

4.2 Epidemiological properties of resistant organisms

Since bacteria are under antimicrobial pressure during treatment of an infection, chances are higher that organisms causing these infections are not only virulent, but also resistant to antimicrobials as well.
Reasons can be given for an evolutionary link between antimicrobial resistance and host-to-host transmission. Antimicrobial treatment will result in overgrowth of resistant bacterial populations that are in the minority under normal competitive circumstances (Martinez & Baquero, 2002). The best colonizers among the remaining (resistant) bacteria will have an advantage for re-colonisation. Success in colonizing the host will be reflected in a corresponding success in between-host transmission ability. This perspective may have some exceptions: there are bacterial species that can only survive in specific niches, e.g. due to dependence on other local bacterial populations. This means that the success of transmission depends on the ability of the organism to cross ecological or physiological barriers. This also includes transmission of the more epidemic strains.

Epidemicity ensures peeking multiplication rates that may be needed for the acquisition of resistance (chances of acquiring mutations leading to bacterial resistance are higher when the organism has a higher multiplication rate). Increasing their absolute numbers and consequently their chances of becoming transmitted efficiently also enhances the spread of resistance. Antimicrobial chemotherapeutics should thus only be prescribed in a way that eradication of the bacterial pathogen occurs. Any survival gives the organism an opportunity to evolve and spread in a more efficient way! Acquiring antimicrobial resistance is likely to indirectly help micro-organisms in their transmission, which again enhances the spread of antimicrobial resistance genes (Martinez & Baquero, 2002).

In hyper acute infections, where death follows quickly after the occurrence of symptoms, treatment is often not reached. As a consequence, extremely virulent strains will not be exposed to antimicrobial agents, and will seldom become resistant. Other infections evolve subclinically and will also not be treated. We can conclude that bacteria with intermediate levels of bacterial virulence have a greater probability of being exposed to and develop resistance against antimicrobials than both the lower and upper class of virulent strains, which sadly represent only the minority of strains.
Epidemic micro-organisms possibly evade antimicrobial treatments more easily because they move to another host (usually non-treated) more rapidly than a non-epidemic micro-organism (Martinez & Baquero, 2002).

Finally, it can be mentioned that the resistant normal bacterial flora might protect virulent bacteria from antimicrobial action. If a mixed population of resistant and susceptible bacteria is exposed to e.g. β-lactam antibiotics, the resistant bacteria will inactivate these antibiotics with the result that they will no longer be effective against the target population.

4.3 Strategies against antimicrobial resistance

In some cases, the pathogenic mechanism is essential for the lifestyle of the bacteria. Elimination of the pathogenic factor will reduce the overall presence of the pathogen. E.g. certain toxins contribute to the successful growth of the bacterial pathogen inside the host. Antitoxin vaccination will in this case eradicate the bacterial species. Thus, strategies against virulence may reduce antimicrobial resistance because a lower number of pathogenic bacteria implies less antibiotic exposure. This also lowers the possibility of acquiring resistance genes from other bacterial pathogens.

Reducing host-to-host transmission by using vaccination and good hygiene practices, also contributes to reducing spread of antimicrobial resistance. E.g., vaccination against pathogens that are known to show resistance frequently educes resistance in other members of a closed community. The principle applies not only to human, but also to animal infections and may have an important influence on the development of antimicrobial resistance in light of public health management!

A theoretical possibility would be to develop antimicrobial agents with an extremely narrow spectrum, directed exclusively against a specific organism. This way, only virulent bacteria will be killed, and commensal bacteria can be left unharmed and would as such not develop resistance mechanisms against these compounds. Horizontal gene transfer from commensal organisms to pathogenic organisms could this way be excluded. Unfortunately, it is not easy to find a lethal target, present only in pathogenic bacteria.
A latter strategy could be to use antimicrobials that are active against resistant organisms. A drug that is only active after modification by e.g. an enzyme of the resistant organism could then lead to lysis of the resistant cell (Martinez & Baquero, 2002).

Until now, the best strategy against resistance is a well over thought policy for the prescription and use of antimicrobials. Only use them when needed: for the treatment of infectious diseases only (Martinez & Baquero, 2002).

4.4 Discussion

Excessive use of antimicrobial drugs could lead to their spread in the environment. This could cause the development of environmental resistant organisms (as is already the case in surroundings of e.g. chicken farms). Furthermore, the use of animal growth promoters in the food industry leads to resistant bacteria in food animals. These two factors would actually cause a constant exposure of humans to antibiotics, meaning a constant stress factor for the susceptible organisms of our normal flora. These organisms would in an extreme case not be able to survive due to this constant exposure to antibiotics. The result could thus be that human-adapted strains would be replaced by animal-adapted strains, changing the human flora. The evolutionary consequences are of course totally unknown.

Not using antibiotics any longer in fighting bacterial infections would recreate micro-organisms susceptible to antibiotics. This, however, would bring us back to the preantibiotic era and would restore the problem of infectious diseases. Furthermore, this would not make

any sense because re-use of these agents after regain of bacterial susceptibility would again lead to antibiotic resistance. Novel antibiotics are thus required.

Although several antibiotics are currently used in therapy, these all belong to a few structural families, so that resistance to an antibiotic usually means resistance to the other agents of the same family. New antibiotics, belonging to new classes of antibiotics need to be developed to escape current resistance mechanisms. Two approaches can be combined in the search for novel antibiotics. One is the search for novel inhibitors of bacteria, second is the search for inhibitors of resistance mechanisms with the aim of recovering a susceptible phenotype in a previously resistant population. An example of the latter strategy is the β-lactamase inhibitor.

5. ESBL

The first plasmid-mediated β-lactamase found in gram-negative bacteria was TEM-1. This was found in an *Escherichia coli* strain, isolated from a blood culture from a patient named Temoniera (hence the designation TEM). Another common plasmid-mediated β-lactamase found in *E. coli* and *Klebsiella pneumoniae* was SHV-1.
With the years, many new β-lactam antibiotics were designed to be resistant to the hydrolytic action of β-lactamases and this gave rise to the existence of expanded-spectrum β-lactam antibiotics. It was of no surprise that resistance to these antibiotics emerged quickly. Because of their increased spectrum of activity, these enzymes were called extended-spectrum β-lactamases (ESBLs). ESBLs confer resistance to most β-lactam antibiotics, including oxyimino-β-lactams, such as ceftazidime, ceftiofur and aztreonam, but are not active against cephamycins, like cefoxitin, and carbapenems and they can be inactivated by clavulanic acid. ESBLs contain a number of mutations in the original β-lactamase gene, that allow them to hydrolyze expanded-spectrum β-lactam antibiotics. However, the expansion of the active site that allows the increased activity against expanded-spectrum β-lactams, may also result in the increased susceptibility of ESBLs to β-lactamase inhibitors (Bradford, 2001).

Four major groups of ESBLs that are relevant for this thesis will be discussed briefly: TEM, SHV, CTX-M and OXA.

5.1 TEM

TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. The enzyme is able to hydrolyze penicillins and prior generation cephalosporins. TEM-2, the first derivative of TEM-1, differs in one amino acid from the original β-lactamase. This caused a shift in the isoelectric point, but did not change the substrate profile. TEM-3 was the first TEM-type ESBL.
The amino acid substitutions that occur within the TEM enzyme, occur at a limited number of positions. The combinations of these amino acid changes result in various subtle alterations in the ESBL phenotypes (substrate and isoelectric point properties) (Bradford, 2001). A number of amino acid substitutions are important for expressing the ESBL phenotype: glutamate to lysine at position 104, arginine to either serine or histidine at position 164,

glycine to serine at position 238 and glutamate to lysine at position 240. This is illustrated in the figure below.

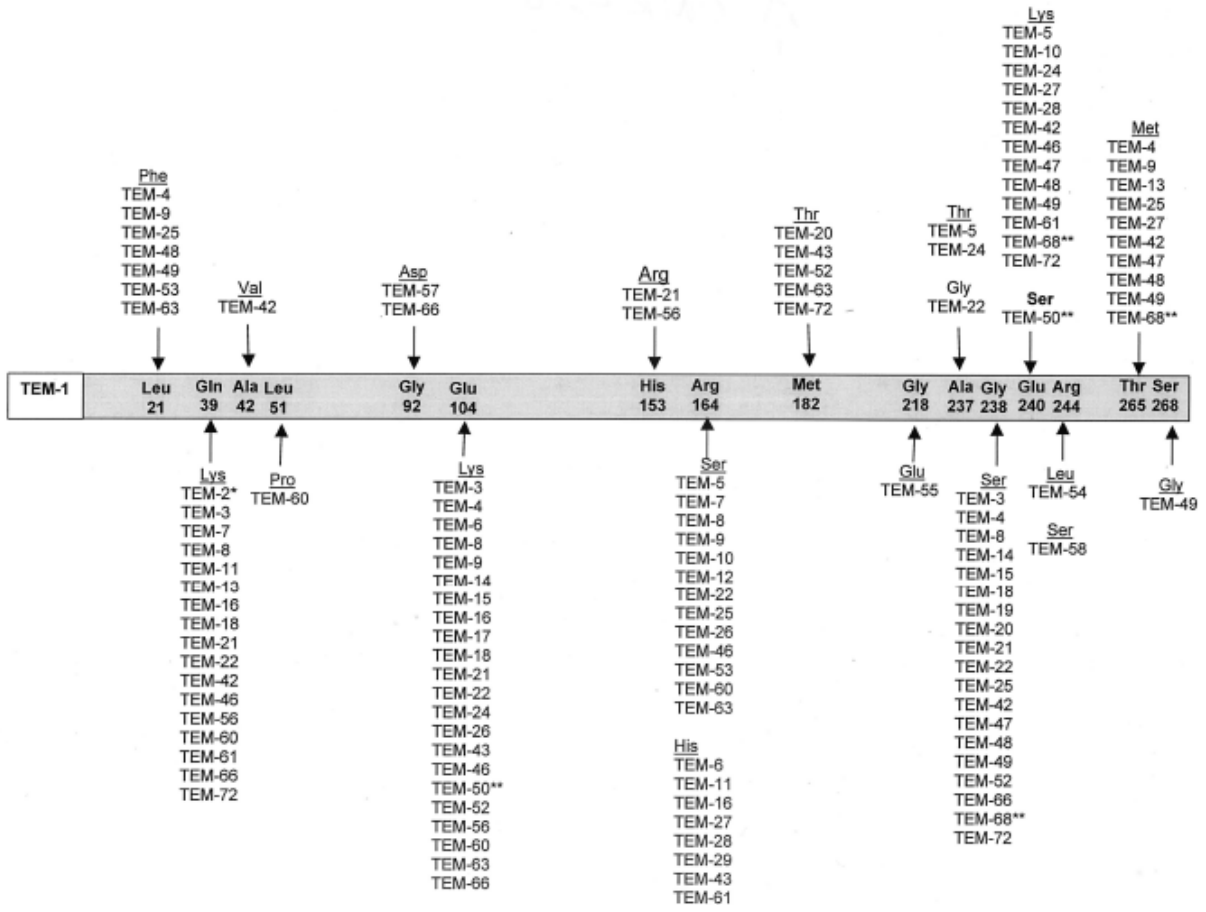

Fig. 6: Amino acid substitutions in the TEM-gene (Bradford, 2001).

It is important to remark, however, that ESBLs should not be confused with inhibitor-resistant β-lactamases, because they are also derivatives of the classical TEM- or SHV-type enzymes. These enzymes were initially designated IRT for inhibitor-resistant TEM β-lactamase, however, all have subsequently been renamed with numerical TEM designations. IRT's confer only resistance to beta-lactamase inhibitors like clavulanic acid. An overview is given in table 1.

pI	Enzymes	Enzyme type		
		Broad spectrum	ESBL	IRT
5.2	TEM-12, TEM-55, TEM-57, TEM-58		X	
	TEM-30, TEM-31, TEM-35, TEM-36, TEM-37, TEM-38, TEM-41, TEM-45, TEM-51, TEM-73, TEM-74			X
5.3	TEM-25		X	
5.4	TEM-1	X		
	TEM-7, TEM-19, TEM-20, TEM-65		X	
	TEM-32, TEM-33, TEM-34, TEM-39, TEM-40, TEM-44			X
5.42	TEM-29		X	
5.55	TEM-5, TEM-17		X	
5.59	TEM-9		X	
5.6	TEM-2	X		
	TEM-10, TEM-11, TEM-13, TEM-26, TEM-63		X	
	TEM-50		X	X
	TEM-59			X
5.7	TEM-68		X	X
5.8	TEM-42		X	
5.9	TEM-4, TEM-6, TEM-8, TEM-27, TEM-72		X	
6.0	TEM-15, TEM-47, TEM-48, TEM-49, TEM-52, TEM-66, TEM-92		X	
6.1	TEM-28, TEM-43		X	
6.3	TEM-3, TEM-16, TEM-21, TEM-22		X	
6.4	TEM-56, TEM-60		X	
6.5	TEM-24, TEM-46, TEM-61		X	
Not determined	TEM-14, TEM-53, TEM-54		X	
	TEM-76, TEM-77, TEM-78, TEM-79, TEM-81, TEM-82, TEM-83, TEM-84			X

Table 1: Overview of TEM type β-lactamases (Bradford, 2001).

5.2 SHV

The SHV-1 β-lactamase is mostly found in *Klebsiella pneumoniae* and is responsible for 20% of the plasmid-mediated resistance to ampicillin in this species.

Unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1. This is illustrated in the figure below.

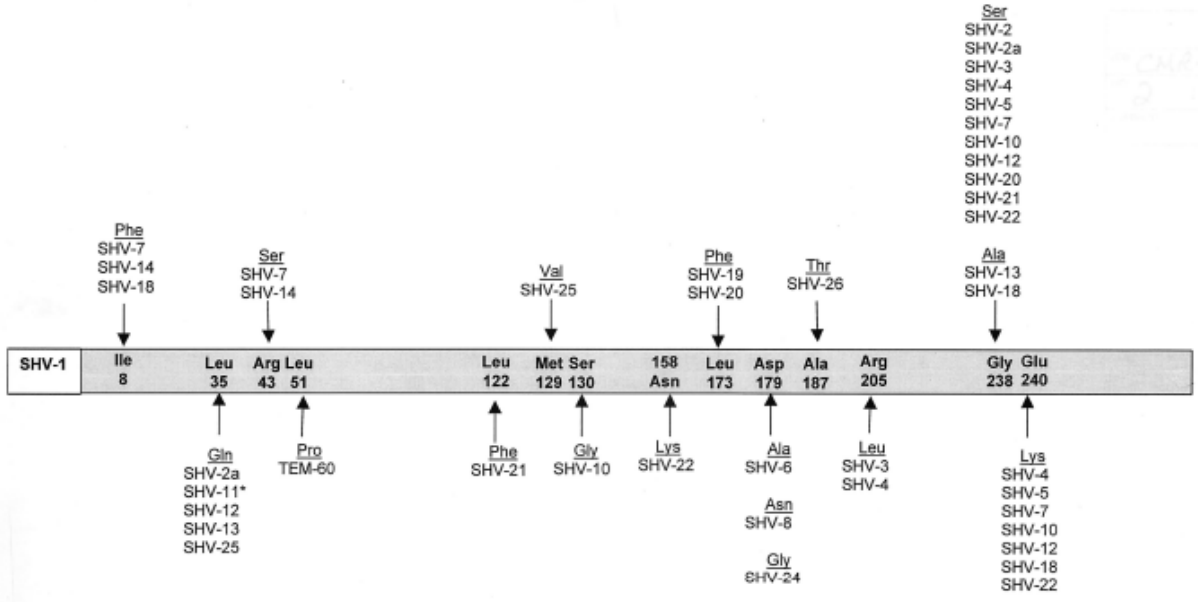

Fig. 7: Amino acid substitutions in the SHV-gene (Bradford, 2001).

The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for a glycine at position 238. The majority of SHV-type derivatives possess the ESBL phenotype (table 2).

pI	Enzymes	Enzyme type		
		Broad spectrum	ESBL	Inhibitor resistant
7.0	OHIO-1, LEN-1	X		
	SHV-3, SHV-14		X	
7.5	SHV-24		X	
7.6	SHV-1, SHV-11	X		
	SHV-2, SHV-2a, SHV-6, SHV-8, SHV-13, SHV-19, SHV-20, SHV-21, SHV-22		X	
7.8	SHV-4, SHV-7[b], SHV-18		X	
8.2	SHV-5, SHV-9, SHV-12		X	
	SHV-10			X

Table 2: Overview of SHV-type β-lactamases (Bradford, 2001).

5.3 CTX-M

CTX-M is a new family of plasmid-mediated ESBLs. These enzymes are not very closely related to TEM or SHV β-lactamases; they show only 40% identity with these enzymes. There is, however, a high degree of homology with the chromosomal AmpC enzyme of *Kluyvera ascorbata*. This suggests that the CTX-M-type enzymes originated from this species.

CTX-M genes can be subclassified based on amino acid sequence similarities. A recent review by Bonnet (2004) and new data within GenBank revealed five major groups of CTX-M enzymes: (1) the CTX-M-1 group, which includes six plasmid-mediated enzymes (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15 and FEC-1) and the enzymes CTX-M-22, CTX-M-23 and CTX-M-28; (2) the CTX-M-2 group, which includes plasmid-mediated enzymes (CTX-M-2, CTX-M-4, CTXM-5, CTX-M-6, CTX-M-7, CTX-M-20, and Toho-1); (3) the CTX-M-8 group, which contains one plasmid mediated member; (4) the CTX-M-9 group, which contains nine plasmid-mediated enzymes (CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27, and Toho-2) and the enzyme CTX-M-24; (5) the CTX-M-25 group, which includes the CTX-M-25 and CTX-M-26 enzymes. The CTX-M phylogeny is illustrated in figure 8.

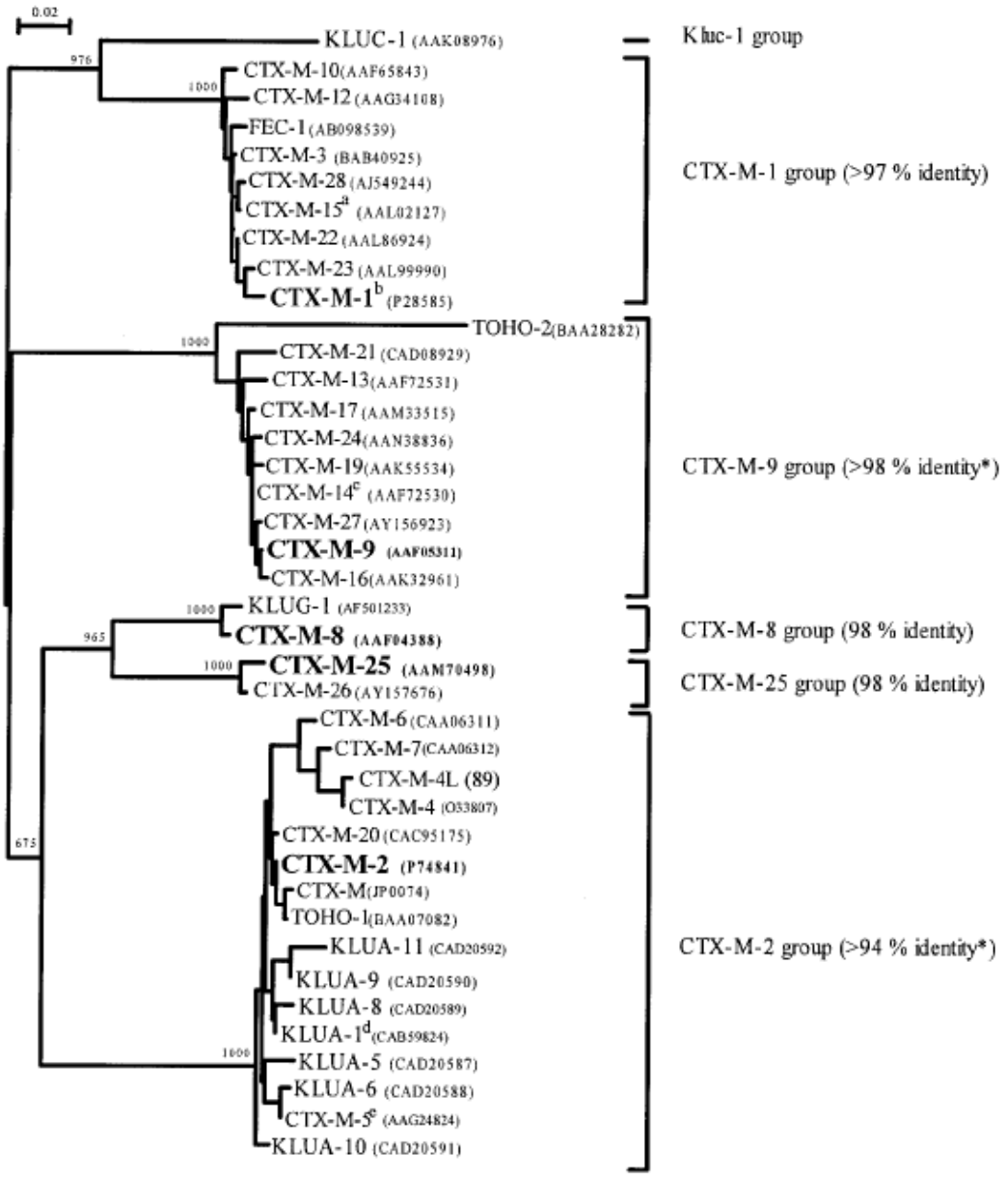

Fig. 8: Dendrogram of CTX-M family (Bonnet, 2004).

Remark: To avoid any confusion: in this thesis, the CTX-M positive strains are subtyped with *three* different primer sets (chapter 2, part 2: Materials & Methods), namely CTX-M-1_3, CTX-M-2_5 and CTX-M-9. These subtype primers can amplify the different CTX-M-genes.

5.4 OXA

The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and show high hydrolytic activity against oxacillin and cloxacillin. They are also poorly inhibited by clavulanic

acid! The OXA-type ESBLs have mainly been found in *Pseudomonas aeruginosa*. Several of the OXA-type ESBLs have been derived from OXA-10 (table 3).

β-Lactamase	Derivation	pI	Amino acid substitutions vs. OXA-10
OXA-11	OXA-10	6.4	Asn143Ser, Gly157Asp
OXA-13	OXA-10	8.0	Ile10Thr, Gly20Ser, Asp55N, Asn73Ser, Thr107Ser, Tyr174Phe, Glu229Gly, Ser245Asn, Glu259Ala
OXA-14	OXA-10	6.2	Gly157Asp
OXA-15	OXA-2	8.7, 8.9 doublet	NA[a]
OXA-16	OXA-10	6.2	Ala124Thr, Gly157Asp
OXA-17	OXA-10	6.1	Asn73Ser
OXA-18	OXA-9, OXA-12	5.5	NA
OXA-19	OXA-10	7.6	Ile10Thr, Gly20Ser, Asp55Asn, Thr107Ser, Gly157Asp, Tyr174Phe, Glu229Gly, Ser245Asn, Glu259Ala
OXA-28	OXA-10	7.6	Ile10Thr, Gly20Ser, Thr107Ser, Trp154Gly, Gly157Asp, Tyr174Phe, Glu229Gly, Ser245Asn, Glu259Ala

Table 3: Characteristics of OXA-type ESBLs (Bradford, 2001).

Part II

Materials & Methods

1. Sampling and identification

1.1 Sampling

Broiler chicken neck skin and intestine samples were taken at the slaughter house and immediately transported to the lab, where the isolation of *Escherichia coli* took place.
The neck skins are first enriched in Oxoid® Brain Heart Infusion broth (BHI) for 18 hours (composition is given in table 4). BHI is a general-purpose liquid medium that is used in the cultivation of fastidious and non-fastidious micro-organisms. It is a nutritious, buffered culture medium that contains infusions of brain and heart tissue and peptones to supply protein and other nutrients necessary to support the growth of a variety of micro-organisms.
After the enrichment period, 10 µl of the medium is grafted on Oxoid® MacConkey agar plates.

Component	Concentration (g/l)
Beef heart infusion solids	17,5
Proteose peptone	10
Glucose	2
NaCl	5
Na_2HPO_4	2,5

Table 4: Composition of Oxoid® Brain Heart Infusion broth.

The intestine is sampled by opening the cecum under sterile conditions. The sample is taken with a sterile swab, and brought directly onto Oxoid® MacConkey agar plates. The typical composition of MacConkey agar is given in table 5.
MacConkey is a selective and differential medium that contains crystal violet, which is inhibitory to gram-positive bacteria and bile salts that select for the enteric gram-negative bacteria. It is thus a selective medium for enteric gram-negative bacteria, of which *Escherichia coli* is part.
Bacteria able to ferment lactose release acid into the medium. The neutral red soaks into bacterial colonies, and will thus cause a colour change of the fermenting bacteria. *Escherichia coli* is one of these and will become dark pink. Other bacteria that are slow lactose fermenters will become slightly pink and lactose-non-fermenters will have uncoloured colonies.

Component	Concentration (g/l)
Peptone from casein	20
NaCl	5
Lactose	10
Bile salt mixture	1,5
Neutral red indicator	0,05
Crystal violet	0,001
Agar	15

Table 5: Composition of MacConkey agar.

1.2 Identification

E. coli colonies, grown on MacConkey agar, have a circular and pink phenotype (fig. 9). Such colonies are grafted on a new MacConkey agar plate to obtain a pure one strain culture. After incubation and confirmation of the purity of the culture, identification is the next step. This is necessary because organisms other then *E. coli* may have the same phenotype on MacConkey agar. Three tests are applied: the indole reaction, Bile aesculin and Kligler Iron agar.

Fig. 9: *E. coli* colonies on MacConkey agar.

1.2.1 The indole reaction

Indole production is a common diagnostic marker for the identification of *Escherichia coli* (Wang, Ding & Rather, 2001). Tryptophanase, an enzyme present in *E. coli*, can hydrolyze the amino acid tryptophan to indole, pyruvic acid and ammonia. p-

Dimethylaminocinnamaldehyde (DMACA) has been shown to be sensitive to the production of indole; a blue-green colour appears when indole is produced.
Spot indole testing is performed by picking up a colony with a swab and then adding a drop of indole reagent to the swab. A positive test is indicated by the appearance of a blue-green colour. It is a rapid test, useful in differentiating *E. coli* from the other coliforms *Enterobacter* and *Klebsiella*, which are indole-negative.

Becton Dickinson DIFCO™ DMACA Indole Reagent Droppers are used in this thesis.

1.2.2 Bile Aesculin Agar

Aesculin is β-glucose-6,7-dihydroxycoumarin, a compound derived from the horse chestnut tree (Edberg *et al.*, 1976). The compound can be enzymatically hydrolyzed at the β-glucose linkage to yield the products aesculetin and glucose. The aesculin hydrolysis test is thus used to determine the ability of an organism to hydrolyse the glycoside aesculin to aesculetin and glucose, and this in the presence of 10-40% bile. The aesculetin combines with ferric ions in the medium to form a black complex.
E. coli isolates do not have the ability to perform this conversion and thus show a negative result. Results are obtained after overnight incubation.
The next table gives the composition of the Oxoid® Bile Aesculin Agar.

Component	Concentration (g/l)
Peptone	8
Bile salts	20
Ferric citrate	0,5
Aesculin	1
Agar	15
pH 7,1 $\pm$ 0,2	

Table 6: Composition of Bile Aesculin Agar.

1.2.3 Kligler Iron Agar

The Kligler test provides information about four characteristics: lactose- (slant surface) and glucose fermentation (bottom), gas formation and H_2S formation.
The Kligler tubes, containing the medium, are coloured red. If the micro-organism we are identifying is able to ferment one or both of the sugars (lactose and glucose), the pH will drop

due to the acidic products that are formed as a result of the fermentation process. This will cause a colour change of the phenol red indicator from red to yellow. If both components are fermented, the tube will be completely yellow. If only one of the sugars is fermented, only the top or bottom of the tube will be yellow, depending on the sugar that has undergone fermentation.

E. coli isolates will ferment both sugars, and the possibility of gas-formation exists. H_2S formation is not the case for *E. coli*; the kligler tube will thus not be blackened due to the formation of FeS.

The next table gives the composition of the Oxoid® Kligler agar.

Component	Concentration (g/l)
'Lab-Lemco' powder	3
Yeast extract	3
Peptone	20
NaCl	5
Lactose	10
Glucose	1
Ferric citrate	0,3
Sodium thiosulphate	0,3
Phenol red	0,05
Agar	12
pH 7,4 $\pm$ 0,2	

Table 7: Composition of Kligler medium (source: SIFIN, 2005).

1.2.4 Rep-PCR

To reliably distinguish bacterial strains or clones, microbial genotyping techniques are necessary. Genotyping methods include plasmid analysis, restriction endonuclease analysis, PCR assays, multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), DNA sequencing, ribotyping, PCR ribotyping, restriction fragment length polymorphism studies, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and repetitive sequence-based PCR (rep-PCR) (Healy *et al.*, 2005).

Current molecular typing methodologies have limitations. Plasmid typing is simple to implement, but often it cannot discriminate because many bacterial species have either few or no plasmids or maintain similar plasmids. MLEE is useful only at providing an estimate of the overall genetic relatedness and diversity. Ribotyping and PCR ribotyping often have difficulty distinguishing among different subtypes. Chromosomal restriction fragment length

polymorphism and AFLP yield complex DNA profiles that can be challenging to interpret. RAPD has high discriminatory power; however, it has poor inter- and intralaboratory reproducibility, due to short random primer sequences and generally low PCR annealing temperatures. MLST data are electronically portable, and MLST can be used as a non-culture-based typing method; however, MLST can be labour intensive and costly. PFGE is highly discriminatory and is considered the “gold standard”; but it has difficulty resolving bands of similar size and there have been issues with interlaboratory reproducibility. Essentially, very few typing methods assess outbreaks in real time, provide comprehensive surveillance or epidemiological data, and have data-archiving capability, all of which are required to build libraries and share data among laboratories (Healy *et al.*, 2005).

Rep-PCR has the advantages of being a fast technique that is relatively cheap and easy to perform. It makes use of repetitive elements that are dispersed throughout the bacterial genome. This dispersion is typical for a certain bacterial strain.
A differentiation between three different repetitive elements should be made: the 36-bp REP (Repetitive Extragenic Palindromic) sequences, the 126-bp ERIC (Enterobacterial Repetitive Intragenic Consesus) sequences and BOX sequences (Olive & Bean, 1999).
Rep-PCR can be performed with DNA extracted from bacterial colonies and primers targeting the non-coding repetitive sequences (in this thesis, primers REP-2I & REP-IR). REP or ERIC amplification can be performed with a single primer, a single set of primers or multiple sets of primers. The technique is easy to perform. The principle is illustrated in the figure below.

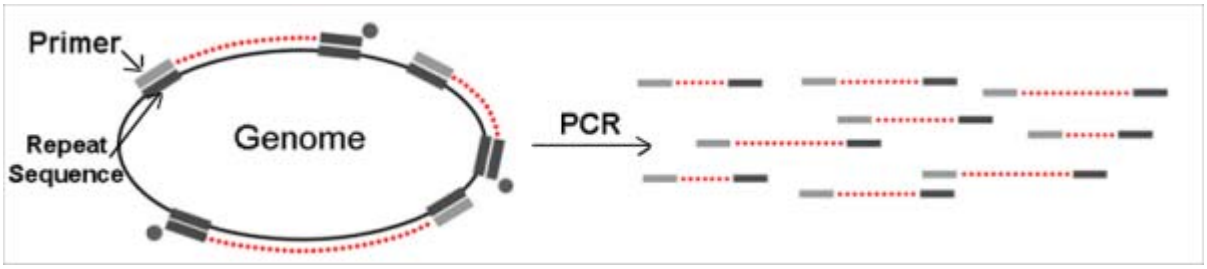

Fig. 10: Rep-PCR principle.

The rep-PCR primers bind to the repetitive sequences dispersed throughout the genome and the DNA within these non-coding repetitive sequences is amplified. Because the arrangement of these sequences shows distinct differences based on the organism being genotyped, this technique can be used for typing purposes. The reaction results in amplicons with lengths depending of the genomic distance between the repetitive elements in the bacterial genome, and thus reflects whether or not two organisms belong to the same strain. These amplicons are subsequently separated using agarose gel electrophoresis, and the

resulting banding pattern will provide the information needed. Two organisms belonging to the same strain will produce a similar banding pattern.
Rep-PCR is an established approach for subspecies classification and strain delineation of bacteria.

Remark: In this thesis, no standardized conditions are used because it is not the purpose to compare the obtained profile to a database. In this case, rep-PCR is solely performed to differentiate between the organisms within one brood and between broods for it is only to look at the antimicrobial resistance profile. The subspecies and strain level is of less importance.

2. Determining the resistance profile

2.1 Antibiogram

An antibiogram is the result of in vitro testing of the susceptibility of an isolated bacterial strain to different antimicrobials. In this thesis, the Kirby-Bauer method was used: small discs containing different antimicrobials are placed in different zones of the culture in the Petri dish. The antimicrobial will diffuse in the area surrounding each disc, and an inhibition zone around the antimicrobial agent will arise when the investigated strain is susceptible to the agent. The diameter of the inhibition zone is a direct measure for the susceptibility of the strain.

2.1.1 Working method

Bacteria grown on the slant surface of the Kligler tube are swabbed and suspended in 3 ml PBS buffer. The bacterial suspension has to fulfil standardised criteria, therefore, the tube containing the PBS buffer is placed in a turbidity measuring apparatus. Initially, a certain turbidity will be displayed. Suspending the bacteria will cause a rise in turbidity. The bacteria will be suspended in the buffer until the turbidity has risen 0,5 McFarland. This solution is then dispersed with a sterile swab on an Oxoid® Iso sensitest medium in three directions to ensure that the surface of the medium is fully covered with bacteria. In the next step, the discs containing the antimicrobials (Rosco Neo-Sensitabs™) are placed on the medium using a Disk dispenser.
The antibiogram is incubated overnight at 37 °C. The result is illustrated in figure 11.

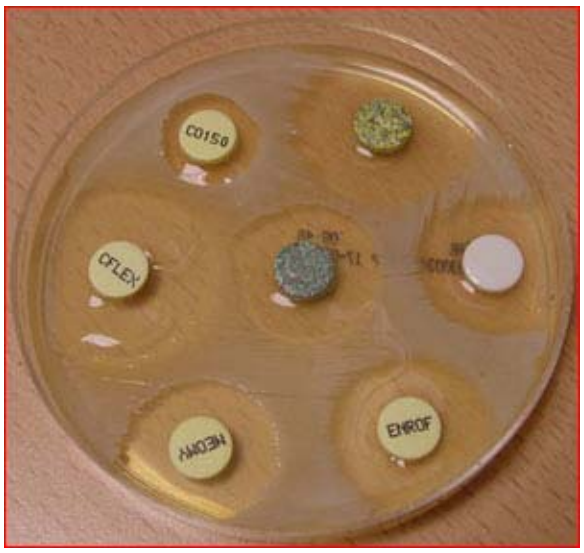

Fig. 11: Result of an antibiogram.

Remark: Standardized criteria are necessary to make comparison of resistance profiles of different bacterial strains possible. If there would not be a standardisation, the obtained antibiograms would be dependant of different variables, which makes comparing and granting of the grades 'resistant', 'intermediately resistant' and 'susceptible' useless.

2.1.2 Interpretation of results

The radius of the inhibition zone is measured and provides information about the susceptibility of the researched strain to the antimicrobial agents tested. Three categories are applied: strains can be resistant, susceptible or intermediately resistant to antimicrobial agents. Criteria were obtained from the manufacturer of the antimicrobial tablets (Rosco), in accordance with the CLSI guidelines.

Agent	Susceptible	Intermediately resistant	Resistant
Amoxycillin-Clavulanic acid	radius > 19	16 < radius ≤ 19	radius ≤ 16
Ampicillin	radius > 19	16 < radius ≤ 19	radius ≤ 16
Apramycin	radius> 22	19 < radius ≤ 22	radius ≤ 19
Ceftiofur	radius > 22	20 < radius ≤ 22	radius ≤ 20
Chloramphenicol	radius > 24	20 < radius ≤ 24	radius ≤ 20
Enrofloxacin	radius > 22	16 < radius ≤ 22	radius ≤ 16
Flumequin	radius > 19	16 < radius ≤ 19	radius ≤ 16
Florphenicol	radius > 19	16 < radius ≤ 19	radius ≤ 16
Gentamicin	radius > 22	19 < radius ≤ 22	radius ≤ 19
Nalidixin	radius > 24	20 < radius ≤ 24	radius ≤ 20
Neomycin	radius > 22	19 < radius ≤ 22	radius ≤ 19
Streptomycin	radius > 25	22 < radius ≤ 25	radius ≤ 22
Tetracyclin	radius > 22	19 < radius ≤ 22	radius ≤ 19
Trimetoprim-sulfonamide	radius > 27	23 < radius ≤ 27	radius ≤ 23

Table 8: Inhibition zone interpretation criteria.

Criteria depend on the minimal inhibitory concentration (MIC) of the agent and the concentration of the antimicrobial agent in the disc. They are not necessarily the same for different bacteria.

2.2 Molecular

In a second phase, isolates are screened for the presence of ESBL's (Extended Spectrum Beta-Lactamases). The DNA of the *E. coli* isolates is liberated (chapter 3, part II: Materials & Methods) and screened for the presence of the genes CTX-M, TEM, OXA and SHV. The screening is performed with the following primers: MA1 & MA2 for detection of the CTX-M gene, OT3 & OT4 for detection of the TEM gene, OS5 and OS6 for detection of the SHV gene and OXA-1A & OXA-1B for detection of the OXA gene. If the gene is present, it will be amplified during the PCR-reaction, and a band will appear during gel electrophoresis.
If a strain gives a positive result for CTX-M, subtyping is necessary (primers MA1 and MA2 detect the three different subtypes of CTX-M genes). This is performed with three different sets of primers: M13up & M13low for detection of CTX-M-1_3 genes, M25up & M25low for detection of CTX-M-2_5 genes and M9up & M9low for detection of CTX-M-9 genes.
A summary is given in table 9.

Remark: Primer MA1 contains S and Y: S= C or G
Y= C or T
Primer MA2 contains R: R= A or G

Gene	Primer	Primer sequence
CTX-M	MA1	5' SCSATGTGCAGYACCAGTAA 3'
	MA2	5' CCGCRATATGRTTGGTGGTG 3'
CTX-M-1_3	M13up	5' GGTTAAAAAATCACTGCGTC 3'
	M13low	5' TTGGTGACGATTTTAGCCGC 3'
CTX-M-2_5	M25up	5' ATGATGACTCAGAGGATTCG 3'
	M25low	5' TGGGTTACGATTTTCGCCGC 3'
CTX-M-9	M9up	5' ATGGTGACAAAGAGAGTGCA 3'
	M9low	5' CCCTTCGGCGATGATTCTC 3'
TEM	OT3	5' ATGAGTATTCAACATTTCCG 3'
	OT4	5' CCAATGCTTAATCAGTGAGG 3'
OXA	OXA-1A	5' ATGAAAAACACAATACATATCAACTTCGC 3'
	OXA-1B	5' GTGTGTTTAGAATGGTGATCGCATT 3'
SHV	OS5	5' TTATCTCCCTGTTAGCCACC 3'
	OS6	5' GATTTGCTGATTTCGCTCGG 3'

Table 9: Primers used for amplification of CTX-M, TEM, OXA and SHV genes.

After gene detection, a sequencing reaction is performed. The PCR-amplified gene products are purified using the Invitek MSB® Spin PCRapace kit (chapter 3, part II: Materials and Methods). Two sequencing PCR-reactions are performed for every gene: one using the

forward primer and one using the reverse primer. Sequencing of the CTX-M genes is performed with three different primers; using an extra primer that is complementary to an internal sequence of the particulate gene.
The products of the sequence PCR are cleaned up using the QIAGEN DyEX 2.0 Spin Kit (chapter 3, part II: Materials and Methods) and sequence analysis of the ESBL genes is performed with an Applied Biosystems Hitachi 3100 Genetic Analyzer. Sequences are assembled manually when necessary using the alignment tool from the following internet address http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

In order to draw conclusions about the exact type of gene, a BLAST is performed at the following internet address http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

3. Protocols

3.1 DNA preparation

One colony, obtained from the antibiogram, is suspended and grown overnight at a temperature of 37 °C in 1 ml Lurea Broth (medium for maintenance and propagation of *E. coli*). After incubation, the suspension is centrifuged for five minutes at 13 000 rpm. The supernatant is then removed and the pellet is resuspended in sterile ultrapure water.
The obtained suspension is subsequently heated at 95 °C, in order to elicit lysis of the cells and liberation of the DNA. Finally, the suspension is centrifuged for five minutes at 13 000 rpm. The supernatant contains the DNA.
The DNA is preserved at -20 °C.

3.2 PCR

The PCR apparatuses used are Eppendorf Mastercycler epgradient and MJ Research PTC-200 Peltier Thermal Cycler.

3.2.1 PCR mix

The PCR mix is prepared with the QIAGEN Quality Taq PCR Master mix Kit (1000 units). The components are mixed in the following quantities:

- 12,5 µl PCR master mix (contains *Taq* DNA Polymerase, QIAGEN PCR buffer and 400 µM of each dNTP)
- 7,5 µl RNase free water
- 1,5 µl 10 µM forward primer
- 1,5 µl 10 µM reverse primer

- 2 µl sample DNA

3.2.2 PCR programmes

REP-PCR:

94 °C, 6 min

30 cycles 94 °C, 1 min
40 °C, 1 min
65 °C, 8 min

65 °C, 16 min

TEM:

94 °C, 5 min

30 cycles 94 °C, 1 min
55 °C, 1 min
72 °C, 1 min

72 °C, 10 min

CTX-M:

94 °C, 5 min

30 cycles 94 °C, 1 min
55°C, 1 min
72 °C, 1 min

72 °C, 10 min

SHV:

94 °C, 5 min

30 cycles 94 °C, 1 min
56 °C, 1 min
72 °C, 1 min

72 °C, 10 min

OXA

94 °C, 5 min

30 cycles 94 °C, 1 min
62 °C, 1 min
72 °C, 1 min

72 °C, 10 min

3.3 Gel electrophoresis

3.3.1 Preparation of the gel

3 grams of SeaKem® LE Agarose (powder) is dissolved in 200 ml 1x TBE (Tris-Boric acid EDTA) buffer; a 1,5 % gel will thus be obtained. The solution is subsequently heated in a microwave oven until boiling. 400 µl Gelred™ is then added to the boiling solution and this is poured into a bearer (figure 12).

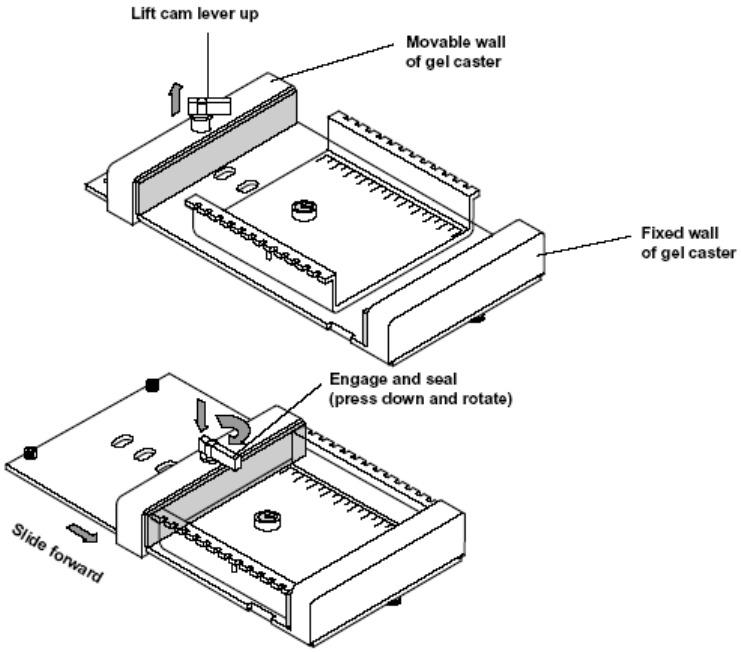

Fig. 12: Bearer used for making an agarose gel.

Combs are placed to form the holes in which the PCR-sample will be brought in. This gel is suited for the separation of DNA fragments varying in length from 0,2 to 3 kb.

GelRed™ is a red fluorescent nucleic acid dye. It has a combination of desirable properties: high sensitivity, high stability and low toxicity. The dye has a major excitation peak at around 300 nm and a red emission at around 595 nm.

TBE (5x)

- 54,5 g Tris base
- 27,8 g Boric Acid
- 2,9 g EDTA
- 800 ml AD
- Adjust the pH to 8 by adding 1 N HCl

- Add AD to a total volume of 1 l

This solution is diluted to a 1x solution.

3.3.2 Preparation of the samples

5 µl of the PCR-samples is added to 3 µl Sample Buffer (1x).
Sample Buffer (5x) contains 50% glycerol, 1 mM cresolred:

- 5 ml glycerol
- 1 ml 10 mM cresolred
- 4 ml AD

The 5x solution is diluted to a 1x solution that contains 10% glycerol and 0,2 mM cresolred.

Cresol red makes the sample visible during loading of the gel and glycerol is used to make the sample sink into the gel slot.

3.3.3 Gel electrophoresis

5 µl of a Fermentas GeneRuler^{TM} 100 bp DNA Ladder Plus is brought in every first hole of the combs in order to inform about the length of the obtained bands. The length of the CTX-M fragment, amplified with the MA1 & MA2 primers is approximately 500 bp, the lengths of the other resistance genes tested in this research are 850 bp.
8 µl of the PCR-samples is added to every hole.
The TEM-, CTX-M-, OXA- and SHV-samples are left to migrate through the gel for 75 minutes, at 170 V. The REP-samples migrate for 7 hours at 70 V in order to obtain a clear separation of the different bands.

3.4 Sequencing PCR

3.4.1 Purification of amplification products

Samples that give a positive result for one of the investigated genes are subsequently sequenced. In order to perform a sequencing reaction, it is necessary to purify the PCR product from excess unreacted primers left over from the PCR reaction. Purification is

performed using the Invitek MSB® Spin PCRapace kit. This kit provides a tool for efficient purification of PCR products from 80 bp up to 30 kbp from amplification reactions.
The principle is the following: the DNA fragments are bound onto the surface of a Spin Filter in a first step. In a second step, the fragments will be eluted with low salt buffer or water.

Step 1: Binding of the PCR-fragments

Add 250 µl Binding Buffer to the PCR sample and mix intensely by pipetting or vortexing.
Transfer the sample completely onto an Spin Filter and centrifugate for 3 min at 10 000 rpm.

Step 2: Elution of the PCR-fragments

Place the Spin Filter into a new 1,5 ml Receiver Tube.
Add 20 µl Elution Buffer directly onto the center of the Spin Filter.
Incubate for 1 min at room temperature. Centrifugate for 1 min at 10 000 rpm.
The eluate contains the purified PCR-product.

3.4.2 PCR mix

The components are mixed in the following quantities:

- 2 µl BigdyeTerm RR mix
- 2,75 µl RNase free water
- 1 µl Bidye seq. buffer
- 2,5 µl 2 µM primer
- 1,75 µl sample DNA

3.4.3 Sequencing PCR programme

Heating to 94°C

24 cycles	96 °C, 10 s
	50 °C, 5 s
	60 °C, 4 min

4°C, for ever

3.4.4 Purification of Sequencing PCR products

Purification of sequencing PCR products is performed using the QIAGEN DyEX 2.0 Spin Kit. This step is necessary for the removal of unincorporated dye terminators. The kit uses gel-filtration technology for allowing cleanup of sequencing reactions in about seven minutes.
Removal of dye terminators is important to prevent the unincorporated dye terminators from interfering with analysis of sequencing results. The DyeEx gel-filtration material consists of spheres with uniform pores and separates molecules according to molecular weight. When sequencing reaction mixtures are applied to DyeEx columns, dye terminators diffuse into the pores and are retained in the gel-filtration material, while labelled DNA fragments are excluded and recovered in the flow-through (figure 13).

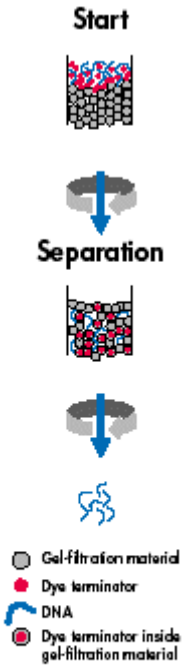

Fig. 13: DyeEX separation principle.

Procedure (figure 14):

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn to avoid vacuum inside the spin column.
3. Snap off the bottom closure of the spin column and place the spin column in a 2 ml collection tube.

4. Centrifuge for 3 min at 3 000 rpm. After centrifugation a solid gel will have formed.
5. Dilute the PCR product by adding 10 µl sterile ultrapure water.
6. Transfer the spin column to a clean 1,5 ml receiver tube. Slowly apply the PCR product to the gel bed.
7. Centrifuge for 3 min at 3 000 rpm.
8. Remove the spin column from the centrifuge tube, the eluate contains the purified DNA.

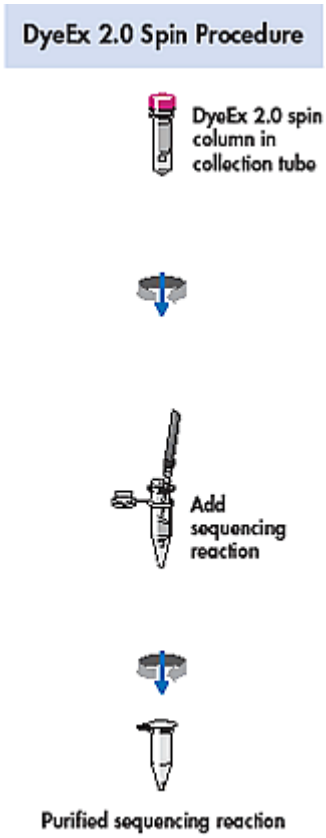

Fig. 14: DyeEx 2.0 Spin Kit procedure.

Part III

Results & Discussion

1. Interpretation of results

The purpose of this chapter is to inform about how results were interpreted in this thesis. Because the same working method was applied for the interpretation of all samples, only one brood (S16) will be discussed in this chapter, this to reduce the amount of data.

1.1 Antibiogram

The diameters of the inhibition zones are measured and a resistance profile is set up for every isolate.

Sample	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracycline	Trimetoprim-sulfonamide
1			22	26		28	24	25	26	15	23	24		39
2	20	10	22	25	28	10	10	26	24	10	22	12	10	10
3	24	10	23	11	30	30	30	24	26	27	24	24	26	10
5	20	10	24	26	30	10	10	20	24	10	22	12	10	10
8	26	10	24	28	28	23	16	24	26	11	25	27	30	33
9	22	10	12	11	28	10	10	24	24	10	22	15	10	10
14	22	10	24	26	32	34	32	25	26	30	25	27	10	10
16	24	10	24	28	29	30	31	26	28	29	24	28	10	10
17	27	27	23	26	32	32	30	26	26	27	24	26	28	40
19	25	10	25	28	29	32	29	25	28	27	21	18	10	10
20	30	31	26	29	32	34	30	27	29	29	26	27	10	10
25	24	10	25	11	29	23	18	24	27	11	24	25	29	10
26	23	10	26	13	30	24	18	26	29	11	28	15	30	10
27	22	10	24	28	10	10	10	24	26	10	26	21	27	10
29	23	10	26	28	10	11	10	26	28	10	26	18	10	10
30	22	10	26	29	10	11	10	26	28	10	26	19	10	10
d1	24	10	26	29	32	11	10	25	28	10	26	28	30	29
d2	28	30	26	30	32	26	21	28	27	12	26	22	28	40
d3	26	10	26	30	32	26	20	27	28	11	26	18	30	10
d4	22	10	26	28	28	32	31	24	28	30	26	26	10	10
d5	25	10	26	30	30	30	30	25	29	29	25	30	28	32
d6	25	10	26	26	30	10	10	25	28	10	24	18	10	10
d7	28	32	26	29	32	26	20	28	27	12	24	20	29	39
d8	26	10	29	12	32	26	21	28	30	11	28	28	32	10
d9	16	10	24	16	30	12	10	26	26	10	24	11	10	10
d10	25	28	26	28	30	30	30	25	28	30	26	28	28	36

Table 10: Antibiogram results S16: inhibition zone diameters.

The values are converted in accordance with the criteria given in part two Materials and Methods (chapter 2, part II: Materials & Methods). The following susceptibilities are then obtained (table 11).

Sample	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracycline	Trimetoprim-sulfonamide
1			I	S		S	S	S	S	R	S	I		S
2	S	R	I	S	S	R	R	S	S	R	I	R	R	R
3	S	R	S	R	S	S	S	S	S	S	S	I	S	R
5	S	R	S	S	S	R	R	S	S	R	I	R	R	R
8	S	R	S	S	S	S	R	S	S	R	S	S	S	S
9	S	R	R	R	S	R	R	S	S	R	I	R	R	R
14	S	R	S	S	S	S	S	S	S	S	S	S	R	R
16	S	R	S	S	S	S	S	S	S	S	S	S	R	R
17	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19	S	R	S	S	S	S	S	S	S	S	I	R	R	R
20	S	S	S	S	S	S	S	S	S	S	S	S	R	R
25	S	R	S	R	S	S	I	S	S	R	S	I	S	R
26	S	R	S	R	S	S	I	S	S	R	S	R	S	R
27	S	R	S	S	R	R	R	S	S	R	S	R	S	R
29	S	R	S	S	R	R	R	S	S	R	S	R	R	R
30	S	R	S	S	R	R	R	S	S	R	S	R	R	R
d1	S	R	S	S	S	R	R	S	S	R	S	S	S	S
d2	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d3	S	R	S	S	S	S	S	S	S	R	S	R	S	R
d4	S	R	S	S	S	S	S	S	S	S	S	S	R	R
d5	S	R	S	S	S	S	S	S	S	S	S	S	S	S
d6	S	R	S	S	S	R	R	S	S	R	S	R	R	R
d7	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d8	S	R	S	R	S	S	S	S	S	R	S	S	S	R
d9	R	R	S	R	S	R	R	S	S	R	S	R	R	R
d10	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 11: Converted antibiogram results.

1.2 Molecular

The isolates are screened for the genes CTX-M, TEM, OXA and SHV. A PCR reaction with primers targeting these regions of the bacterial genome results in amplicons if the gene is present. These amplicons are visualized by gel electrophoresis: a band will appear under UV-light. The picture below illustrates this.

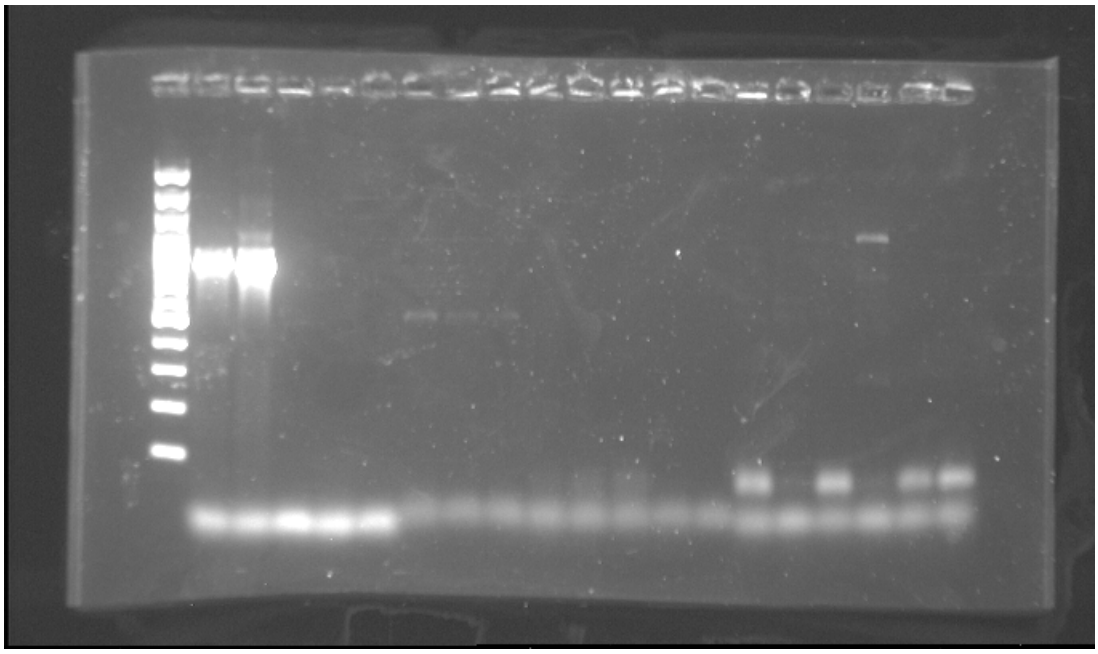

Fig. 15: Visualized amplicons after gel electrophoresis.

The first lane contains the 100 bp ladder: the lowest band has a length of 100 bp, the second lowest band a length of 200 bp, etc. The second and third lane represent isolates that are positive for the investigated gene; a band with a length of approximately 850 bp is clearly visible. Lanes 4, 5, 6 to 20 represent isolates that show negative results for the investigated genes.

Mutations in the ESBL-genes occur and create subtypes of every gene. The subtype is indicated by a number (chapter 5, part I: Literature). Subtyping the genes requires a sequencing PCR reaction. The result of this latter reaction is illustrated in the picture below.

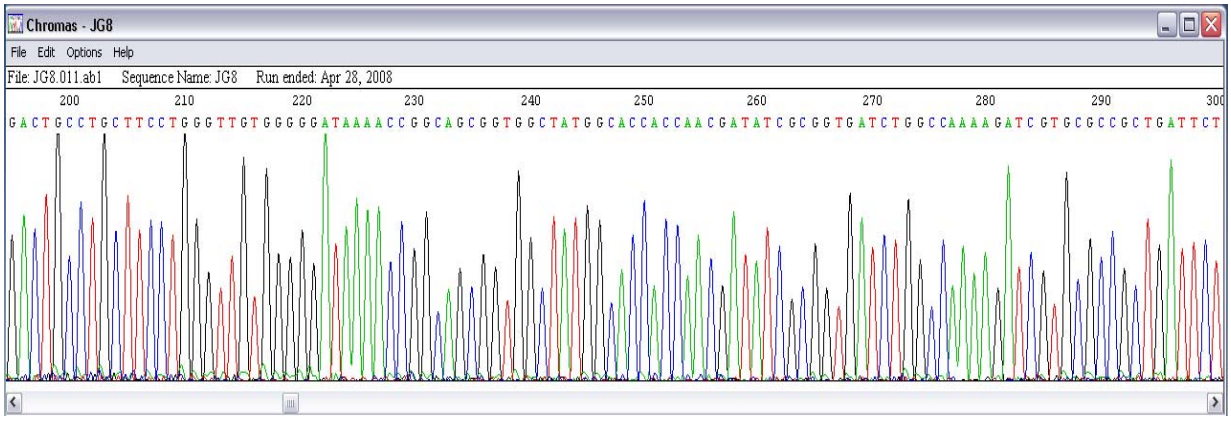

Fig. 16: Result of a sequencing reaction, visualized with Chromas Version 1.45 (32-bit).

Chromas Version 1.45 (32-bit) is used to visualize the gene sequence and convert it to a format that can be BLASTed.

1.3 Genotypic-phenotypic relationships

In this research, molecular detection methods were used for screening of the isolates for ESBLs. Presence of a gene encoding a β-lactamase or ESBL, will result in the phenotypical resistance to β-lactam antibiotics. For this research, the β-lactam antibiotics ampicillin and ceftiofur were used. A relationship between the presence of ESBL genes and resistance to ampicillin and ceftiofur should thus occur.
We need to remark, however, that this relationship can be disturbed by hyperexpression of the chromosomal AmpC β-lactamase, which, in normal circumstances, is only produced at very low levels. AmpC production depends mostly on the strength of the *ampC* promoter. Mutations have been described in the *ampC* promoter that change the strength of this promoter, and thus change the level of transcription (Caroff *et al.*, 2000). Hyperexpression causes a rise of the MIC of a number of β-lactam antibiotics.

Results are in accordance to the statement in part 1 (chapter 5, part I: Literature) that says that TEM-1 is responsible for 90% of the ampicillin resistance in *Escherichia coli*. Table 12 shows the results obtained from brood S16 and confirms the latter thesis. Moreover, an ESBL phenotype (resistance to ceftiofur), is clearly in correlation with presence of the CTX-M gene. In this aspect, samples 25 and d9 are an exception, but this can be explained with possible occurrence of hyperexpression (which was not investigated).
It is also shown that samples 17, 20, d2, d7 and d10, that do not show the β-lactamase encoding gene TEM-1 or any ESBL gene are susceptible to the β-lactam antibiotics used in this work. Again there are two exceptions: samples 1 and 25; that can also be explained with the before mentioned remark about occurring hyperexpression.

The samples indicated in green (table 12) show a clear genotypic-phenotypic relationship; this is in 88,46% the case for the S16 brood.

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracycline	Trimetoprim-sulfonamide
Carcass S16			-															
1	-	-	-	-	R	R	I	S	R	S	S	S	S	R	S	I	R	S
2	-	TEM-1	-	-	S	R	I	S	S	R	R	S	S	R	I	R	R	R
3	CTX-M-1	-	-	-	S	R	S	R	S	S	S	S	S	S	S	I	S	R
5	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	I	R	R	R
8	-	TEM-1	-	-	S	R	S	S	S	S	R	S	S	R	S	S	S	S
9	CTX-M-1	TEM-1	-	-	S	R	R	R	S	R	R	S	S	R	I	R	R	R
14	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
16	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
17	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	I	R	R	R
20	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	R	R
25	-	-	-	-	S	R	S	R	S	S	I	S	S	R	S	I	S	R
26	CTX-M-1	TEM-1	-	-	S	R	S	R	S	S	I	S	S	R	S	R	S	R
27	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	S	R
29	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	R	R
30	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	R	R
d1	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	S	S	S	S
d2	-	-	-	-	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d3	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	S	R
d4	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
d5	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	S	S
d6	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	S	R	R	R
d7	-	-	-	-	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d8	CTX-M-1	-	-	-	S	R	S	R	S	S	S	S	S	R	S	S	S	R
d9	-	TEM-1	-	-	R	R	S	R	S	R	R	S	S	R	S	R	R	R
d10	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 12: Overview results brood S16.

2. Veterinary samples

2.1 Resistance profiles

From the antibiograms, the resistance percentages to every antimicrobial can be calculated. Results are summarized in the tables and figures.

2.1.1 S16

Agent	% R neck skins	% R intestine
Amoxycillin-Clavulanic acid	6,25	10
Ampicillin	87,5	70
Apramycin	6,25	0
Ceftiofur	25	20
Chloramphenicol	25	0
Enrofloxacin	37,5	30
Flumequin	43,75	30
Florfenicol	0	0
Gentamicin	0	0
Nalidixin	62,5	70
Neomycin	0	0
Streptomycin	50	50
Tetracycline	62,5	30
Trimetoprim-sulfonamide	81,25	50

Table 13: Resistance to antimicrobial agents in broiler chicken *E. coli* isolates (brood S16).

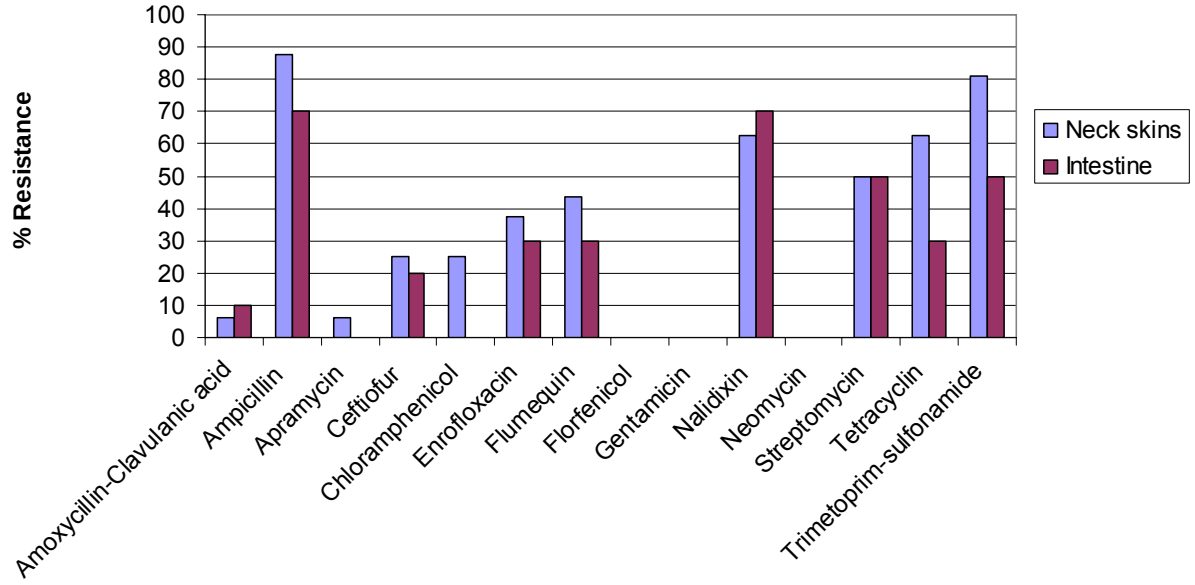

Fig. 17: Resistance to antimicrobial agents in broiler chicken *E. coli* isolates (brood S16).

As can be seen, neck skin isolates and intestine isolates give different resistance profiles. For most of the antimicrobials, higher resistance percentages are obtained for the neck skin samples (exceptions: amoxicillin-clavulanic acid and nalidixic acid). Neck skin isolates are exposed to the environment, thus to a high diversity of micro-organisms, and have full access to the genetic pool presented by them. Intestine isolates, however, have limited access to this genetic pool because they are only exposed to a limited diversity of micro-organisms. Moreover, the exposing can be considered to be limited to the animal's own flora. It is thus clear that chances of receiving resistance genes against antimicrobial agents are higher for neck skin isolates than for intestine isolates, which is confirmed by the results.
This is more clearly illustrated when looking at the number of agents to which isolates show resistance. From our results, it is clear that this number is higher for neck skin isolates than for intestine isolates (11 agents vs. 9 agents).

The reduced susceptibility of intestine isolates to gene transfer infers that intestine isolates represent antibiotic exposure at the chicken farm, whilst neck skin isolates represent the exposure to antimicrobials or antimicrobial resistant organisms during transportation and processing at the slaughter plant.

High resistance percentages are obtained for ampicillin, nalidixic acid, streptomycin, tetracycline and trimethoprim-sulfonamide. No resistance was found to florfenicol, gentamicin and neomycin.

2.1.2 S23

Agent	%R neck skins	%R intestine
Amoxycillin-Clavulanic acid	0	0
Ampicillin	50	26,67
Apramycin	6,25	0
Ceftiofur	0	6,25
Chloramphenicol	0	12,5
Enrofloxacin	12,5	0
Flumequin	18,75	0
Florfenicol	0	0
Gentamicin	0	0
Nalidixin	25	31,25
Neomycin	0	0
Streptomycin	50	80
Tetracycline	56,25	83,33
Trimetoprim-sulfonamide	31,25	6,67

Table 14: Resistance to antimicrobial agents in broiler chicken *E. coli* isolates (brood S23).

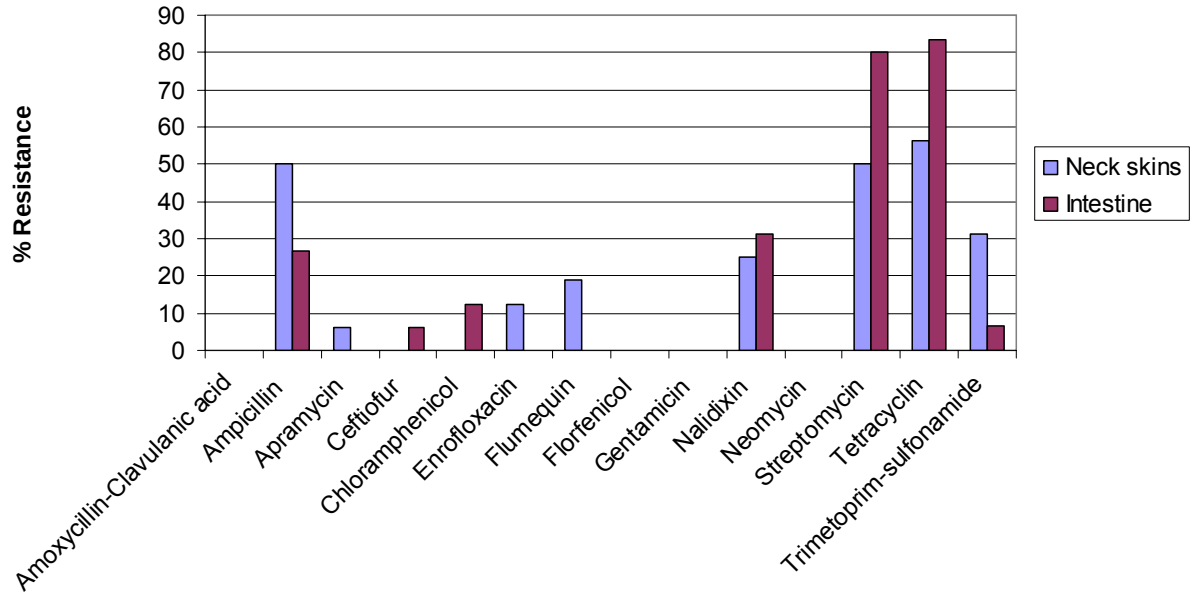

Fig. 18: Resistance to antimicrobial agents in broiler chicken *E. coli* isolates (brood S23).

Again, we can see that the neck skin isolates are resistant to a greater variety of antimicrobial agents than the intestine isolates, which confirms the earlier statement!

High resistance was seen for ampicillin, nalidixic acid, streptomycin, tetracycline and trimethoprim-sulfonamide. This is in accordance to results from brood S16.
No resistance was found for amoxicillin-clavulanic acid and again for florfenicol, gentamicin and neomycin.

2.1.3 Comparing S16 and S23

It is clear that the overall resistance in brood S23 is smaller than in brood S16. In chicken farm S23, very good hygiene was applied and stables remained inhabited for fourteen days between two broods. No antimicrobials were used during approximately four months before sampling.
In chicken farm S16, less good hygiene was applied and the animals were treated with antimicrobials. The stables remained inhabited for 21 days between two broods.

The results clearly show that there is a relationship between antimicrobial use and resistance. Hygiene may also play a crucial role.

2.2 Multiresistance

2.2.1 S16

Number of agents	%R neck skin samples	%R intestine samples
0	6,25	10
1	0	10
2	6,25	20
3	25	10
4	12,5	30
5	12,5	0
6	0	0
7	18,75	10
8	12,5	0
9	6,25	10
10	0	0
11	0	0
12	0	0
13	0	0

Table 15: Multiresistance in broiler chicken *E. coli* isolates (S16).

Remark: Multiresistance differs from the earlier mentioned resistance to a variety of antimicrobials in this sense that multiresistance is about resistance to different antimicrobials in one isolate, whilst resistance to a variety of antimicrobials is about a population!
Multiresistant isolates are a problem because of their resistance to a variety of antimicrobial agents, which makes eradication of the organism more difficult.

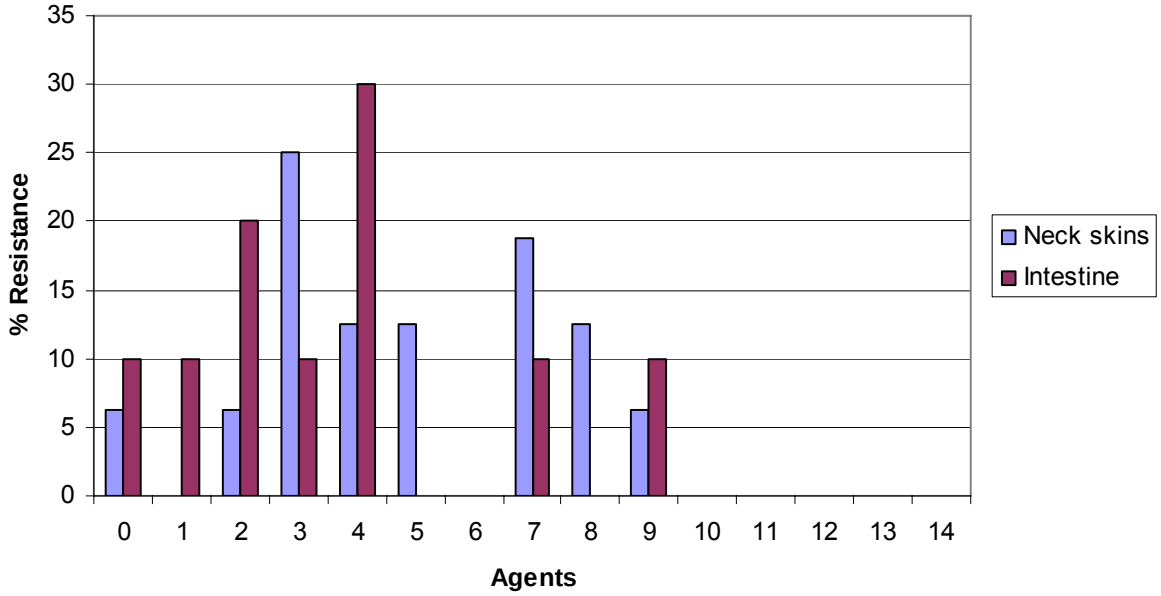

Fig. 19: Multiresistance in broiler chicken *E. coli* isolates (S16).

Figure 19 shows that the highest percentage of the intestine isolates show resistance to four different antimicrobials. Results in the appendix learn that this concerns resistance to ampicillin, enrofloxacin, flumequin and nalidixic acid.

The largest number of neck skin isolates, however, show resistance to three different agents. No uniformity about resistance genes occurring together was found in this case.

It has to be noticed that a number of isolates, neck skin isolates as well as intestine isolates, show resistance against up to nine antimicrobials out of fourteen tested.

2.2.2 S23

Number of agents	%R neck skins	%R intestine
0	6,67	13,33
1	13,33	3,33
2	13,33	56,67
3	6,67	3,33
4	6,67	20,00
5	0,00	0,00
6	3,33	3,33
7	0,00	0,00
8	3,33	0,00
9	0,00	0,00
10	0,00	0,00
11	0,00	0,00
12	0,00	0,00
13	0,00	0,00

Table 16: Multiresistance in broiler chicken *E. coli* isolates (S23).

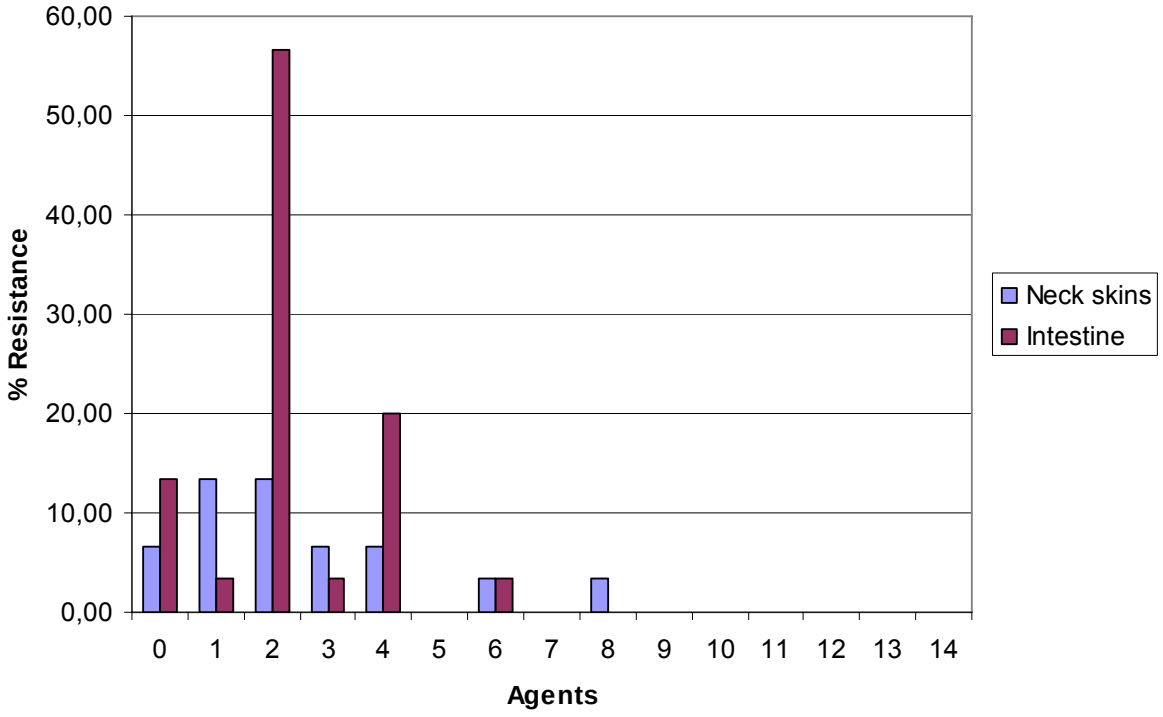

Fig. 20: Multiresistance in broiler chicken *E. coli* isolates (S23).

It is remarkable that the isolates from brood S23 were resistant to both tetracyclin and streptomycin in 80% of the cases for the intestine isolates and 50% of the cases for the neck skin isolates.

2.3 ESBL resistance profile

2.3.1 S16

Gene	% neck skin samples	% intestine samples
CTX-M-1	18,75	10
TEM-1	68,75	60
SHV	0	0
OXA	0	0
None	25	30

Table 17: ESBL resistance genes in broiler chicken *E. coli* isolates (brood S16).

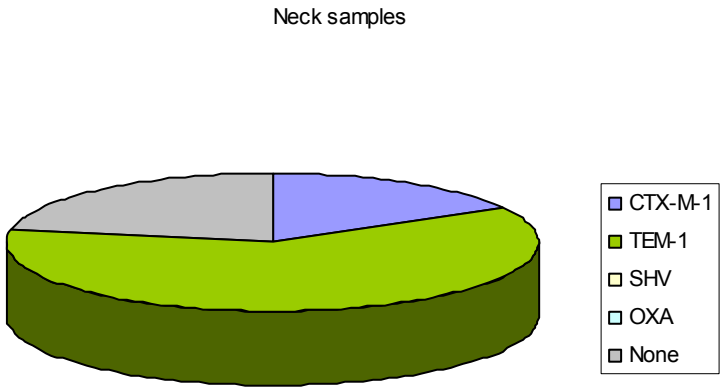

Fig. 21: ESBL resistance genes in broiler chicken neck skin *E.* coli isolates (brood S16).

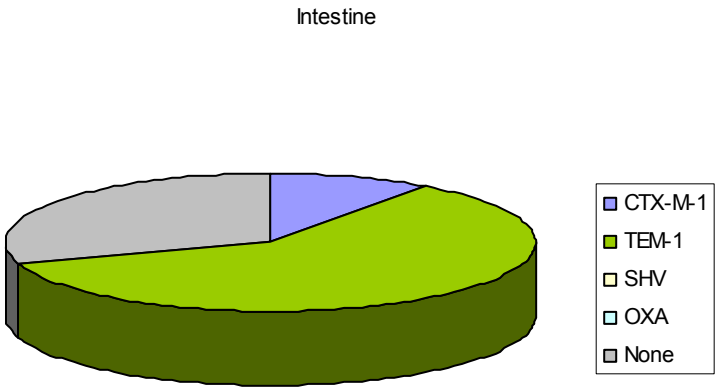

Fig. 22: ESBL resistance genes in broiler chicken intestine *E.* coli isolates (brood S16).

High TEM-1 percentages can be linked to high ampicillin resistance in brood S16. No OXA- or SHV-genes were found in the broiler chicken *Escherichia coli* isolates. 18,75% of the neck skin isolates and 10% of the intestine isolate show an ESBL genotype because of the presence of the CTX-M-1 gene. Normally, this should be linked to the ESBL phenotype, but in a few cases, the earlier mentioned hyperexpression could have occurred, which explains this deviation.

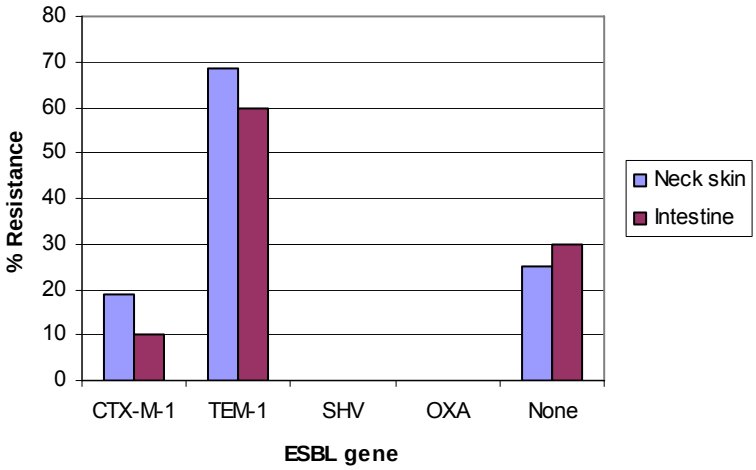

Fig. 23: Comparing ESBL resistance of broiler chicken intestine and neck skin *E. coli* isolates (brood S16).

Neck skin isolates show higher ESBL resistance than intestine isolates. This again can be explained by exposure of the neck skin isolates to a larger genetic pool than intestine isolates. No OXA- or SHV- genes have been observed.

2.3.2 S23

Gene	% neck skin samples	% intestine samples
CTX-M-1	0	3,33
TEM-1	43,75	23,33
SHV	0	0
OXA-1	0	3,33
None	56,25	73,33

Table 18: ESBL resistance genes in broiler chicken *E. coli* isolates (brood S23).

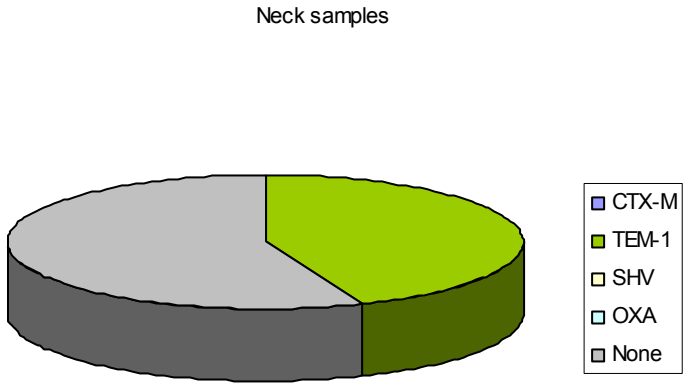

Fig. 24: ESBL resistance genes in broiler chicken neck skin *E.* coli isolates (brood S23).

No ESBL genotype and phenotype has been observed for S23 neck skin isolates. TEM-1 genes, however, were observed and can be related to ampicillin resistance. One isolate (20, see appendix) shows no β-lactam resistance genes, but does show phenotypic resistance to ampicillin. Again, hyperexpression of the AmpC β-lactamase gene can be mentioned here.

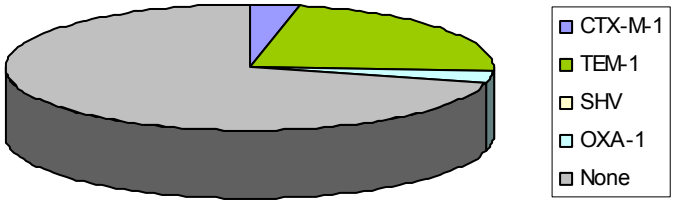

Fig. 25: ESBL resistance genes in broiler chicken intestine *E.* coli isolates (brood S23).

One intestine isolate (d30, see appendix) contained an OXA-1 gene, but this was not in accordance with its phenotypic properties; the isolate seemed to be susceptible to ceftiofur. The possibility exists that contamination took place, because OXA-genes are seldom amongst broiler chicken *Escherichia coli* isolates. This was not reanalyzed.

The other results (appendix) underline the phenotypic-genotypic relationship between presence of the ESBL gene CTX-M and ceftiofur resistance and presence of TEM-1 and ampicillin resistance (except for d28).

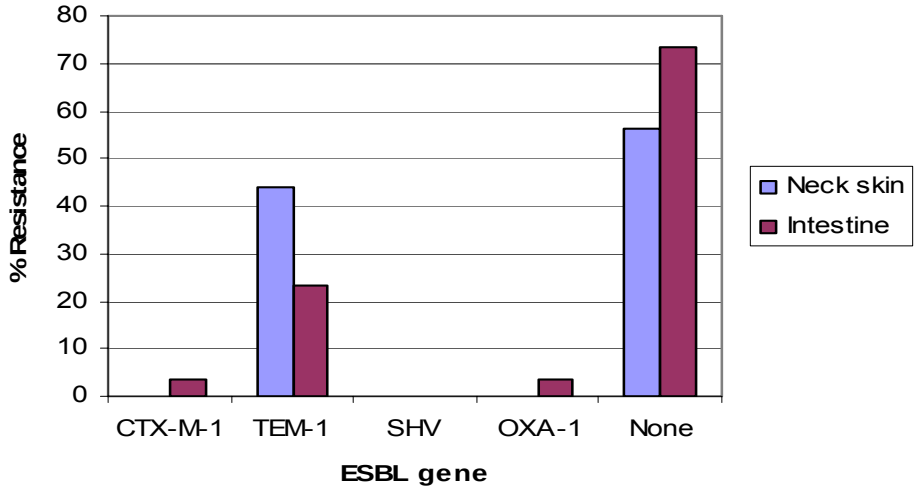

Fig. 26: Comparing ESBL resistance of broiler chicken intestine and neck skin *E. coli* isolates (brood S23).

The ESBL resistance in this brood is very limited. One isolate seemed to have a CTX-M-gene and one an OXA-1 gene, both of them were intestine isolates. Again, more ESBL genes were observed in neck skin isolates (fig. 26, latter bar 'None').

3. Human samples

3.1 Resistance profiles

Agent	%R community acquired	%R hospital acquired
Amoxycillin-Clavulanic acid	6,25	10,20
Ampicillin	100	100,00
Apramycin	0	0,00
Ceftiofur	91,67	97,96
Chloramphenicol	16,67	51,02
Enrofloxacin	70,83	63,27
Florfenicol	4,17	26,53
Gentamicin	12,50	8,16
Nalidixin	91,67	67,35
Neomycin	4,17	22,45
Streptomycin	4,17	38,78
Tetracycline	60,42	73,47
Trimetoprim-sulfonamide	45,83	73,47
Amoxycillin-Clavulanic acid	6,25	10,20

Table 19: Resistance to antimicrobial agents in human *E. coli* isolates.

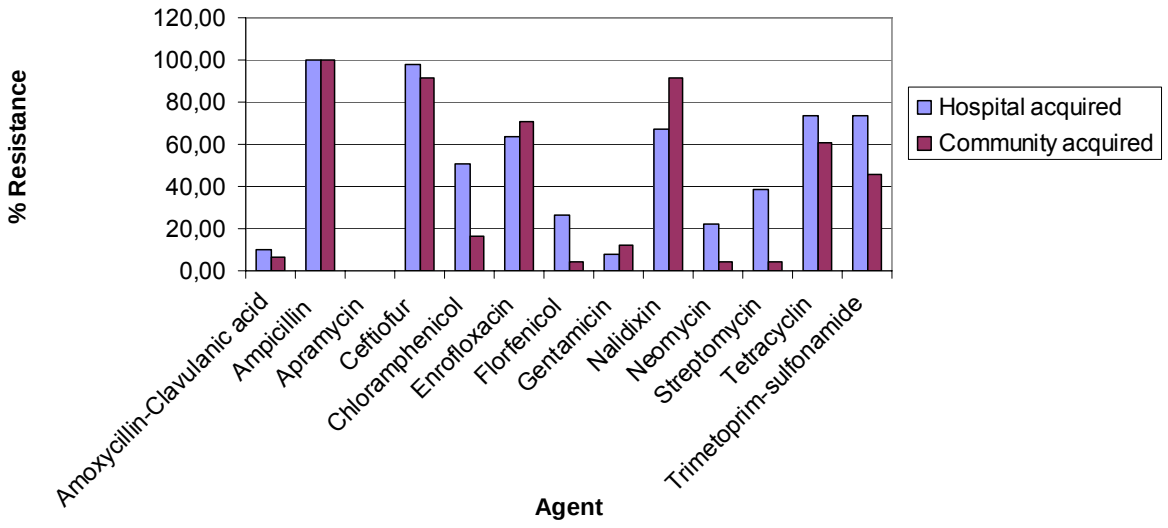

Fig. 27: Resistance to antimicrobial agents in human *E. coli* isolates.

The results obtained from human isolates differ from these of veterinary isolates. It is remarkable that there is a 100% resistance against ampicillin. Also, very high resistance

against the third generation cephalosporin ceftiofur was observed. Antimicrobial resistance in human *Escherichia coli* isolates is thus a major problem that may not be underestimated.

It can also be mentioned that, in contrast to veterinary isolates, there are no agents, except for apramycin, to which there is zero resistance.

With a few exceptions, hospital acquired isolates show higher resistance percentages than community acquired isolates. This is in accordance to the fact that higher antibiotic pressure (as is the case in hospitals) leads to selection of resistance genes, with the result that these will not be lost over time. This means higher exchange of genetic material and consequently, higher resistance percentages. Exceptions to this rule are enrofloxacin, gentamicin and nalidixic acid.

3.2 Multiresistance

Number of agents	%R community acquired	%R hospital acquired
0	0	0
1	0	0
2	4,17	8,16
3	4,17	6,12
4	31,25	12,24
5	25	10,20
6	16,67	16,33
7	12,5	14,29
8	6,25	10,20
9	0	10,20
10	0	8,16
11	0	4,08
12	0	0
13	0	0

Table 20: Multiresistance in human *E. coli* isolates.

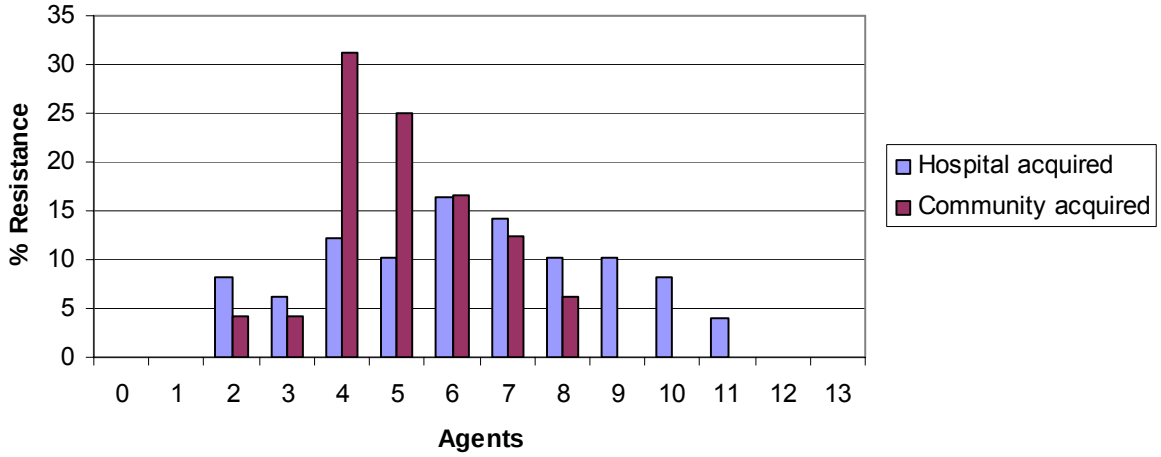

Fig. 28: Multiresistance in human *E. coli* isolates.

In human isolates, multiresistance is the case; no strains were found that showed no resistance or resistance to only one antimicrobial agent. Hospital acquired isolates show multiresistance to up to eleven antimicrobials. This is a known problem and is a bottleneck in antimicrobial treatments. No community acquired isolates were found to be resistant to nine or more antimicrobial agents.

The highest fraction of the community acquired isolates showed resistance to four different antimicrobial agents, whilst the highest percentage of hospital acquired isolates showed resistance to six antimicrobials. We can thus conclude that multiresistance is a problem that ought to be monitored.

3.3 ESBL resistance profile

Gene	% present in community acquired isolates	% present in hospital acquired isolates
CTX-M	74,42	50
TEM-1	67,44	39,29
TEM-24	/	14,29
TEM-52	/	3,57
OXA-1	32,56	21,43
SHV-11	2,33	/
SHV-12	4,65	17,86
SHV-38	2,33	/
None	2,33	21,43

Table 21: ESBL resistance genes in human *Escherichia coli* isolates.

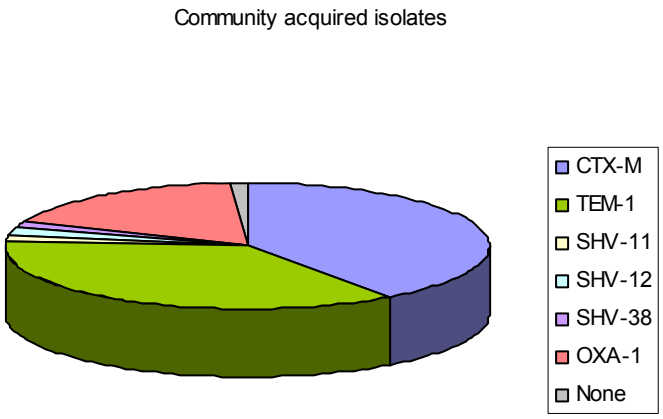

Fig. 29: ESBL resistance genes in community acquired *Escherichia coli* isolates.

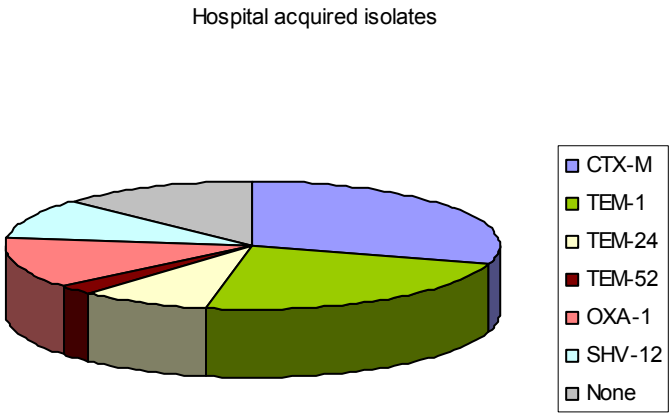

Fig. 30: ESBL resistance genes in hospital acquired *Escherichia coli* isolates.

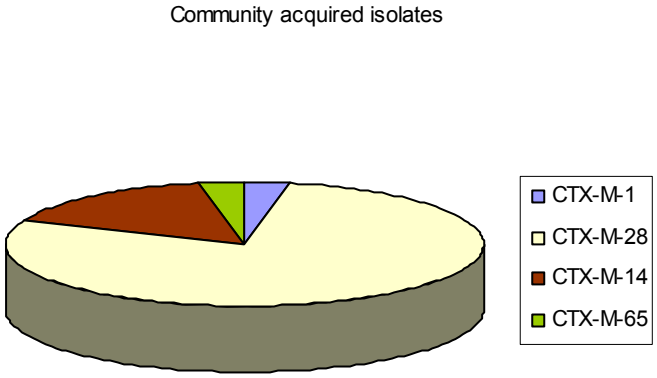

Fig. 31: Share of each CTX-M subtype within CTX-M positive community acquired *Escherichia coli* isolates.

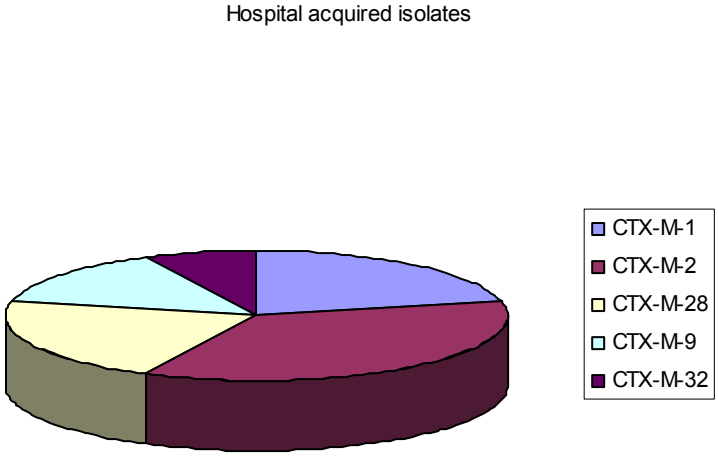

Fig. 32: Share of each CTX-M subtype within CTX-M positive hospital acquired *Escherichia coli* isolates.

A large variety in CTX-M genes was observed, both in hospital and community isolates. This is in contrast to veterinary isolates, where only CTX-M-1 type CTX-M genes were found.

Fig. 33 compares ESBL resistance in community acquired and hospital acquired *E. coli* isolates.

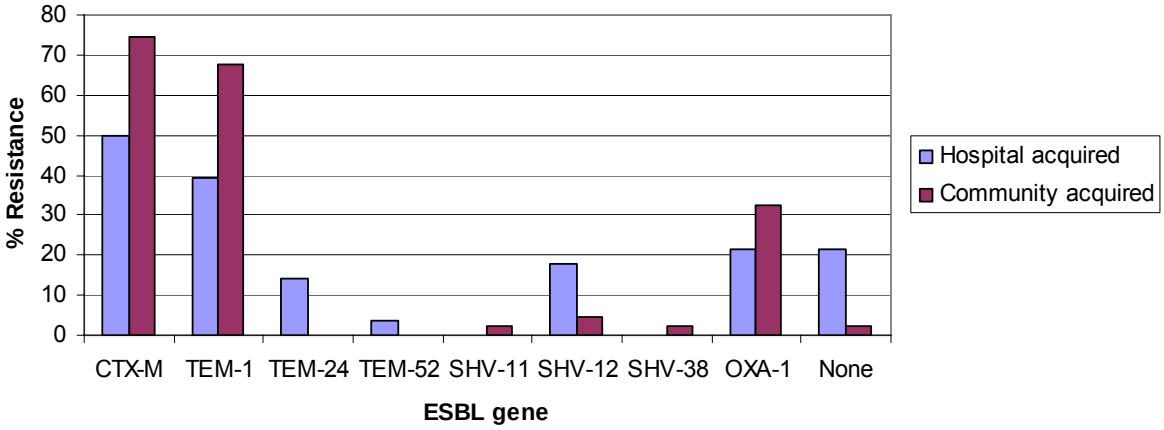

Fig. 33: Comparing ESBL resistance in community acquired and hospital acquired *E. coli* isolates.

Drawing a straight line here is difficult. There seems to be a greater variety in TEM-genes in hospital samples, whilst the variety in SHV-genes is larger in community samples.
A bit of a surprise is that the overall presence of ESBL genes is smaller in hospital samples. There are also more hospital isolates carrying no ESBL gene than community isolates. This might mean that ESBL resistance genes are widespread in the environment and that environmental transfer of ESBL genes is an important aspect.

4. Veterinary versus human samples

In this chapter, resistance in veterinary and human *Escherichia coli* isolates is compared. For every antimicrobial agent, conclusions will be drawn about whether or not there is a significant difference in resistance against the agent between veterinary and human isolates.

This part will contain a number of figures in which blue bars represent the number of susceptible organisms and green bars the number of intermediately resistant and resistant organisms. The first groups represent the veterinary isolates, whilst the second groups represent human isolates.

The Student's T-test was applied for the statistical evaluation of differences in resistance phenotypes of veterinary and human *Escherichia coli* isolates.

4.1 Amoxycillin-clavulanic acid

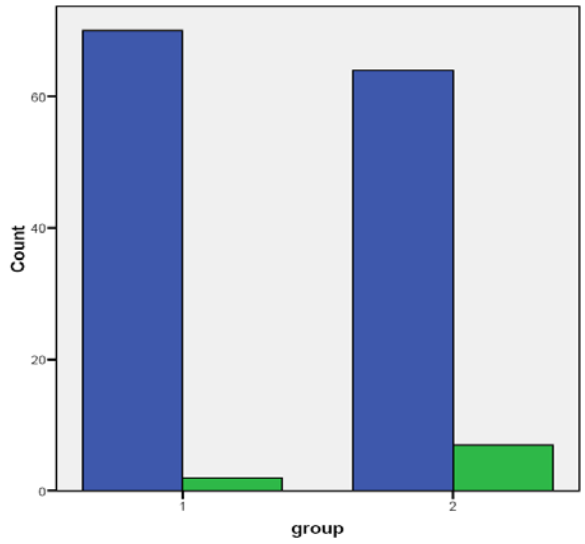

Fig. 34: Resistance against amoxycillin-clavulanic acid.

No significant differences in resistance against amoxycillin-clavulanic acid between human and veterinary *E. coli* isolates was observed (P<0,05).

4.2 Ampicillin

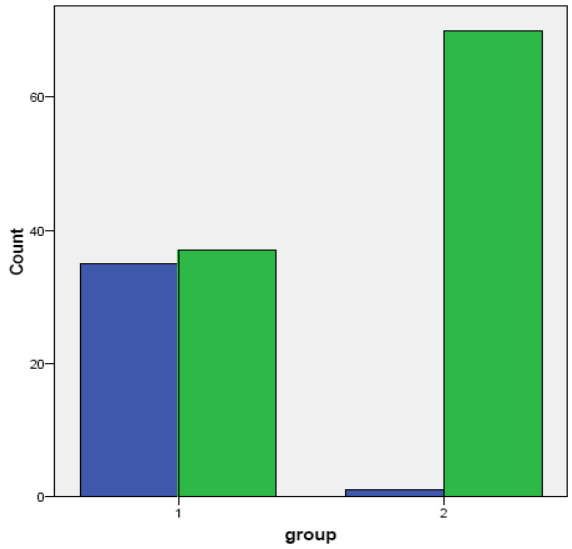

Fig. 35: Resistance against ampicillin.

Significant differences in resistance against ampicillin between human and veterinary *E. coli* isolates was observed (P<0,001). Higher ampicillin resistance in human *E. coli* isolates.

4.3 Apramycin

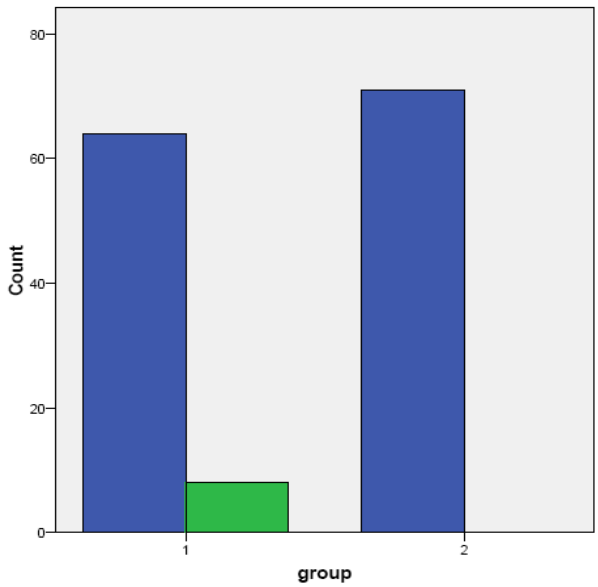

Fig. 36: Resistance against apramycin.

Significant differences in resistance against apramycin between human and veterinary *E. coli* isolates was observed (P<0,01). Higher apramycin resistance in veterinary *E. coli* isolates.

4.4 Ceftiofur

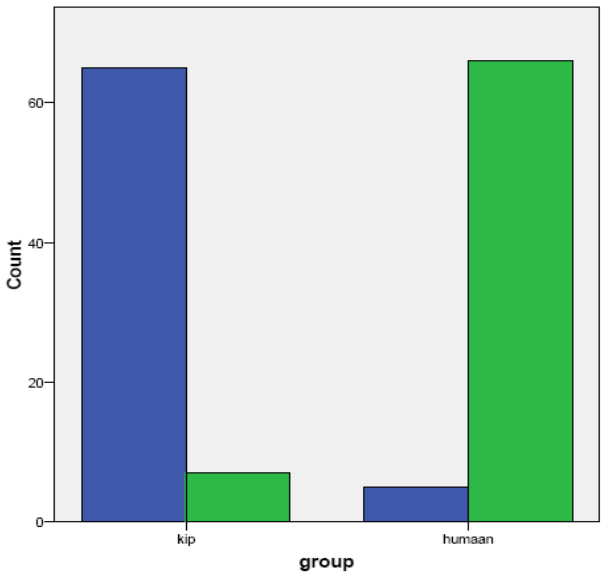

Fig. 37: Resistance against ceftiofur.

Significant differences in resistance against ceftiofur between human and veterinary *E. coli* isolates was observed (P<0,001). Higher ceftiofur resistance in human *E. coli* isolates.

4.5 Chloramphenicol

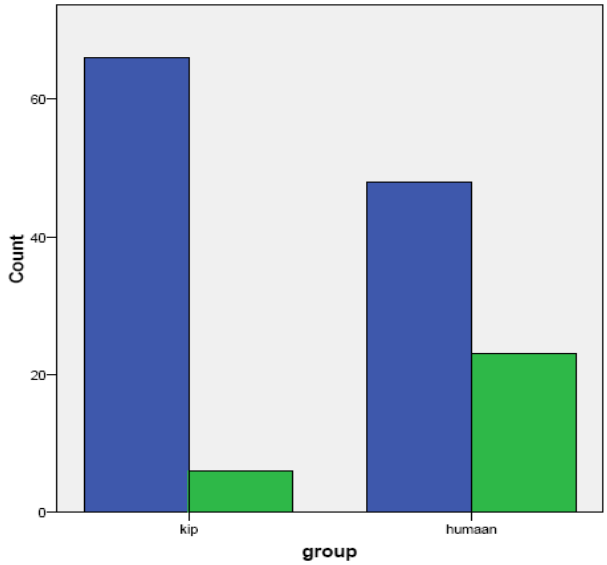

Fig. 38: Resistance against chloramphenicol.

Significant differences in resistance against chloramphenicol between human and veterinary *E. coli* isolates was observed (P<0,001). Higher chloramphenicol resistance in human *E. coli* isolates.

4.6 Enrofloxacin

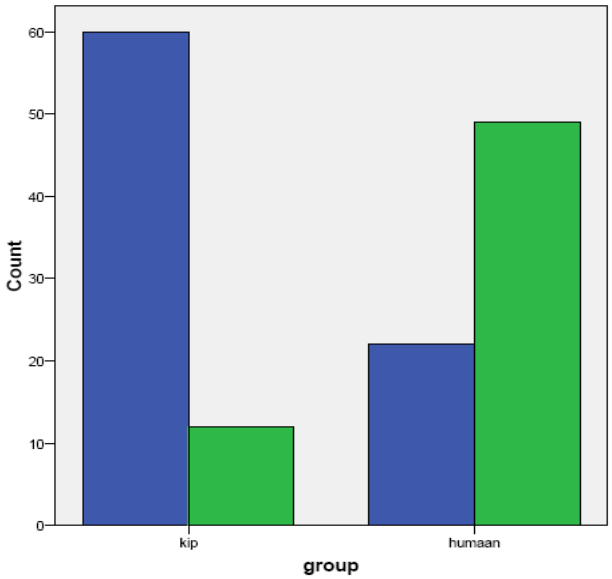

Fig. 39: Resistance against enrofloxacin.

Significant differences in resistance against enrofloxacin between human and veterinary *E. coli* isolates was observed (P<0,001). Higher enrofloxacin resistance in human *E. coli* isolates.

4.7 Flumequin

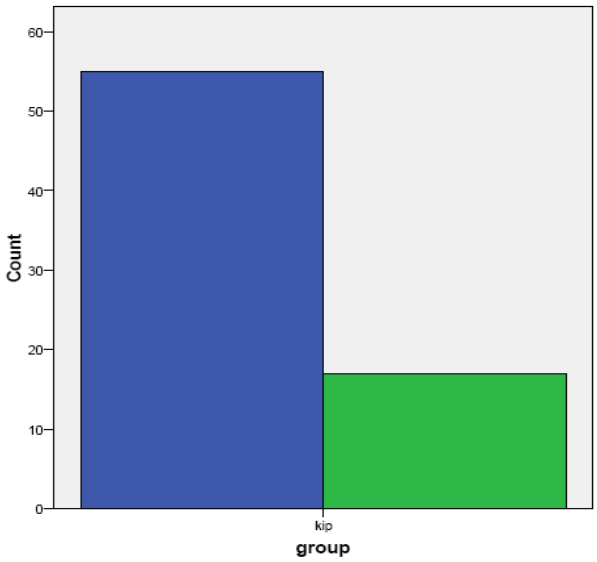

Fig. 40: Resistance against flumequin.

No comparison made because flumequin is an agent that is for veterinary use only. Looking at the resistance in human isolates is not necessary because it is not used as curing agent in humans.

4.8 Florfenicol

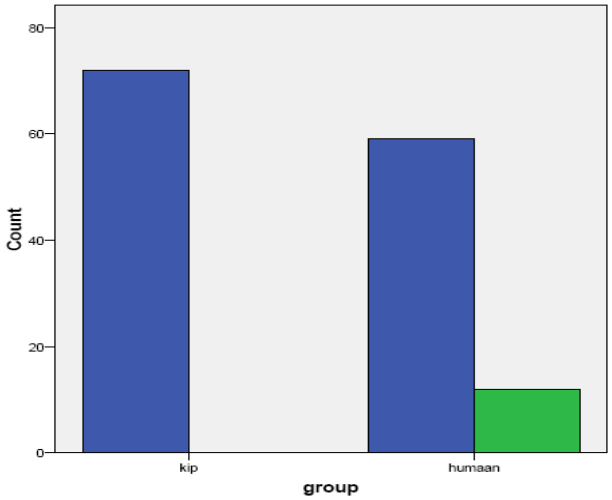

Fig. 41: Resistance against florfenicol.

Significant differences in resistance against florfenicol between human and veterinary *E. coli* isolates was observed (P<0,001). Higher florfenicol resistance in human *E. coli* isolates.

4.9 Gentamicin

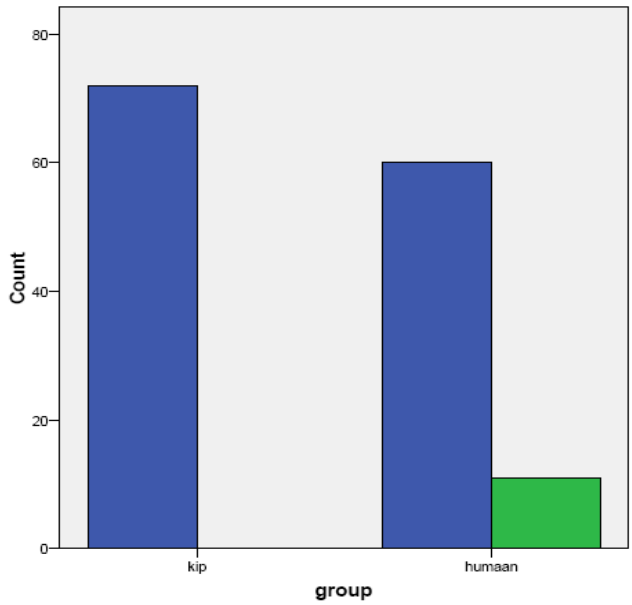

Fig. 42: Resistance against gentamicin.

Significant differences in resistance against gentamicin between human and veterinary *E. coli* isolates was observed (P<0,01). Higher gentamicin resistance in human *E. coli* isolates.

4.10 Nalidixic acid

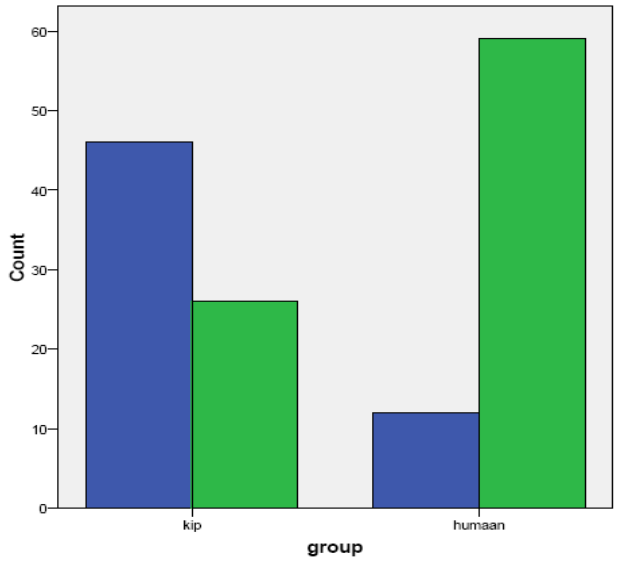

Fig. 43: Resistance against nalidixin.

Significant differences in resistance against nalidixin between human and veterinary *E. coli* isolates was observed (P<0,001). Higher nalidixic acid resistance in human *E. coli* isolates.

4.11 Neomycin

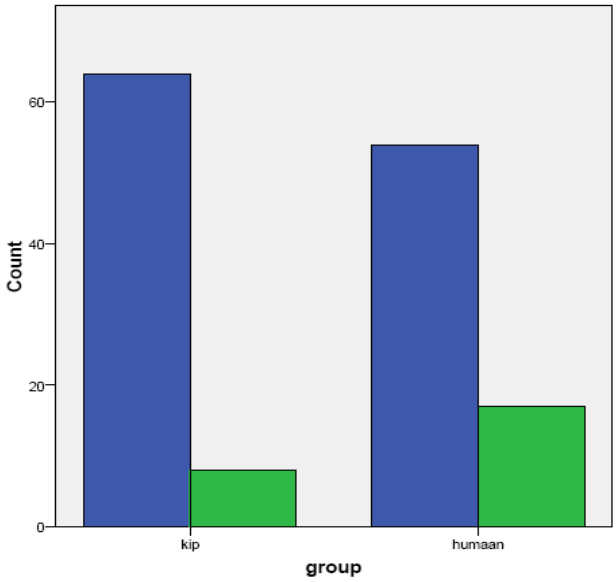

Fig. 44: Resistance against neomycin.

Significant differences in resistance against neomycin between human and veterinary *E. coli* isolates was observed (P<0,05). Higher neomycin resistance in human *E. coli* isolates.

4.12 Streptomycin

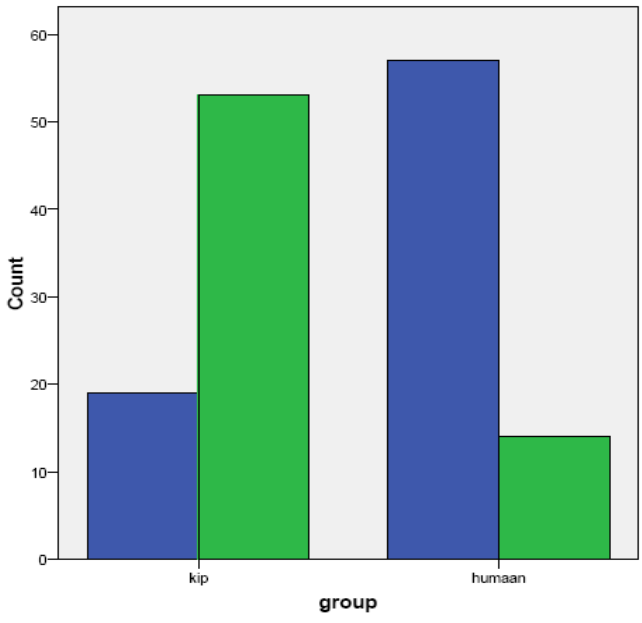

Fig. 45: Resistance against streptomycin.

Significant differences in resistance against streptomycin between human and veterinary *E. coli* isolates was observed (P<0,001). Higher streptomycin resistance in veterinary *E. coli* isolates.

4.13 Tetracycline

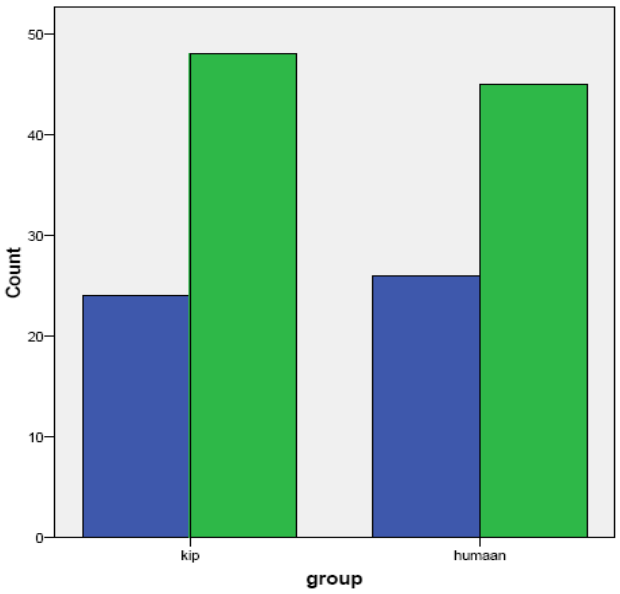

Fig. 46: Resistance against tetracycline.

No significant differences in resistance against tetracycline between human and veterinary *E. coli* isolates was observed (P>0,05).

4.14 Trimethoprim-sulfonamide

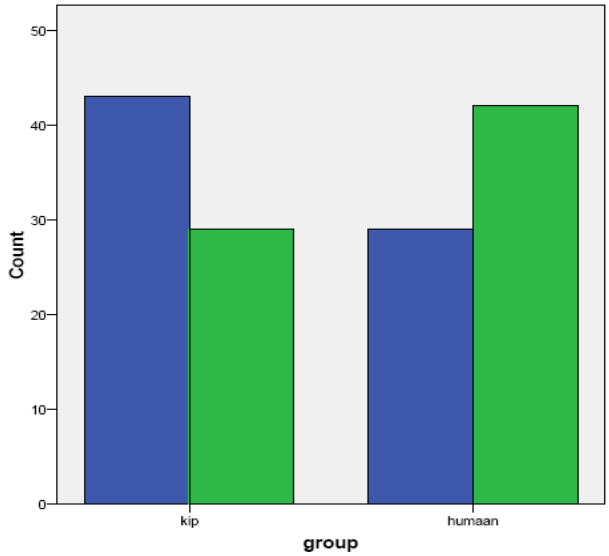

Fig. 47: Resistance against trimethoprim-sulfonamide.

Significant differences in resistance against trimethoprim-sulfonamide between human and veterinary *E. coli* isolates was observed (P<0,05). Higher trimethoprim-sulfonamide resistance in human *E. coli* isolates.

5. Typing

In this chapter, the results of the REP-PCR from brood S23 are displayed. Interpretation of the obtained REP-profiles occurred manually. We have to remark, however, that no conclusions can be drawn from these pictures due to contamination. Results were not reanalyzed because of the duration of the gel electrophoresis. Nevertheless, the results can be applied as a demonstration of how results were interpreted.

As mentioned before, the REP-PCR gel electrophoresis was not performed under standard conditions because it was not our goal to identify the organisms. The goal was solely to look at the variety within the bacterial populations, and whether identical resistance profiles were obtained for organisms displaying identical electrophoresis banding patterns.
REP-PCR provides information about the bacterial chromosome. In most of the cases, strains with identical banding patterns show identical resistance-profiles. It is possible, however, that certain strains with identical REP-profiles differ in resistance profile, because these resistance genes are encoded on a plasmid.

5.1 S23

Profiles for brood S23 are shown in this part.

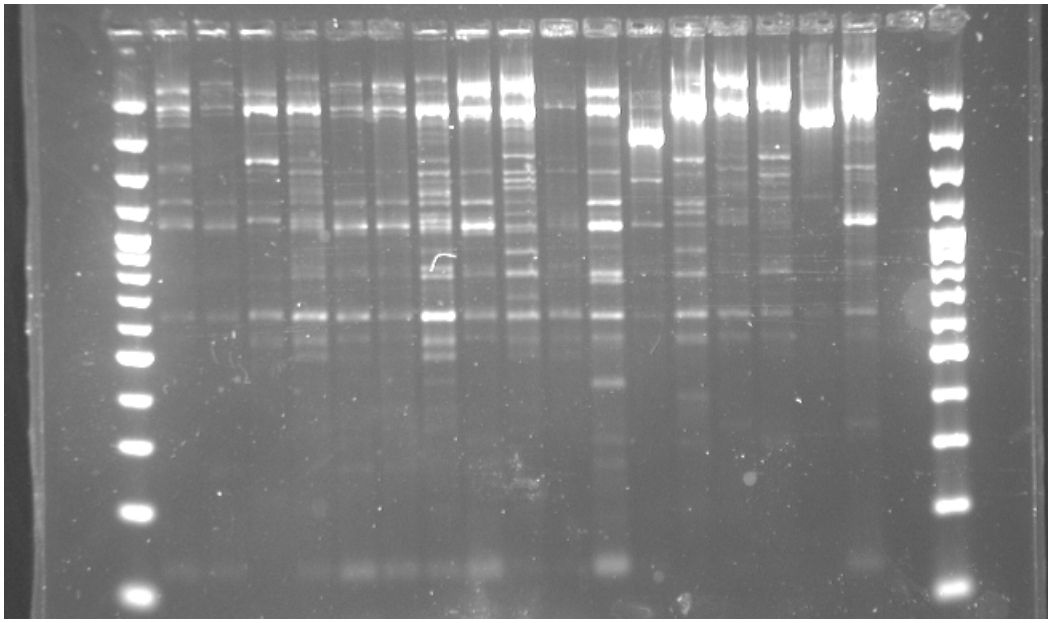

Fig. 48: REP-profile 1 of brood S23.

Identical banding patterns indicate organisms belonging to the same strain. Interpreting these banding patterns means searching for similarities.

Identical banding patterns in this picture: lanes 5 and 8
lanes 6, 7 and 9

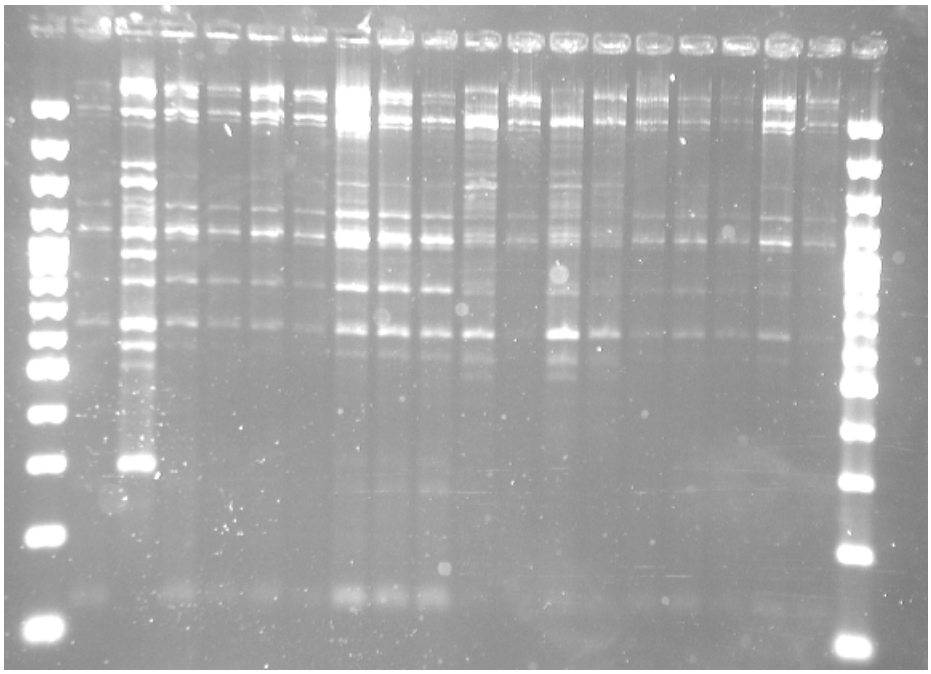

Fig. 49: REP-profile 2 of brood S23.

In this picture, all lanes show identical banding patterns, except for lane 2, lane 11, lane 12 and lane 13.

Contamination is visible in this picture: lane 19 contained the negative control, and bands are clearly visible. We can see the pattern reappearing in every lane.

5.2 S16

The same method was used for brood S16. No contamination occurred in this case.

Isolates belonging to the same strain:

2 – 5 – 19 – 27 – d2

3 – 17

14 – 16

9 – 26 – 29 – 30

25 – d1

When comparing the resistance profiles, it can be concluded that for most of the antimicrobials, identical categories (resistant/susceptible) are obtained for organisms belonging to the same strain. However, some exceptions have occurred. This can be due to the fact that certain resistance genes are plasmid encoded, or due to faults during manual interpretation of results.

Part IV
Conclusions

1. Conclusions

1.1 Technical aspects

- PCR is an interesting tool for detection of ESBL genes. Results are obtained in approximately 4,5 hours, it is thus a fast technique that is relatively easy to perform. Interpretation of results is easy: if a band appears, the gene is present, if no band appears, no gene was present.

  An important drawback of this technique is that no real-time registration is possible. Contamination can only be registered after gel electrophoresis; the whole procedure needs to be carried out before any conclusions about utility of results can be made. This can be problematic when performing a REP-PCR because gel electrophoresis takes 7 hours. This implies a loss of one day when results have to be rejected.
  Real-time PCR could be an interesting tool in this aspect.

- An antibiogram is a cheap and easy to perform method for susceptibility testing of a bacterial strain to a number of antimicrobial agents. Results are obtained in approximately 18 hours.

  A drawback of this technique is the interpretation of results. Inhibition zones are not always a hundred percent symmetrical, and this can cause slight variations in measured diameters. Moreover, sometimes inhibition zones are observed in which separate colonies (mutants) had grown. This of course makes interpretation more difficult.

1.2 Veterinary *Escherichia coli*

- Clear genotypic-phenotypic relationships were observed with few exceptions. These exceptions could be due to hyperexpression of the chromosomal AmpC β-lactamase. This was not further researched in this thesis, nevertheless, it would be useful to trace presence of mutations in the *ampC* promoter, leading to this hyperexpression.

- Differences in antimicrobial resistance of neck skin *E. coli* isolates and intestine *E. coli* isolates were observed. Neck skin *E. coli* isolates seemed to show resistance to a

larger variety of antimicrobial agents. This is due to the exposure of these organisms to a larger genetic pool than intestine isolates, the latter being only exposed to the intestinal flora of the broiler chickens.

Therefore, it can be concluded that intestinal *E. coli* isolates represent antibiotic exposure at the chicken farm, whilst neck skin isolates represent the exposure to antimicrobials or antimicrobial resistant organisms during transportation and processing at the slaughter house.

- High resistance percentages in broiler chicken isolates were observed for ampicillin, nalidixic acid, streptomycin, tetracyclin and trimethoprim-sulfonamide. An explanation can be found in the long term use of these agents. This gave the organisms the possibility to create biochemical mechanisms that convert the agents into harmless substances, and transfer these resistance genes to other organisms.

- No resistance in broiler chicken isolates was found against florfenicol, gentamicin and neomycin. Florfenicol is an antimicrobial that is for veterinary use only; its use is thus limited, explaining the lower resistance levels. Gentamicin and neomycin are relatively new agents.

  Low resistance was found for amoxicillin-clavulanic acid.

- A relationship between antimicrobial use and hygiene at the chicken farm, and antimicrobial resistance in broiler chicken *E. coli* isolates was shown. The overall antimicrobial resistance was smaller in chicken farms harvesting good hygiene and no administration of antimicrobial agents.

- Multiresistance occurred frequently. This has practical implications for the treatment of infections.

1.3 Human *Escherichia coli*

- 100% resistance to ampicillin and very high percentages to ceftiofur were observed.

  Overall resistance seemed to be greater for human *E. coli* isolates.

- Multiresistance is a major problem in human *E. coli* isolates; no isolates were observed showing resistance to zero or only one antimicrobial agent. This is a problem that ought to be monitored.

- A large variety in ESBL genes was observed in human *E. coli* isolates. E.g. in veterinary isolates, only CTX-M-1 type CTX-M was found whilst in human *E. coli* isolates, seven CTX-M types were observed. This was also the case for TEM and SHV genes. No variety was observed in OXA-genes; only OXA-1 occurred.

- A bit of a surprise was that the overall presence of ESBL genes was smaller in hospital samples than in community samples. There were also more hospital isolates carrying no ESBL genes than community isolates. A possibility is that ESBL resistance genes are widespread in the environment and that environmental transfer of ESBL genes is an important aspect.

- Significant differences in resistance were observed between veterinary and human *E. coli* isolates for all agents, except for amoxicillin-clavulanic acid and tetracyclin. In most cases, resistances were higher for human *E. coli* isolates.

1.4 General conclusion

Increasing antibiotic drug resistance is a major problem with global proportions and that has practical implications for the treatment and outcome of invasive infections from *E. coli* and other bacteria. Clinicians and researchers are now acknowledging the importance of preventing resistant infections through appropriate use of antibiotics and vaccines. Surveillance data are needed to monitor the success of these campaigns and to raise awareness of the problem.

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Appendix

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
Karkas S16			-															
1	-	-	-	-	R	R	I	S	R	S	S	S	S	R	S	I	R	S
2	-	TEM-1	-	-	S	R	I	S	S	R	R	S	S	R	I	R	R	R
3	CTX-M-1	-	-	-	S	R	S	R	S	S	S	S	S	S	S	I	S	R
5	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	I	R	R	R
8	-	TEM-1	-	-	S	R	S	S	S	S	R	S	S	R	S	S	S	S
9	CTX-M-1	TEM-1	-	-	S	R	R	R	S	R	R	S	S	R	I	R	R	R
14	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
16	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
17	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
19	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	I	R	R	R
20	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	R	R
25	-	-	-	-	S	R	S	R	S	S	I	S	S	R	S	I	S	R
26	CTX-M-1	TEM-1	-	-	S	R	S	R	S	S	I	S	S	R	S	R	S	R
27	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	S	R
29	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	R	R
30	-	TEM-1	-	-	S	R	S	S	R	R	R	S	S	R	S	R	R	R
d1	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	S	S	S	S
d2	-	-	-	-	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d3	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	S	R
d4	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
d5	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	S	S
d6	-	TEM-1	-	-	S	R	S	S	S	R	R	S	S	R	S	R	R	R
d7	-	-	-	-	S	S	S	S	S	S	S	S	S	R	S	R	S	S
d8	CTX-M-1	-	-	-	S	R	S	R	S	S	S	S	S	R	S	S	S	R
d9	-	TEM-1	-	-	R	R	S	R	S	R	R	S	S	R	S	R	R	R
d10	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
Humane 1			-															
LON 1	-	TEM-1	-	-	S	R	S	R	S	S		S	S	R	S	S	S	R
LON 2	CTX-M-14	TEM-1	-	-	S	R	S	R	R	R		S	S	R	S	S	R	R
LON 3	CTX-M-14	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	S	R
LON 4	CTX-M-14	TEM-1	-	SHV-11	S	R	S	R	R	R		R	S	R	S	S	S	S
LON 5	CTX-M-28	-	-	-	S	R	S	R	S	S		S	R	S	S	S	S	R
LON 6	CTX-M-28	-	-	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 7	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 8	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	S
LON 10	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	S
LON 11	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 12	CTX-M-14	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 13	-	TEM-1	-	-	S	R	S	S	S	S		S	S	R	S	S	S	R
LON 14	-	TEM-1	-	-	S	R	S	S	R	S		S	R	R	R	R	R	R
LON 15	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	S		S	R	R	S	S	R	S
LON 16	-	TEM-1	OXA-1	-	S	R	S	R	S	S		S	S	R	S	S	R	S
LON 17	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	S		S	S	R	S	S	R	S
LON 18	CTX-M-28	TEM-1	-	-	S	R	S	R	S	S		S	R	R	S	S	S	S
LON 20	CTX-M-28	TEM-1	-	-	R	R	S	R	S	S		S	S	R	S	S	S	S
LON 22	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	S
LON 24	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	R	R	S	S	R	R
LON 25	CTX-M-28	-	-	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 26	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		R	S	R	S	S	S	S
LON 27	CTX-M-14	TEM-1	-	-	S	R	S	R	R	R		S	S	R	R	I	R	R
LON 28	-	-	-	SHV-12	S	R	S	S	S	S		S	S	S	S	S	R	S
LON 29	CTX-M-1	-	-	-	S	R	S	R	S	R		S	S	R	S	S	R	S
LON 31	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	S	S

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
LON 32	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 33	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	R	S
LON 34	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 35	-	-	-	-	S	R	S	R	I	R		S	S	R	S	S	S	R
LON 36	-	TEM-1	-	-	S	R	S	R	S	S		S	S	R	S	S	S	R
LON 37	CTX-M-28	TEM-1	OXA-1	-	R	R	S	R	S	R		S	S	R	S	S	R	R
LON 38	CTX-M-28	-	-	-	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 40	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	R		S	I	R	S	S	R	S
LON 41	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 43	CTX-M-65	TEM-1	-	-	S	R	S	R	R	R		S	R	R	I	S	R	R
LON 44	-	-	-	SHV-38	S	R	S	R	S	R		S	S	R	S	S	R	R
LON 45	CTX-M-28	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 46	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	S	S
LON 47	-	TEM-1	-	SHV-12	S	R	S	R	R	R		S	S	R	S	S	R	R
LON 48	-	TEM-1	-	-	S	R	S	R	R	R		S	S	R	S	S	R	R
LON 49	-	TEM-1	-	-	S	R	S	S	S	S		S	S	R	S	R	S	S
LON 51	CTX-M-28	-	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	S
BUT 12	-	-	-	-	S	R	S	R	R	R		S	R	R	R	R	R	R
BUT 19	-	-	-	SHV-12	S	R	S	R	R	S		R	S	S	I	R	R	S
BUT 20	-	TEM-24	OXA-1	?	S	R	S	R	R	R		R	S	R	I	R	R	R
BUT 21	-	-	-	-	R	R	S	R	R	R		S	S	R	I	R	S	S
BUT 22	CTX-M-1	-	-	-	S	R	S	R	S	S		S	S	S	I	S	S	R
BUT 23	-	TEM-24	OXA-1	SHV-12	R	R	S	R	R	R		R	I	R	R	R	R	R
BUT 24	CTX-M-28	TEM-1	OXA-1	-	R	R	S	R	R	R		I	S	R	I	R	R	S
BUT 28	-	TEM-1	-	-	R	R	S	R	R	R		I	S	R	I	S	S	R
BUT 29	-	-	-	-	R	R	S	R	R	R		R	S	R	R	R	R	R

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
BUT 31	-	TEM-52	-	-	S	R	S	R	S	S		S	S	S	S	S	S	S
BUT 36	CTX-M-2	TEM-1	-	-	S	R	S	R	S	R		S	S	R	S	S	R	R
BUT 37	CTX-M-2	TEM-1	-	-	S	R	S	R	S	S		S	S	S	I	S	R	R
BUT 38	CTX-M-9	-	-	SHV-12	S	R	S	R	I	I		S	S	I	S	S	S	R
BUT 40	CTX-M-9	-	-	SHV-12	S	R	S	R	I	I		S	I	I	S	I	R	R
BUT 41	-	TEM-1	-	SHV-12	S	R	S	R	R	R		R	I	R	I	R	R	R
BUT 42	CTX-M-1	TEM-1	-	-	S	R	S	R	S	S		S	S	S	S	R	R	R
BUT 43	-	-	-	-	S	R	S	R	S	R		S	S	R	S	I	S	R
BUT 45	CTX-M-1	TEM-1	-	-	S	R	S	R	S	S		S	S	S	I	S	R	R
BUT 46	-	-	-	-	S	R	S	R	S	R		S	S	R	S	S	R	R
BUT 47	CTX-M-32	-	-	-	S	R	S	R	R	R		I	S	R	I	S	R	R
BUT 50	CTX-M-28	TEM-1	OXA-1	-	S	R	S	R	S	R		S	S	R	S	S	R	R
BUT 51	CTX-M-2	TEM-1	OXA-1	-	S	R	S	R	R	R		S	S	R	S	S	R	R
BUT 54	CTX-M-2	TEM-1	-	-	S	R	S	R	S	S		S	S	S	R	S	R	R
BUT 55	-	TEM-24	-	-	S	R	S	R	R	R		R	S	R	S	S	R	R
BUT 56	-	-	-	-	S	R	S	R	S	S		S	S	S	S	S	S	S
BUT 57	-	TEM-24	-	-	S	R	S	R	R	R		R	S	R	S	S	R	R
BUT 58	CTX-M-2	TEM-1	-	-	S	R	S	R	S	S		S	S	S	S	S	R	R
BUT 92	CTX-M-28	-	OXA-1	-														
Karkas S23																		
d1	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	I
d2	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d3	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	R	S
d4	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	R	S
d5	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d6	CTX-M-1	TEM-1	-	-	S	R	S	R	R	S	S	S	S	S	S	R	R	R
d7	-	TEM-1	-	-	S	R	S	S	S	S	I	S	S	R	S	R	R	I

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
d9	-	-	-	-	S	S	S	S	S	S	S	S	S	S	I	R	R	S
d8	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d10	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d11	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d12	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
d13	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d14	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d15	-	-	-	-	S	S	S	S	S	S	S	S	S	S	I	R	R	I
d16	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d17	-	-	-	-	S	S	I	S	S	S	S	S	S	S	I	I	R	S
d18	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	R	S
d19	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
d20	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d21	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d22	-	-	-	-	S	R	S	S	S	S	S	S	S	S	S	R	R	R
d23	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
d24	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	R	S	R	R	S
d25	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d26	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d27	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d28	-	TEM-1	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
d29	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
d30	-	-	OXA-1	-	S	R	S	S	R	S	S	S	S	S	S	I	R	S
1	-	TEM-1	-	-	S	R	I	S	S	S	S	S	S	S	S	I	S	S

Sample	CTX-M	TEM	OXA	SHV	Amoxycillin-Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Enrofloxacin	Flumequin	Florfenicol	Gentamicin	Nalidixin	Neomycin	Streptomycin	Tetracyclin	Trimetoprim-sulfonamide
3	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	R	R	S
5	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	R	R
6	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	I	S	S
7	-	-	-	-	S	S	S	S	S	R	R	S	S	R	S	R	R	R
8	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	S	S	S
9	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	S
2	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	R	R	R
15	-	-	-	-	S	S	S	S	S	S	I	S	S	R	S	S	S	S
19	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	I	S	S
20	-	-	-	-	S	R	R	S	S	R	R	S	S	R	S	R	R	R
21	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S
26	-	-	-	-	S	S	I	S	S	S	S	S	S	S	S	R	R	S
27	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	R	R	I
28	-	-	-	-	S	S	I	S	S	I	R	S	S	R	I	S	S	S
29	-	TEM-1	-	-	S	R	S	S	S	S	S	S	S	S	S	R	R	R