Quinolone resistance and ESBL/AmpC's in commensal *Escherichia coli* in veal calves

Prevalence and molecular characterization

Joost Hordijk

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Chinolonenresistentie en ESBL/AmpC's in commensale *Escherichia coli* in vleeskalveren

Prevalentie en moleculaire karakterisatie
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

Veal calf sector

The veal calf sector in the Netherlands is a relatively large player in livestock-production industry, with a strong international orientation. In the Dutch quality assurance scheme (SKV), animals are not allowed to be transported before two weeks of age. The calves are transported to distribution centres from where the fattening farms are populated with newly assembled herds. In 2011 there were 1,929 veal calf farms, annually producing 1.6 million calves, of which 1.5 million were brought to slaughter in the Netherlands [1]. Approximately 50% of the animals originate from domestic dairy farms. The remaining 50% is imported from mainly Germany, Poland and Belgium/Luxembourg, the Baltic States and other European countries. Approximately 90% of the veal produced is exported to mainly Italy, Germany and France, but also other countries [1]. Veal calves are divided into two categories, white and rose veal calves. White veal calves are fed mainly with milk replacer and are slaughtered before eight months of age. They are mostly maintained in all-in, all-out production systems. Rose veal calves are slaughtered between seven and twelve months of age. They are fed milk-replacer during the first approximately six to eight weeks, and subsequently switch to mainly roughage, which consists of pelleted feed and silage. On rose veal calf farms, often multiple age groups are present. Rose veal calf farms can be subdivided in specialised starter farms and specialised fattening farms and farms that combine starting and fattening of calves. At starter farms, the animals stay until 10 to 12 weeks of age. At fattening farms animals are present from 10 to 12 weeks of age until slaughter. The majority of veal calves produced in the Netherlands is white veal calves (approximately 70%).

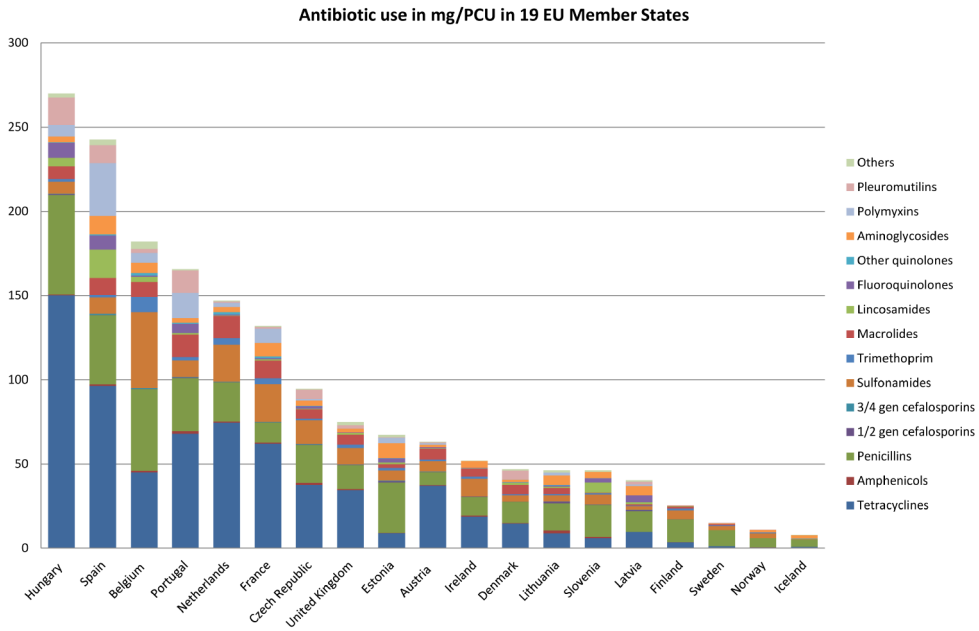
Regulations

The increasing prevalence and complexity of antimicrobial resistance in both humans and animals, is to a certain extent related to antimicrobial use in food-producing animals. The emergence of multi-drug resistant MRSA and ESBL-producing organisms in food producing animals, led to increased awareness in the society and among (scientific) authorities, which resulted in increased political pressure to act. In response, the veal calf sector composed a Masterplan for Prudent use of Antimicrobials in the Veal Calf Sector in 2007. The plan aimed to describe the responsibilities of the farmer, the veterinarian and advisor of the veal calf integration in prescription and application of antibiotics. Also scientific research on MRSA and multi-drug resistance was part of the Masterplan. In 2008 memoranda of understanding were signed by the four main food animal production sectors: pigs, broilers, dairy/beef cattle and veal calves. The goal was to reduce antimicrobial resistance and to achieve a more prudent and rational use of antimicrobials in food-producing animals. In addition, the parliament demanded a reduction in antibiotic usage of 20% in livestock production in 2011 and 50% in 2013 compared to 2009 in all sectors. Treatment plans based on national guidelines for therapy are mandatory on each farm, and all antibiotic usage is registered in a national database (starting from 2011). The result is that antibiotic usage is transparent, farmers and veterinarians can benchmark themselves and can be benchmarked. Moreover, as an element of the Masterplan, research was initiated, aimed at epidemiological and molecular aspects of antimicrobial resistance in veal calves.

Antimicrobial use in food-producing animals

In Europe a large variation in antimicrobial use in food-producing animals exists between countries. In a study analysing sales data from ten countries in 2007, The Netherlands was shown to use the highest amount of antibiotics, expressed in mg active substance/ kg biomass [2]. A recent study analysed the sales data from 2010 [3]. These results were reported mg antibiotics used per Population Correction Units (PCU), not only taking animal weight at slaughter into account, but also correcting for imported and exported animals. This study confirmed a relatively high amount of antimicrobials was used in the Netherlands (Figure 1.1). However, this study also showed that large variations exist in classes of antibiotics used in different countries (Figure 1.1). Important classes in human medicine are 3rd/4th generation cephalosporins and fluoroquinolones. The use of 3rd/4th generation cephalosporins in food-producing animals is relatively low in all countries. In contrast, a large variation exists in the use of fluoroquinolones.

Figure 1.1: Percentages of sales for food-producing animals (including horses), in 2010

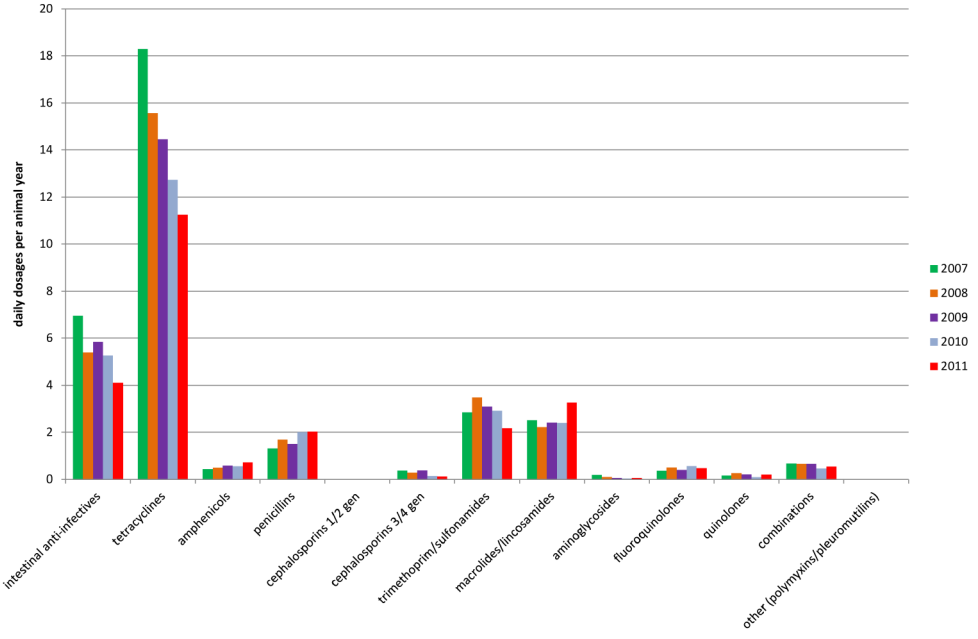


This Figure was modified from data reported by the European Medicines Agency [3] (Table 6)
PCU: Population Correction Units. Calculated by taking into account animal weight at slaughter, but also correcting for imported and exported animals that either did, or did not receive antibiotics, and were subsequently transported to another country.

Antimicrobial use in food-producing animals in the Netherlands has been reported since 2002 in the Monitoring of Antimicrobial Resistance and antibiotic Usage in Animals in the Netherlands (MARAN) [4]. As from 2012 the Animal Drug Authority (SDa) monitors the antimicrobial use in food-producing animals based on more detailed information, and also defines benchmark indicators for responsible use of antibiotics in the different animal sectors.

All food-producing animal sectors monitored in MARAN (broilers, pigs, dairy cattle and veal calves), showed a decrease in antimicrobial usage (defined as animal daily dosage per year; add/yr) over the last several years (www.maran.wur.nl/UK/; Last accessed 25 October 2012). This unit of measurement is used to calculate the number of days per year an average animal on a farm is exposed to antimicrobials. This type of antimicrobial usage measurement provides insight in how the antimicrobials sold in a country are administered to different animal species. To date only in Denmark and the Netherlands this type of usage and exposure data is available. Based on add's in fattening pigs a decrease was observed since 2008, in sows/piglets and broilers since 2009. In veal calves, antimicrobial use was monitored since 2007. Since then, a decreasing trend in use was observed. In dairy cattle, antimicrobial usage in 2010 and 2011 is lower compared to 2008, but higher compared to 2009. The overall decrease since 2008 is relatively small. However, when taking the actual exposure into account, veal calves generally receive more antimicrobials compared to dairy cattle. In 2011 the average dose in veal calves was 25 add/yr, compared to 6.1 add/yr in dairy cattle. Broilers and pigs received 16 add/yr (broilers), 13 add/yr (sows/piglets) and 8 add/yr (fattening pigs) respectively in 2011. A more detailed presentation of antimicrobial exposure in veal calves is given in Figure 1.2. The main classes of antimicrobials used in 2011 were tetracyclines, penicillins, trimethoprim/sulfonamides, macrolides/lincosamides and intestinal anti-infectives (e.g. colistin and neomycin). The important classes fluoroquinolones and 3rd/4th generation cephalosporins were used relatively little.

Figure 1.2: Antimicrobial use in veal calves in daily dosages per animal per year (add/yr) in the Netherlands



This table was obtained from: www.maran.wur.nl/UK/

Antimicrobial resistance in food-producing animals

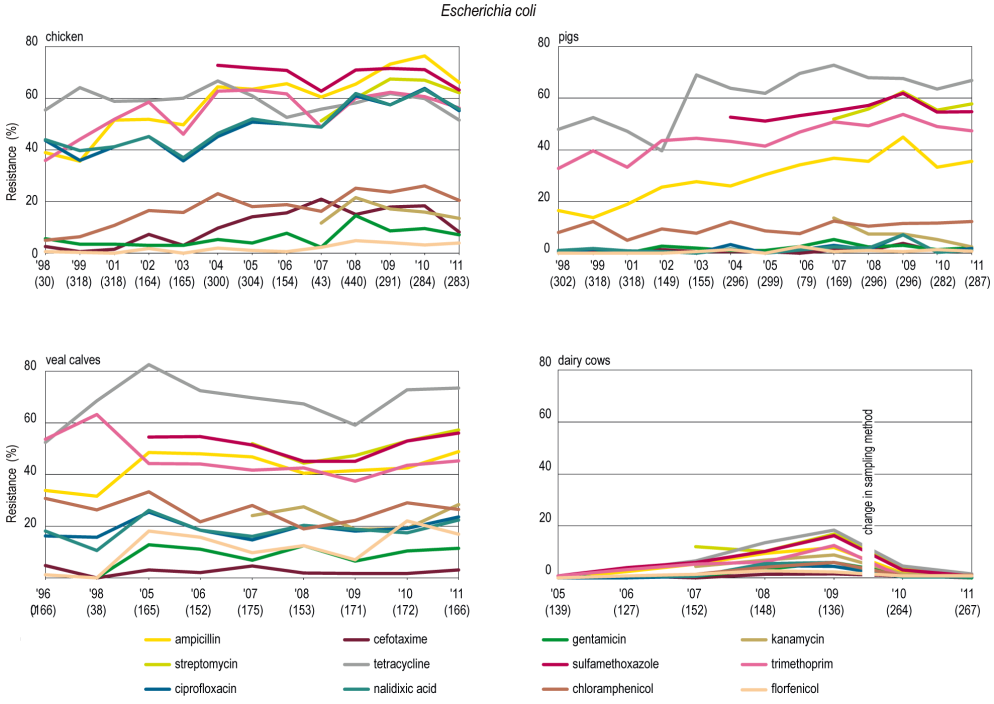
The susceptibility of bacteria to antimicrobials is determined by the Minimal Inhibiting Concentration (MIC) which is reported in mg/L. At this concentration of antimicrobials, the bacteria are no longer able to replicate. Resistant bacteria have a higher MIC-value than susceptible bacteria. To determine when bacteria are considered resistant to a certain antimicrobial, MIC cut-off values were determined for many different antimicrobials. When bacteria have a MIC-value above this cut-off value for a certain antimicrobial, it is considered resistant. The susceptibility cut-off values used in the in the surveillance were defined for epidemiological purposes [5]. For clinical interpretation, break points are determined [5, 6]. In general epidemiological cut-offs are lower compared to break point values defined for clinical interpretation. In this thesis, if available, data were interpreted using epidemiologic cut-off values. When epidemiological cut-offs are used, the term resistance should be interpreted as non-wild type susceptibility [7] or reduced susceptibility.

Antimicrobial resistance in food-producing animals and food products of animal origin is reported worldwide. In order to monitor trends in prevalence of antimicrobial resistance, many surveillance systems are in place. The European Food Safety Authority (EFSA) reported surveillance data from several European countries (using epidemiological cut-off values) as part of an on-going surveillance. A part of this report provides quantitative MIC data on antimicrobial resistance in *Escherichia coli* (*E. coli*), which was used as a commensal indicator organism [8]. For cefotaxime, used as an indicator for resistance to 3rd generation cephalosporins, a relatively low prevalence was shown of approximately 5% or less in cattle (dairy cattle, beef cattle and veal calves combined) from 2005 to 2010 in the Netherlands, Austria, Estonia, Spain and Denmark. Germany and Switzerland were the only countries that reported data from veal calves separately in 2010, and showed a prevalence of 10% and 1% respectively. For fluoroquinolones, all countries except Germany, showed a relatively low prevalence below 10% in cattle. In Germany (only reporting veal calves) prevalence was 42%. In preceding years prevalence in cattle in the Netherlands and Estonia was higher (over 40%) compared to 2010, in Germany prevalence was lower (approximately 20%) compared to 2010, and in the remaining countries it was approximately similar compared to 2010. In general, even though all countries showed large differences in prevalence compared to each other, for all countries reduced susceptibility levels to ampicillin, streptomycin, sulfonamides and tetracyclines were relatively high. In broilers, the Netherlands and Germany showed the highest levels in reduced susceptibility to all antimicrobials (except fluoroquinolones in Austria), compared to Austria, Denmark, France, Sweden and Switzerland. Compared to the data from cattle, levels of all antimicrobial classes were higher. The largest difference is the higher level of reduced susceptibility to fluoroquinolones. Especially in Austria, 80% of the isolates were reduced susceptible. In pigs, differences between countries are not as big is shown for cattle and broilers. Also in pigs, the highest levels of reduced susceptibility were observed for ampicillin, streptomycin, sulfonamides and tetracyclines. Levels of reduced susceptibility to 3rd generation cephalosporins and fluoroquinolones were relatively low (5% or lower) in all countries (Austria, Denmark, Estonia, Finland, France, the Netherlands and Switzerland).

Also in the United States (US) [9] and Canada [10] surveillance of antimicrobial resistance is performed in food-producing animals and food products of animal origin. In the US and

Canada different standards for interpretation of resistance data are used compared to European countries [6]. This makes comparing data to European countries rather difficult. Furthermore, in the US only retail meat products and chicken carcasses are samples for monitoring antimicrobial susceptibility among *E. coli*. Nonetheless, also in chickens from the US, resistance to ampicillin, streptomycin, sulfonamides and tetracyclines were relatively high, compared to other antimicrobials, as was observed in Europe. As was shown in European countries, in Canada, also resistance levels in poultry to all classes of antimicrobials were higher compared to cattle and pigs, with exception of penicillins and tetracyclines, which were higher in pigs.

Figure 1.3: Trends in resistance (%) of *E. coli* isolated from broilers, slaughter pigs, veal calves and dairy cattle in the Netherlands from 1998 – 2011



This figure was obtained from MARAN 2012 [4]. For dairy cattle, sampling strategy changed between 2009 and 2010 from sample collection at farm level to individual animals randomly sampled at slaughterhouses. Susceptibility data were interpreted using cut-off values defined for epidemiological purposes as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [5]

Next to the monitoring of antimicrobial use in food-producing animals in the Netherlands, surveillance of antimicrobial resistance in both food-borne pathogens (*Salmonella*, *Campylobacter* and Shiga-toxin producing *Escherichia coli* O157), as well as commensal indicator organisms (*Escherichia coli* and *Enterococcus faecalis* and *E. faecium*) is performed[4]. As can be seen in Figure 1.3, randomly selected commensal *E. coli*, isolated from

poultry, pigs and veal calves, all showed reduced susceptibility to tetracycline, trimethoprim, sulfamethoxazole, streptomycin and ampicillin (except for pigs) in 40% or more of the isolates from 2001 (poultry), 2002 (pigs) or 2005 (veal calves). A clear dissimilarity in these animal groups is the difference in reduced susceptibility to (fluoro)quinolones (ciprofloxacin and nalidixic acid). In poultry this varies from approximately 40% to 60%, in veal calves it remains approximately 20% and in pigs it remains below 10%. Also the reduced susceptibility to 3rd/4th generation cephalosporins (cefotaxime) differs between the different animal groups. In pigs and veal calves it remains below 5%. In contrast, in poultry it increased from 2003 to 2007, than stabilized from 2007 to 2010, and subsequently decreased after 2010. A reason for these differences may be that a relatively large amount of fluoroquinolones were used in poultry in the past, compared to pigs and veal calves. This was also the case for the use of ß-lactam antibiotics in poultry compared to fattening pig. Usage of ß-lactams was also higher in poultry compared to veal calves and sows/piglets, but these differences were relatively small [11]. In contrast to what was described above for poultry, pigs and veal calves, is what was observed in dairy cattle. Levels of reduced susceptibility to all antimicrobials were relatively low (Figure 1.3). An increase was observed from 2005 to 2009 for all antimicrobials, however, after the sampling strategy was changed from farm level to individual animals being sampled at slaughter, prevalence decreased again (Figure 1.3). The status on farm level after 2009 is not known.

Mechanisms of antimicrobial resistance

From the 1930s, many different antimicrobial classes have become available for human medicine, especially between 1940 and 1970 (Figure 1.4) [12, 13]. The various classes of antimicrobials have different mechanisms of action. Important bacterial targets are: 1 interference with cell wall synthesis; 2 Inhibition of protein synthesis; 3 Interference with DNA and RNA replication; 4 Inhibition of the metabolic pathway; 5 Disruption of the bacterial membrane structure [14]. Figure 1.5 shows the bacterial targets of different classes of antimicrobials [13].

Figure 1.4: Introduction of new antimicrobial classes

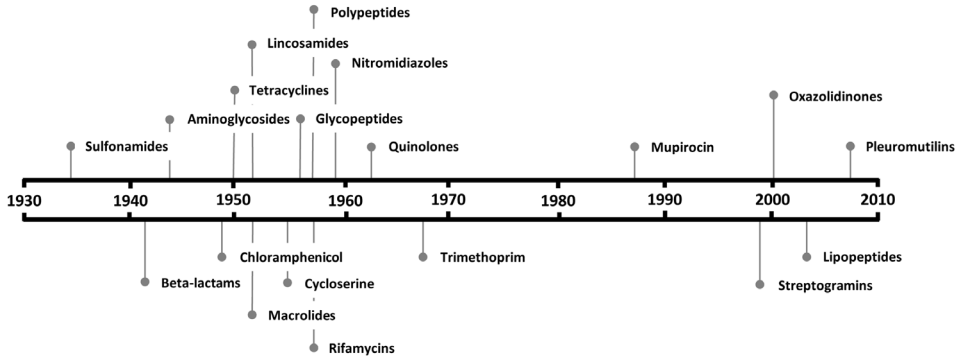
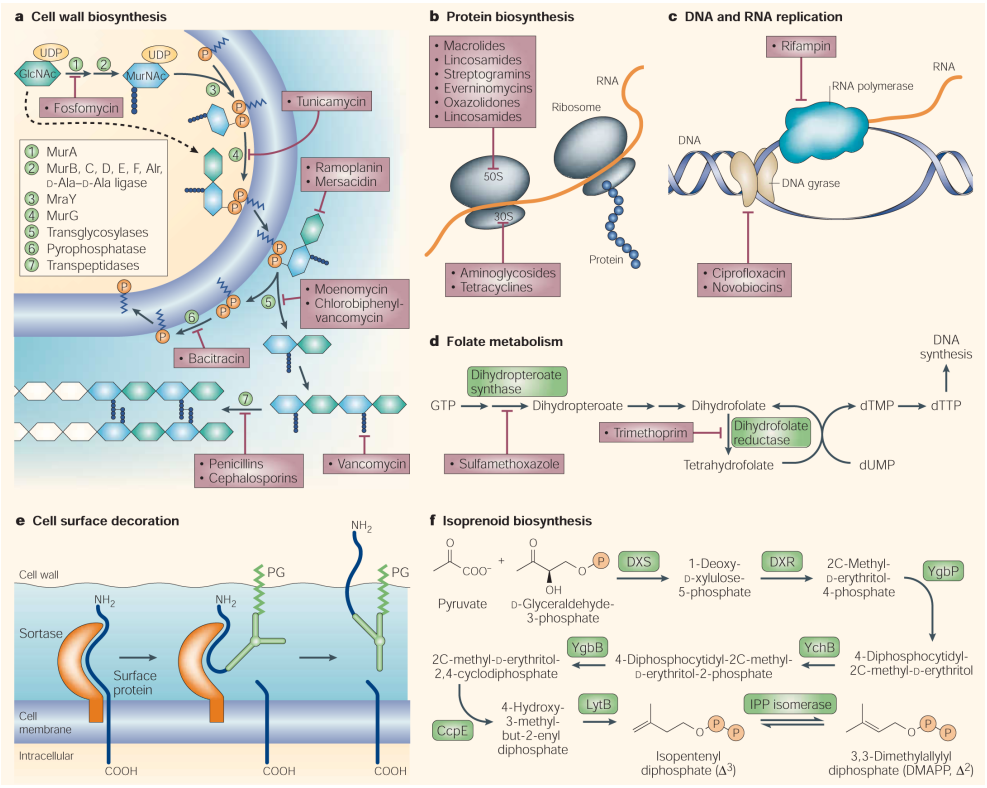


Figure modified from Colson 2008 [12]

Figure 1.5: Schematic overview of antimicrobial targets



Walsh et al [13]

Resistance to all classes of antimicrobials has emerged, often quite rapidly after their introduction on the market [13, 15]. Clinically relevant antimicrobial resistant pathogens were not only found in hospital settings, but also in the community [16]. Resistance to penicillin was already discovered before the drug was used in a clinical setting [17]. Resistance to antimicrobials can either be innate or acquired [18]. Especially acquired resistance determinants are a cause for concern. In case of acquired resistance, bacteria originally susceptible to antimicrobials can become resistant by either incorporation of extra-chromosomal genetic material (horizontal gene transfer through conjugative plasmids or transposons), or by mutations in the DNA. Bacteria can become resistant trough several mechanisms: 1 permeability changes in the bacterial cell wall; 2 Active efflux of antimicrobials; 3 Enzymatic modification/degradation of antimicrobials; 4 Modification of drug target; 5 Acquisition of alternative metabolic pathways[18-21].

Almost all classes of antimicrobials are used in both human and veterinary medicine. This results in cross-resistance of bacteria to antimicrobials used in humans due to exposure of antimicrobials used in veterinary medicine. The prevalence of antimicrobial resistance and diversity of resistance determinants is increasing worldwide [22]. This in combination with

multi-drug resistance may complicate the treatment of bacterial infection, or may even lead to treatment failure. Therefore, the World Health Organization (WHO) categorized several classes of antimicrobials as 'critically important for human medicine' [23]. Classes of antimicrobials were designated critically important if they were: 1; sole therapies or one of few alternatives to treat serious human disease, and 2; used to treat diseases caused by organisms that may be transmitted via non-human sources or diseases caused by organisms that may acquire resistance genes from non-human[23]. For two critically important groups of antimicrobials, quinolones and ß-lactam antimicrobials, further discussed in this thesis, an overview of the resistance mechanisms is given.

Quinolone resistance

Nalidixic Acid was the first quinolone introduced in human medicine in the late 1960s, and was used for treatment of urinary tract infections [24]. From the 1980s onward, quinolones were also used to treat infections at multiple body sites [24]. Further modification of this antimicrobial class led to new structures with a wider spectrum of efficacy. The introduction of a fluorine substituent at the 6-position of the core quinolone ring led to the formation of fluoroquinolones [24]. An important fluoroquinolone in human medicine is ciprofloxacin. A homologue enrofloxacin (Baytril®), is used in veterinary medicine.

Mechanism of action

The target of (fluoro)quinolones in a bacterial cell are the DNA gyrase and topoisomerase IV enzymes [25]. The DNA gyrase enzyme consists of two GyrA and two GyrB subunits, encoded by the *gyrA* and *gyrB* genes. The topoisomerase IV enzyme is homologous to the gyrase enzyme and consists of two ParC and two ParE subunits, encoded by the *parC* and *parE* genes [26]. Both enzymes are involved in DNA replication (Figure 1.5), and play a distinct role in the formation and relaxation of negative supercoils of the bacterial chromosome, and decatenation of daughter chromosomes to allow segregation into daughter cells [26, 27]. These are essential steps in DNA replication and subsequent cell division. Interfering with this process prevents a bacterial cell from replicating.

Resistance determinants

Altered drug target enzymes, caused by mutations in the chromosomally located DNA gyrase and topoisomerase IV genes are reported commonly. Within these gyrase and topoisomerase genes, mutations associated with resistance are clustered in a specific region called the Quinolone Resistance Determining Region (QRDR) [26]. In *E. coli*, GyrA is thought to be the primary target of quinolones, since mutations in *parC* and *parE* have shown only to contribute to quinolone resistance when mutations in *gyrA* were present [28, 29]. For Gram-positive bacteria it was demonstrated vice versa, mutations in *gyrA* were not observed in the absence of mutations in *parC* or *parE* [30, 31]. Mutations associated with resistance in *gyrB* generally occur less frequent than those in *gyrA*. The same applies for *parE* versus *parC* [26]. Additionally, multiple mutations in the QRDR region of the various genes increases the level of resistance [26].

Another important group of various resistance genes are the Plasmid Mediated Quinolone Resistance genes (PMQR). The first PMQR was described by Martinez-Martinez in 1998 [32]. These PMQR genes comprehend a distinct set of resistance genes that can be transferred between bacteria. In general, presence of these genes result in a reduced susceptibility to

ciprofloxacin (MIC ≥ 0.06 mg/L) and nalidixic acid (MIC 4 - 32 mg/L) [33, 34]. Three different transferrable (fluoro)quinolone resistance mechanisms have been described: 1 Protection of DNA gyrase and topoisomerase enzyme by steric hindrance; 2 Efflux of the drug; 3 Modification of the drug. A large family within this group of PMQR genes are the *qnr* genes [35]. The *qnr* genes code for proteins that are able to bind and protect the DNA gyrase and topoisomerase IV enzymes from inhibition by ciprofloxacin [36]. To date, five subgroups have been reported: *qnrA* [37], *qnrB* [38], *qnrC* [39], *qnrD* [40] and *qnrS* [41]. For *qnrA*, *qnrB* and *qnrS*, many different allelic variations have been reported and is this number is still expanding (http://www.lahey.org/qnrStudies/; last accessed 25 October 2012). Efflux of the drugs is facilitated by efflux pumps, which are protein structures located in the bacterial cell wall. Two variants are encoded by genes that were described to be plasmid mediated. One is the quinolone specific efflux pump A (QepA) [42], another is the multi-drug exporting pump OqxAB [43, 44]. Finally, an enzyme encoded by a modified aminoglycoside resistance gene, *aac(6')Ib-cr*, was shown also to reduce quinolone susceptibility by *N*-acetylation of ciprofloxacin [45].

In addition to the plasmid mediated efflux pumps mentioned above, various chromosomally encoded efflux pumps and porins may also play a role in reducing susceptibility to quinolones [18, 46, 47]. Efflux pumps through up regulation of expression, decreasing drug concentrations within the bacterial cell, and porins by down regulation of expression, decreasing bacterial influx of the drug. Both systems are not substrate specific [18], as was shown for the QepA efflux pump [42].

ESBL/AmpC producing Escherichia coli

The class of ß-lactam antimicrobials include monobactams, penicillins, cephalosporins, cephamycins and carbapenems. These are also used for bacterial infections at multiple body sites. The groups of cephalosporins can be further subdivided into several generations of cephalosporins (1 to 5). Third to fifth generation cephalosporins are also referred to extended spectrum cephalosporins or extended spectrum ß-lactams. Important antibiotics within these groups are: aztreonam (monobactam), amoxicillin (penicillin), cefotaxime (3rd generation cephalosporin), cefoxitin (cephamycin) and ertapenem (carbapenem).

Mechanism of action

The target for ß-lactam antimicrobials is disruption of the bacterial cell wall by inhibiting the formation of a peptide bond in the biosynthesis of the peptidoglycan layer (Figure 1.5) [13]. The ß-lactam ring of the antibiotic is 'mistakenly' incorporated as a building block, preventing cross-linkage of adjacent glycan strands [48], thereby destabilizing the cell wall. The most common resistance mechanism for ß-lactam antibiotics is enzymatic hydrolysis of the ß-lactam ring, resulting in inactive antimicrobial compounds [49].

Extended Spectrum ß-Lactamases (ESBL) and AmpC ß-Lactamases are two distinct types of enzymes, both of which confer reduced susceptibility to 3rd generation cephalosporins, penicillins and monobactams. They can be differentiated based on a different susceptibility towards 4th generation cephalosporins, inhibitors (e.g. clavulanic acid) and cephamycins. ESBL-type enzymes cause resistance to 4th generation cephalosporins (e.g. cefepime), are susceptible to cephamycins (e.g. cefoxitin) and show a synergistic effect when a 3rd generation cephalosporin is potentiated with clavulanic acid. AmpC-type enzymes show the opposite

phenotype, being susceptible to 4th generation cephalosporins, are resistant to cephamycins and inhibit the synergistic effect of a 3rd generation cephalosporin potentiated with clavulanic acid. [6, 50, 51].

Resistance determinants
Over the years, but especially in the last decade, increasing prevalence of ESBL/AmpC producing bacteria have been reported in clinical settings and surveillance studies [52, 53]. In *Enterobacteriaceae* ESBL/AmpC genes are often carried on plasmids, which can be transferred horizontally between bacteria of either the same or different species [54, 55]. A wide variety exists in ESBL/AmpC enzymes. These enzymes are classified according to either their functional characteristics [49, 56], or to their primary structure [57]. Important gene families within the group of ESBLs are bla_{CTX-M}, bla_{TEM} and bla_{SHV}, but also many other exist [58]. An important gene family within the group of plasmid mediated AmpCs is bla_{CMY}, but also within this group many others are reported [59]. Within each gene family, a high allelic variation exists, and novel variants are continuously reported. Table 1.1 shows different enzyme families with the number of allelic variations reported until 2010 [60]. At present, when all gene families are combined, over 1000 allelic variations have been reported (http://www.lahey.org/studies; last accessed 1 November 2012).

Next to the (in general) plasmid mediated ESBL/AmpC genes, a chromosomally located *ampC* gene has been described. In *E. coli*, the *ampC* gene is constitutively expressed by a weak promoter, which does not result in decreased susceptibility to penicillins, cephalosporins or cephamycins [61, 62]. However, various alterations in the promoter or attenuator region of this *ampC* gene may result in differences in promoter strength, or an altered transcription regulation [62-65]. In some cases this leads to a decreased susceptibiltity to cephmycins and cephalosporins.

Finally, reduced susceptibility has been described as a result of alterations in permeability. In various bacteria, different efflux pumps are thought to play a role in lowering the drug concentration within the bacterial cell [18]. Also the loss of porins may facilitate a reduction in susceptibility, due to a decreased uptake of antimicrobials [66, 67].

Outline of this thesis

This thesis is subdivided in two parts, focusing on the prevalence and molecular characteristics of quinolone resistance (part 1) and ESBL/AmpC-producing *Escherichia coli* (part 2) in veal calves. These studies were conducted as part of the research program Antimicrobial Resistance in Veal Calf Farming (ABRES), funded by the Dutch Ministry of Economic Affairs and the Product Boards for Livestock and Meat (PVV).

Fluoroquinolones are regarded as critically important antimicrobials in human medicine, but are also used in veterinary medicine [23]. Resistance to drugs belonging to this class of antimicrobials are reported worldwide, however little is known about quinolone resistance in veal calves. **Chapter 2** describes the prevalence and molecular characteristics of quinolone resistance in veal calves in a cross sectional study, based on isolates collected in the surveillance

of antimicrobial resistance in food-producing animals. In addition, **chapter 3** will go into further detail by characterizing the flanking region of the PMQR gene *qnrB19*, and these results will be compared to the flanking regions of *qnrB19* genes of human origin, described by other research groups.

As for fluoroquinolones, 3rd/4th generation cephalosporins are regarded as critically important antimicrobials in human medicine [23]. Resistance to this class of antimicrobials due to ESBL/AmpC-producing bacteria is reported increasingly, and given the diversity in resistance genes and associated plasmids, its epidemiology is complex. ESBL/AmpC producing bacteria have been described mainly in dairy and beef cattle. Little is known about its prevalence and molecular characteristics in veal calves. **Chapter 4** describes the occurrence of different ESBL/AmpC genes over a fourteen-year period, based on faecal samples collected at farms. In **chapter 5** results will be shown describing the prevalence at the end of a production cycle, at slaughter. In this study, also the within-herd prevalence was taken into account, as well as the association of different ESBL/AmpC genes to different plasmids. Finally, **chapter 6** describes a longitudinal field-study, in which the dynamics in carriage of different ESLB/AmpC genes in association with different plasmids and *E. coli* will be reported. Individual animals were repeatedly sampled during the first ten weeks of their presence at the fattening farm.

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Part I

Quinolone resistance in commensal *Escherichia coli* in veal calves

Chapter 2

Prevalence and characteristics of quinolone resistance in Escherichia coli in veal calves

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Abstract

Quinolone resistance is studied and reported increasingly in isolates from humans, food-producing animals and companion animals. Resistance can be caused by chromosomal mutations in topoisomerase genes, plasmid-mediated resistance genes, and active transport through efflux pumps. Cross sectional data on quinolone resistance mechanisms in non-pathogenic bacteria from healthy veal calves is limited. The purpose of this study was to determine the prevalence and characteristics of quinolone resistance mechanisms in *E. coli* isolates from veal calves, after more than 20 years of quinolone usage in veal calves. MIC values were determined for all isolates collected as part of a national surveillance program on antimicrobial resistance in commensal bacteria in food-producing animals in the Netherlands. From the strains collected from veal calves in 2007 (n=175) all isolates with ciprofloxacin MIC ≥ 0.125 mg/L (n=25) were selected for this study, and screened for the presence of known quinolone resistance determinants. In this selection only chromosomal mutations in the topoisomerase type II and IV genes were detected. The number of mutations found per isolate correlated with an increasing ciprofloxacin MIC. No plasmid-mediated quinolone resistance genes were found. The contribution of efflux pumps varied from no contribution to a 16-fold increase in susceptibility. No correlation was found with the presence of resistance genes of other antimicrobial classes, even though all quinolone non-wild type isolates were resistant to 3 or more classes of antibiotics other than quinolones. Over twenty years of quinolone usage in veal calves in the Netherlands did not result in a widespread occurrence of plasmid-mediated quinolone resistance, limiting the transmission of quinolone resistance to clonal distribution.

Introduction

Quinolones are considered to be drugs of critical importance to humans. They are also frequently used in veterinary medicine. Quinolones have been used in calves in the Netherlands for therapy of gastro-intestinal and respiratory infections since flumequine was approved in 1981. Fluoroquinolones have been introduced in 1987 (enrofloxacin) for therapeutic use in calves and poultry. In the 1990s other fluoroquinolones besides enrofloxacin have been approved for use in food-producing animals (e.g. marbofloxacin, danofloxacin). Although resistance against fluoroquinolones in microorganisms with a zoonotic potential poses a risk to human health, knowledge of the emergence of quinolone resistance mechanisms in non-pathogenic bacterial isolates from veal calves in specific is limited. Unlike poultry, dairy cattle and to a lesser extent slaughter pigs, veal calf herds in the Netherlands are assembled from animals imported from many different European countries. Therefore herds act as a large melting pot for animals with different antimicrobial resistances obtained from different (foreign) farms or during transport.

Antimicrobial resistance in food-producing animals in The Netherlands has been monitored since 1996 [1]. To study the effects of selection pressure of antibiotic usage in animals, the resistance level of commensal organisms was determined. Commensal *Escherichia coli* was isolated from the intestinal tract of the animals. These surveillance data show that the prevalence of multi-resistant *E. coli* (resistant to 3 or more classes of therapeutic antibiotics) in food-producing animals increased in the last decade [2]. In veal calves the prevalence of multi-resistant *E. coli* is stable since 2006 [2]. In veal calf *E. coli* isolates from 1996 to 2010, the prevalence of non-wild type phenotype to fluoroquinolones varies between 15 and 25 percent.

Quinolones target the topoisomerase type II and IV enzymes [3,4]. Quinolone resistance may arise through different possible mechanisms, of which various point mutations in the quinolone resistance determining region (QRDR) of the topoisomerase type II and IV enzymes are most frequently observed [5]. Plasmid-mediated quinolone resistance (PMQR) mechanisms however, are studied and reported increasingly. These mechanisms include *qnr* genes [6], *qepA* [7], *oqxAB*[8] and *aac(6')-Ib-cr* [9] all result in reduced susceptibility to quinolones.

Finally, quinolone susceptibility can be decreased by altered permeation of the outer membrane and/or active efflux of drugs. For *E. coli* it was shown that the *marRAB* regulon is involved in both the regulation of outer membrane porin OmpF as well as the regulation of the endogenous AcrAB membrane efflux pump [10]. Both play a role in regulation of the cytosolic concentration of certain antimicrobials, including quinolones.

The purpose of this paper was to determine the prevalence and molecular characteristics of quinolone resistance determinants in *E. coli* from veal calves, based on isolates from a national surveillance program, after more than 20 years of usage of quinolones in veterinary medicine in the Netherlands.

Material & Methods

Strain selection
All *E. coli* isolates were obtained from randomly collected pooled fecal samples (one *E. coli* per pooled sample). Each pooled sample was taken from one herd per farm as part of the annual surveillance program on antimicrobial resistance in animals in The Netherlands. The complete 2007 strain collection, stored at -80˚C, includes 175 *E. coli* isolates obtained from veal calves. From all 175 isolates MICs were determined for a customized panel of antibiotics (see section *antimicrobial susceptibility)*. From this collection, all non-wild type isolates with ciprofloxacin MIC ≥ 0.125 mg/L (designated 'quinolone non-wild type (WT) selection') were included in this study (n=25) for further analysis.

Antimicrobial susceptibility
Susceptibility to antimicrobials was determined by broth microdilution according to ISO standards (ISO 20776-1:2006), using microtiter trays with a customized panel of dehydrated antibiotics. This panel included sulfamethoxazole, gentamicin, ampicillin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, kanamycin and colistin (panel EUMVS, Sensititre©, Trek Diagnostics, UK).

Sequence analysis QRDR & PMQR
The twenty five selected quinolone-resistant isolates were screened for the presence of mutations in the QRDR region of *gyrA*, *gyrB*, *parC* and *parE* as described before [11], using *E. coli* ATCC 25922 as a quinolone susceptible control strain.
The presence of PMQR genes was determined as described previously for *qnrA* and *qnrS* [12], *qnrB* [13], *qnrC* [14] and *qnrD* [15],
qepA [16] and *aac(6')-Ib-cr* [17]. All sequence reactions were performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Since the OqxAB efflux pump is not specific to quinolones, its presence was not determined. However, the contribution of efflux pumps in general was determined by a separate assay.

Contribution of efflux pumps
For each isolate of the quinolone non-WT selection with a ciprofloxacin MIC of 0.25 – 2 mg/L growth curves were determined at the following ciprofloxacin (Sigma-Aldrich, Germany) concentrations: 0 (negative control), 0.06, 0.125, 0.25, 0.5, 1 and 2 mg/L. Isolates with ciprofloxacin MIC > 8 mg/L were exposed to ciprofloxacin concentrations of 0, 0.5, 1, 2, 4, 8 and 16 mg/L. To determine the contribution of efflux pumps to quinolone susceptibility, all growth curves were measured with or without the presence of efflux pump inhibitors 1-(1-naphthylmethyl)-piperazine (NMP) (Sigma-Aldrich, Germany) or phenylalanine-arginine-ß-naphthylamide (PAßN) (Sigma-Aldrich, Germany), both at a concentration of 50 mg/L. A cell suspension of $1.5 * 10^6$ CFU/ml in cation adjusted Mueller Hinton broth (CAMHB, Sensititre®, Trek Diagnostics, UK) was prepared for each isolate. Growth was determined by measuring optic density (OD) of 200 µl cell suspension with different ciprofloxacin concentrations with and without an efflux pump inhibitor. Samples were measured at 37°C in a honeycomb multiwell plate, using a wide band filter (420-580 nm) with 10 min intervals for 7 hours, using the Bioscreen C (Oy Growth Curves AB Ltd, Finland). All measurements were

performed in triplicate. The area under the curve (AUC) was determined for all growth curves using equation 1 [18]. One-way analysis of variance (ANOVA) was performed separately on the three treatments with PAβN, NMP or no EPI respectively, to determine at which ciprofloxacin concentration growth was significantly reduced using 0 mg/L ciprofloxacin as negative control. Furthermore, the negative control curves of PAβN, NMP and no inhibitor were also compared at the significance level p=0.05.

Eqation 2.1: Calculation of Area Under the Curve

$$AUC = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_0 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

AUC = area under curve; N_0 = OD of cell suspension at time t_0; N_1 = OD of cell suspension at time t_1; N_n = OD of cell suspension at time t_n; t_1 = time of first measurement after beginning of test; t_n = time of n^{th} measurement after beginning the test

Micro array analysis

To determine the presence of antimicrobial resistance genes of other classes of antibiotics than quinolones, all twenty five isolates from the quinolone non-WT selection were screened using the Amr-ve kit on a miniaturized array platform (Alere, UK). Cell lysis, target labeling, and probe hybridization were performed according to the manufacturers' protocol.

Results

Antimicrobial resistance phenotype

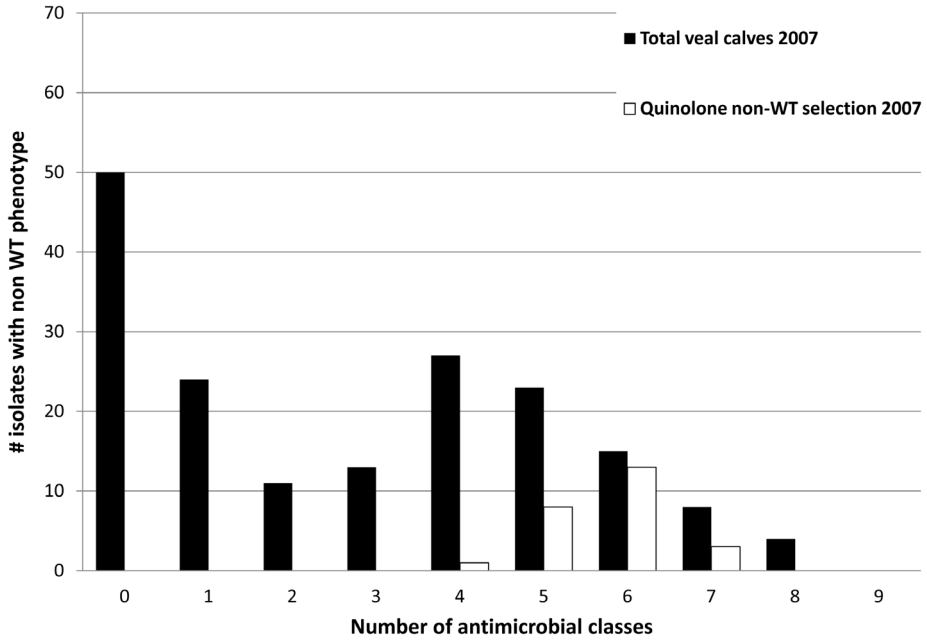
For the total 2007 strain collection (n = 175), non-wild type MICs were determined for ampicillin, cefotaxime, ceftazidime, gentamicin, tetracycline, sulfamethoxazole, trimethoprim, florfenicol, chloramphenicol, streptomycin, kanamycin, ciprofloxacin and nalidixic acid (Table 2.1)**.** Non-wild type MICs for colistin were not observed. From this collection, twenty five isolates showed a non-wild type MIC for ciprofloxacin, ranging from 0.25 to > 8 mg/L, and for nalidixic acid > 64 mg/L (Table 2.1 and 2.2). In these twenty five isolates, designated "quinolone non-WT selection", the percentage of non-wild type MICs for the other antibiotics in the above mentioned panel was 1.5 to 4 fold higher compared to the total strain collection (Table 2.1). As a result, isolates with a non-WT quinolone phenotype were co-resistant to at least 3 additional classes of antimicrobials other than (fluoro)quinolones (penicillins, cephalosporins, tetracyclines, aminoglycosides, sulfonamides or phenicols) (Figure 2.1).

Table 2.1: Percentage of non-wild type MICs in both '2007 total strain collection' and 'quinolone non-WT selection'

	Cip	Nal	Amp	Ctx	Ctz	Gen	Tet	Sul	Tmp	Ffn	Chl	Str	Kan	Col
Total strain collection (n=175)	14	14	47	5	3	7	70	51	42	10	28	52*	24*	0*
Quinolone non-WT selection (n=25)	100	100	84	4	12	36	100	100	88	40	76	88	68	0

*Cip, ciprofloxacin; Nal, nalidixic acid; Amp, ampicillin; Ctx, cefotaxime; Ctz, ceftazidime; Gen, gentamicin; Tet, tetracyclin; Sul, sulfamethoxazole; Tmp, trimethoprim; Ffn, florfenicol; Chl, chloramphenicol; Str, streptomycin; Kan, kanamycin; Col, colistin. * n=75 for these antibiotics*

Figure 2.1: Number of antimicrobial classes to which *E. coli* strains from the present study show non-wild type MIC-values



Distribution of E. coli isolates that are fully susceptible or resistant to one to eight different antimicrobial classes, shown for all veal calf isolates in the database (n=175) and the quinolone non-WT selection (n=25)

Mutations in QRDR

All isolates harboured one or more point mutations in the QRDR. A clear correlation was shown between the increasing MICs of ciprofloxacin and the number of mutations found in the QRDR region of primarily *gyrA* and in addition *parC* and *parE* (Table 2.2). No mutations were found in *gyrB*. Isolates with a ciprofloxacin MIC up to 1 mg/L showed only one point mutation in *gyrA*, except for isolate 43.72, which has a MIC of 0.5 mg/L and also harboured a point mutation in *parE*. Isolate 41.25, with an additional mutation in *parC*, had a ciprofloxacin MIC of 2 mg/L. All remaining isolates, having ciprofloxacin MIC-values > 8 mg/L, harboured two mutations in the the *gyrA* gene and an additional mutation in *parC*. In addition, one mutation in *parE* was observed in three of the highly resistant isolates.

Plasmid-mediated quinolone resistance

All isolates were screened for the presence of PMQR mechanisms. No genes were found belonging to the *qnr* gene family, nor the quinolone specific efflux pump *qepA*. One isolate harboured an *aac(6')-1b gene*, however it did not show the point mutation specific for the modified aminoglycoside acetyltransferase variant *aac(6')-1b-cr*.

Contribution of efflux pumps

The contribution of efflux pumps to the susceptibility of isolates to fluoroquinolones was determined by measuring growth inhibition at ciprofloxacin concentrations varying from 0.06 to 16 mg/L, with or without the presence of efflux pump inhibitors. Growth inhibition was quantified by calculating the AUC at each ciprofloxacin concentration. All AUCs were compared to a negative control (0 mg/L ciprofloxacin) using the same inhibitor. As demonstrated in Table 2.3, in all isolates with a ciprofloxacin MIC up to 2 mg/L, without addition of an inhibitor, significant growth reduction occurred at 2 or 3 dilution steps below the MIC value. For all isolates with MIC > 8 mg/L, significant growth reduction without inhibitor was observed at various ciprofloxacin concentrations ranging from 1 to 8 mg/L. When adding inhibitor NMP to the medium, no shift in susceptibility could be observed for 11 out of 13 isolates with a MIC up to 0.5 mg/L, since these strains were also growth inhibited at 0.06 mg/L without addition of an inhibitor. Furthermore, adding NMP to isolates with MIC > 0.5 mg/L resulted in no effect for three isolates and an increase in ciprofloxacin susceptibility of 1 to 4 dilution steps in all remaining isolates. When adding inhibitor PAßN to the medium, isolates with a MIC up to 0.5 mg/L showed similar results compared to the addition of NMP. Adding PAßN to isolates with MIC > 0.5 mg/L resulted in no effect for five isolates and an increase in ciprofloxacin susceptibility of 1 to 3 dilution steps for the remaining isolates. Comparing the negative controls (0 mg/L ciprofloxacin) of the treatments without an inhibitor, to the treatments with either NMP or PAßN, shows that adding an inhibitor without ciprofloxacin also causes some growth reduction. In medium supplemented with NMP, 24 out of 25 isolates show a reduction in growth of less than 25% compared to the medium lacking an inhibitor. With PAßN 15 out of 25 isolates show a growth reduction of less than 25%, the remaining 10 exceed 25%.

Micro array analysis

As shown in Table 2.2, all quinolone non-WT isolates harboured genes for resistance to three or more classes of antibiotics other than quinolones. The number of resistance genes detected for each isolate varied from 3 to 12. Most abundant determinants detected were the aminoglycoside resistance gene *aadA1* (75% of the isolates), single or multiple variants per isolate of trimethoprim resistance gene *dfr* (83%), one or more variants per isolate of the sulfamethoxazole resistance gene *sul* (75%) and ß-lactamase bla_{TEM} (75%). No *qnr* genes were detected using the array. The gene *qepA* was not present on the chip. One *aac(6')-1b* gene was detected, however as mentioned above, it did not show the point mutation specific for the *aac(6')-1b-cr* variant. Furthermore, in 14 isolates *str* genes were detected, of which 5 were positive for both *strA* and *strB,* and 9 were only positive for *strB*. Since the presence of only *strB* is rather uncommon, this was verified by PCR. The PCR results show that all isolates harbouring *strB* also harbour *strA* (data not shown), indicating the array showed several false negative results for *strA*.

Table 2.2: Mutations in QRDR-region of topoisomerase II and IV genes and the presence of resistance genes to antibiotics other than quinolones

	MIC mg/L		Mutations in QRDR			
Isolate	CIP[1]	NAL[1]	*gyrA*[2]	*gyrB*	*parC*[3]	*parE*[4]
37.22	0.25	> 64	S83L	-	-	-
42.50	0.25	> 64	D87N	-	-	-
42.57	0.25	> 64	S83L	-	-	-
43.64	0.25	> 64	S83L	-	-	-
44.19	0.25	> 64	S83L	-	-	-
44.36	0.25	> 64	S83L	-	-	-
37.11	0.5	> 64	S83L	-	-	-
40.75	0.5	> 64	S83L	-	-	-
41.67	0.5	> 64	S83L	-	-	-
42.09	0.5	> 64	S83L	-	-	-
42.52	0.5	> 64	S83L	-	-	-
43.72	0.5	> 64	S83L	-	-	S458A
45.01	0.5	> 64	S83L	-	-	-
40.10	1	> 64	S83L	-	-	-
41.25	2	> 64	S83L	-	S80R	-
37.38	> 8	> 64	S83L; D87N	-	S80I	-
40.07	> 8	> 64	S83L; D87N	-	S80I	S458A
40.79	> 8	> 64	S83L; D87Y	-	S80I	S458A
41.26	> 8	> 64	S83L; D87N	-	E84K	-
41.59	> 8	> 64	S83L; D87N	-	S80I	-
41.70	> 8	> 64	S83L; D87N	-	S80I	-
42.03	> 8	> 64	S83L; D87N	-	S80I	-
42.31	> 8	> 64	S83L; D87N	-	S80I	-
42.53	> 8	> 64	S83L; D87N	-	S80I	-
43.47	> 8	> 64	S83L; D87N	-	S80I	S458A

Resistance genes of other antimicrobial classes[5]
aadA1, floR,bla$_{OXA-1}$, *tet*(B)
aadA1, dfrA1, sul3, bla$_{TEM-1}$
dfrA17, sul2, bla$_{TEM-1}$, *tet*(B)
aadA1, catA1, dfrA1, sul2, tet(B), *bla*$_{TEM-1}$, *strB*
aadA1, catA1, dfrA1, tet(A), *bla*$_{TEM-1}$, *sul1, strB*
aadA1, tet(A), *dfrA1, sul1*
aadA1, aadA2, cmlA1, sul3, catA1, dfrA1
aadA1, dfrA1, sul3, bla$_{TEM-1}$
sul2, tet(A), *bla*$_{TEM-1}$, *strB*
aadA1, cmlA1, sul3, bla$_{TEM-1}$
aadA1, dfrA1, strB
aadA1, aadA2, catA1, cmlA1, dfrA1, sul1, sul2, sul3, bla$_{TEM-1}$, *tet*(A), *strA, strB*
aadA1, bla$_{TEM-1}$, *sul1, sul2, tet*(A), *floR, dfrA1, strB*
aadA1, catA1, dfrA1, bla$_{TEM-1}$, *tetB*
aadA1, aadA2, catA1, dfrA1, sul3, bla$_{TEM-1}$, *tetB*
catA1, sul2, bla$_{TEM-1}$, *tet*(A), *strA, strB*
aadA1, aadA2, catA1, dfrA1, tet(A), *tet*(B), *strB*
aac6Ib, catA1, dfrA1, bla$_{TEM-1}$, *tet*(B)
aadA1, catA1, dfrA1, sul1, tet(A), *bla*$_{TEM-1}$, *strB*
aadA1, dfrA1, floR, sul1, sul2, bla$_{TEM-1}$, *tet*(B)
dfrA7, dfrA17, sul1, sul2, tet(A), *strB*
aadA1, catA1, dfrA1, sul1, sul2, tet(A), *bla*$_{TEM-1}$, *strA, strB*
aadA1, dfrA1, bla$_{TEM-1}$, *strB*
aadA1, aadA2, catA1, cmlA1, dfrA1, sul1, sul3, bla$_{TEM-1}$, *tet(A), strA, strB*
sul2, tet(B), *dfrA14, bla*$_{TEM-1}$, *strA, strB*

[1] CIP: ciprofloxacine, NAL: nalidixic acid.

[2] S83L, substitution of serine to leucine at amino acid 83; D87N, aspartic acid to asparagine; D87Y, aspartic acid to tyrosine.

[3] S80R, serine to arginine; S80I, serine to isoleucine; E84K, glutamic acid to lysine.

[4] S458A, serine to alanine.

[5] Resistance determinants detected using the Identibac AMR-ve array tubes. The probe for bla$_{OXA-1}$ *does not distinguish between bla*$_{OXA-1, -30, -31}$ *and* $_{-33}$*. The probe for cmlA1does not distinguish between cmlA1, -4, -5, -6 and -7. The probe for bla*$_{TEM-1}$*detects all bla*$_{TEM}$ *subtypes*

Table 2.3: Ciprofloxacin concentrations at which growth was significantly reduced

		Growth inhibition at [ciprofloxacin]* (mg/L)		
Isolate	**MIC (mg/L)**	**No inhibitor**	**NMP**	**PAßN**
37.22	0.25	0.06	0.06	0.06
42.50	0.25	0.06	0.06	0.06
42.57	0.25	0.06	0.06	0.06
43.64	0.25	0.06	0.06	0.06
44.19	0.25	0.06	0.06	0.06
44.36	0.25	0.06	0.06	0.06
37.11	0.5	0.06	0.06	0.06
40.75	0.5	0.06	0.06	0.06
41.67	0.5	0.06	0.06	0.06
42.09	0.5	0.06	0.06	0.06
42.52	0.5	0.12	0.12	0.06
43.72	0.5	0.06	0.06	0.06
45.01	0.5	0.12	0.06	0.06
40.10	1	0.12	0.06	0.06
41.25	2	0.06	0.12	0.06
37.38	> 8	4	2	1
40.07	> 8	8	4	2
40.79	> 8	4	2	8
41.26	> 8	8	0.5	2
41.59	> 8	1	0.5	2
41.70	> 8	2	2	0.5
42.03	> 8	4	2	2
42.31	> 8	4	1	8
42.53	> 8	4	1	8
43.47	> 8	8	8	2

** = Lowest concentration of ciprofloxacin at which significant growth inhibition was observed (p≤ 0.05) compared to the control (same isolate, same inhibitor, 0 mg/L ciprofloxacin added). Growth curves of all isolates were determined in triplicate and quantified by calculating the 'area under the curve' of each growth curve using equation 1. One-way ANOVA was used to determine significant reduction in growth*

Discussion

Resistance levels of *E. coli* to (fluoro)quinolones in veal calves in the Netherlands have leveled off to values between 15 and 25 percent [2]. Compared to several other European countries, this quinolone resistance level is relatively high [19]. The Dutch resistance level of ciprofloxacin and nalidixic acid in *E. coli* from bovine sources is similar to France and Italy, although the EFSA report is based on data of cattle and not specifically veal calves. One of the causes of this relatively high resistance level might be the usage of antimicrobials. In the Netherlands and France both the overall usage of antibiotics and the usage of fluoroquinolones in veterinary medicine is two to ten times higher compared to other European countries [20].

The first quinolone resistance mechanism discovered was mutation of the gyrase genes [3]. In 1998 the first *qnr* gene was discovered [21], followed in the last decade by more *qnr* variants, *qepA* and *aac(6')Ib-cr*, all plasmid-mediated genes specific for quinolone resistance. Even though PMQR mechanisms are studied and reported increasingly, the results in this study show that after screening all quinolone non-WT *E. coli* isolates from 2007, the reduced susceptibility to quinolones was due to chromosomal mutations in the QRDR region. The mutations found in *gyrA*, *parC* and *parE* in this selection (Table 2.2) have been reported previously [11, 22]. No mutations were observed in the *gyrB* gene.

No plasmid-mediated quinolone resistance determinants were found in this selection of strains. Recently, in an ongoing epidemiological study determining prevalence of non-WT antibiotic phenotype on 50 farms (A.B. Bosman; unpublished data) one *qnrB19* gene was detected in veal calves in the Netherlands [23] and one *qnrS1* gene was observed in veal calves in the annual surveillance program on antimicrobial resistance in animals in the Netherlands (Veldman, unpublished data, 2010), indicating that *qnr* prevalence is still low. This is consistent with observations in other food-producing animals in the Netherlands. So far, several *qnr* genes were detected in *Salmonella enterica* and *E. coli* [24, 25], all isolated from poultry. No *qepA* or *aac(6')-Ib-cr* have been detected, indicating that the prevalence is also very low. This low prevalence indicates that the transmission of quinolone resistance within and between animals is limited to clonal spread. In surrounding European countries plasmid-mediated quinolone resistance in food-producing animals has been observed more frequently, however available data is still limited [24, 26-28]. Outside Europe there is a higher prevalence of PMQR determinants in food-producing animals [29-31]. However, in all studies reported outside the Netherlands, only Kirchner *et al*. report a *qnrS1* isolated from cattle. Veal calves at Dutch fattening farms originate from different dairy farms, including dairy farms from other EU-countries. Therefore, a continuous mixture of bacterial flora will occur in veal calf production. Due to selection pressure by usage of antibiotics, ideal circumstances for selection of resistant organisms are present. Still in veal calves to date, PMQR has been detected only two times, indicating that in cattle in the EU PMQR is still virtually absent.

Data presented in Table 2.3 shows that all isolates with a ciprofloxacin MIC > 1 mg/L and two isolates with a MIC of 0.5 mg/L become more susceptible to ciprofloxacin in the presence of an efflux pump inhibitor. These isolates with a detectable reduction in susceptibility (14 isolates) respond differently to the addition of an inhibitor. Four isolates only showed a reduction when adding NMP, three with PAßN and seven in response to both. The exact mechanism of efflux pump inhibitors is still unknown, although NMP has shown to reverse multi-drug resistance

in *E. coli* overexpressing resistance-nodulation-cell division (RND) type efflux pumps, but not in pump deficient mutants. PAßN also shows inhibitory effects without the presence of RND type efflux pumps in *E. coli* [32]. *E. coli* is known to harbour different efflux pumps, of which the RND efflux pump AcrAB associated with TolC is most predominant and best characterized [33]. The difference in response to the addition of an inhibitor suggests that these isolates differ in expression of different efflux pumps, possess different mutations in their efflux pumps or regulator genes, have differences in efflux pump transcription level, or differ in number of copies of membrane porins [33].
Efflux pump inhibitors NMP and PAßN are commonly used inhibitors in various concentrations ranging from 20 to 160 mg/L [34, 35]. This study shows that growth is significantly reduced in all isolates, solely by adding an inhibitor to the medium without ciprofloxacin. One isolate showed only a reduction in growth by adding NMP without ciprofloxacin and not by adding PAßN. Therefore, the observed mild effect of increased susceptibility by adding inhibitors is indicative, not conclusive. Some studies have shown that the MIC values for the inhibitors themselves is lower in wild type strains than efflux pump deficient strains [34, 35], suggesting that the inhibitors themselves may also act as substrate for efflux. Speculating on the results in the present study, the mild increase in susceptibility by adding an inhibitor to the medium and the growth inhibition in all isolates by just adding 50 mg/L NMP or PAßN without ciprofloxacin, suggests that there is no overexpression of efflux pumps. However, this is not confirmed by quantitative PCR.

All twenty five selected isolates in this study were resistant to 3 or more classes of antibiotics other than quinolones. Even though some isolates show similarities, there is great diversity in the presence of resistance determinants between isolates (Table 2.2). Since quinolone resistance in these isolates is mainly caused by mutations in the chromosomal topoisomerase genes, there is no direct linkage between quinolone resistance and the presence of other resistance determinants. However, looking at the 'quinolone non-WT selection' in Table 2.1, the percentages of resistant isolates for all antibiotics tested except colistin are higher than the percentages in the 'total strain collection'. This suggests that co-selection of resistance determinants for antibiotics other than quinolones occurred.

In conclusion, *E. coli* from Dutch veal calves are frequently resistant to quinolones. In the sample selection described in this study quinolone resistance is mainly driven by chromosomal mutations in the QRDR region of *gyrA/B* and *parC/E*. No PMQR determinants were detected. Inhibition of efflux pumps with NMP or PAßN resulted in a mild decrease in MIC values, indicating a limited contribution of efflux pumps to reduced susceptibility to quinolones in the tested isolates.

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Chapter 3

qnrB19 gene bracketed by IS26 on a 40 kb IncR plasmid from an *Escherichia coli* isolated from a veal calf

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QnrB19 genes have been described in *Escherichia coli*, *Escherichia hermannii*, *Salmonella enterica* and *Klebsiella* spp, located on IncN, IncL/M (human isolates) and ColE-like (both human and chicken isolates) plasmids [1-8]. This study describes the characterization of the genetic environment of a plasmid mediated *qnrB19* gene identified in *E. coli* isolated from a veal calf in the Netherlands.

E. coli strain 013.1 was selected from a fecal sample grown on a MacConkey agar plate supplemented with 0.125 mg/L ciprofloxacin. This strain showed MIC values of 0.25 mg/L and 16 mg/L for ciprofloxacin and nalidixic acid respectively, suggesting the presence of a plasmid-mediated quinolone resistance mechanism [9, 10].

PCR as described for *qnrA* and *qnrS* [11], *qnrB* [12], *qnrC* [13], *qnrD* [14], *qepA* [15] and *aac(6')-Ib-cr*[16] and sequence analysis revealed a *qnrB19* gene [17], whereas chromosomal mutations in the topoisomerase genes *gyrA/B* and *parC/E* were absent.[18].

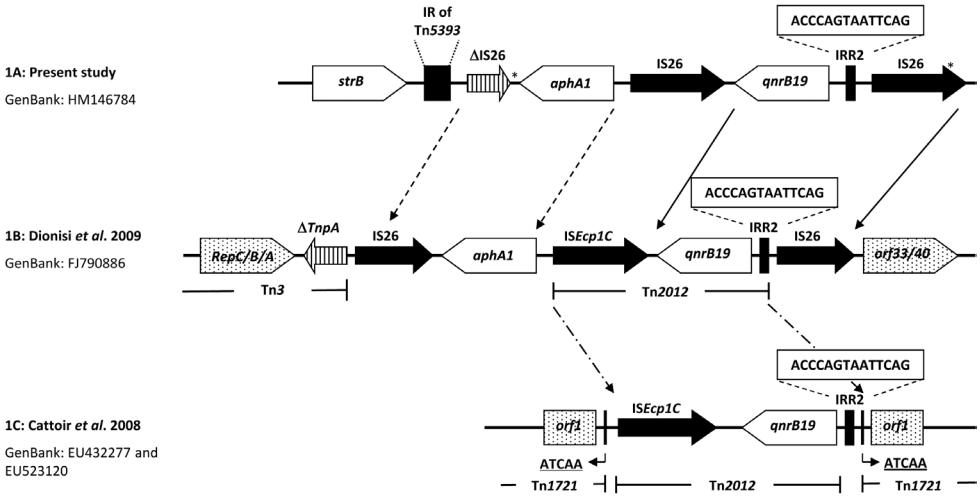
Conjugation experiments using standard broth mating experiments with rifampicin resistant *E. coli* K12 as recipient were not successful. However, DH10B (Gibco Invitrogen) transformants (designated 013.1-T) could be selected on Luria-Bertani agar plates supplemented with 0.03 mg/L ciprofloxacin (Sigma) using plasmids isolated from strain 013.1, indicating that the *qnrB19* gene was plasmid located. Strain 013.1-T showed MIC values for ciprofloxacin and nalidixic acid of 0.12 mg/L and ≤ 4 mg/L, respectively. Untransformed DH10B cells were susceptible to ciprofloxacin (MIC 0.008 mg/L) and nalidixic acid (MIC ≤ 4 mg/L). Strain 013.1-T did not harbour *qepA* or *aac(6')-Ib-cr* genes, or mutations in the chromosomally located *gyrA*, *gyrB*, *parC* or *parE* genes.

Plasmid sizes were determined by S1-PFGE [19], as well as by S1-GE (running at 80V for 4 hours). Strain 013.1 harboured a 40 kb and a 2.6 kb plasmid. Strain 013.1-T harboured a 40 kb plasmid only. Subsequently, the plasmids were identified by PCR-Based Replicon Typing (PBRT) [4, 20] as replicon type IncR and as ColE in 013.1. Transformant 013.1-T harboured only an IncR type plasmid, designated p013.1IncR.

Sequence analysis of the *qnr* gene and its flanking regions was performed by primer walking analysis on purified DNA of p013.1IncR. The *qnrB19* gene was bracketed by two identical insertion sequences IS26 (figure 1A). Further downstream of the *qnrB19* gene an *aphA1* gene was identified, followed by a partial IS26 sequence, which was interrupted by transposable element *Tn5393* harbouring a *strB* gene. Two regions, one comprising of *qnrB19*, IRR2 and a IS26 sequence and a second comprising of *aphA1* and the partial sequence of IS26 (figure 1A and 1B), both showed 1 point mutation difference compared to the sequences published previously [2]. Both full length IS26 elements bracketing the *qnrB19* gene are identical to each other (figure 1A) and to the one previously described downstream *aphA1* (figure 1B) [2]. IS*Ecp1C,* located with *qnrB19* on *Tn2012* as described by Cattoir et al. [1] (figure 1C), was not observed in the present study.

The presence of antimicrobial resistance genes other than *qnr* on p013.1IncR was analyzed by micro array technology, using AMR-ve array tubes (Identibac; Addlestone; UK). The resistance genes *dfr12, sul1, strB* and integrase *intI1* were found in both 013.1 and 013.1-T, indicating that these genes all reside on p013.1IncR. *TetB* was only observed in donor strain 013.1.

Figure 3.1: Genetic environment of the *qnrB19* gene



*Schematic overview of sequencing results (1A) compared with the qnrB19 gene with flanking regions found by Dionisi et al (1B) [2] and Cattoir et al (1C) [1]. Black arrow indicates an insertion sequence or repeat sequence. White arrow indicates an antimicrobial resistance gene. Striped arrow represents a partial sequence. Dotted arrow indicates a gene other than antimicrobial resistance gene. * Sequence harbours point mutation compared to FJ790886. (Figure is not to scale)*

This is the first report of a *qnrB19* gene found in *E. coli* isolated from veal calves and the first *qnrB19* found on an IncR-type plasmid. The association with different transposable units indicates a high potential of spreading.

The sequence presented in this study was published on GenBank under accession number HM146784.

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Part II

ESBL/AmpC producing commensal
Escherichia coli in veal calves

Chapter 4

Increasing prevalence and diversity of ESBL/AmpC-type ß-lactamase genes in *Escherichia coli* isolated from veal calves from 1997 to 2010

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Abstract

Several studies on faecal carriage of ESBL/AmpC producing *E. coli* have been performed in cattle, but little is known about faecal carriage in veal calves. This study describes the prevalence and molecular characteristics of ESBL/AmpC genes in *E. coli* isolated from faecal samples of veal calves from 1997 to 2010. The prevalence of *E. coli* with reduced susceptibility to cefotaxime showed a discontinuous increasing trend, ranging from 4% in 1998 and 1999 to 39% in 2010. Promoter mutations of the chromosomal *ampC* gene were present in all years. In 2000, ESBL genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}52}$ and $bla_{TEM\text{-}20}$, were first observed. Before 2005 the majority of *E. coli* with reduced susceptibility to cefotaxime harboured *ampC* promoter mutants. After 2005 the majority harboured $bla_{CTX\text{-}M}$ genes, of which $bla_{CTX\text{-}M\text{-}1}$ was most abundant, followed by $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$. The diversity of $bla_{CTX\text{-}M}$ genes present gradually increased from one variant in 2000 to six variants in 2010. The prevalence of $bla_{TEM\text{-}52}$ was relatively low, but it was detected from 2000 on. Bla_{CMY} and bla_{SHV} were found sporadically. In summary, this study showed an increasing trend in prevalence and molecular diversity of cefotaxime resistance encoding genes in *E. coli* from veal calves over a fourteen year period. After 2005 $bla_{CTX\text{-}M}$ genes were most abundant, especially $bla_{CTX\text{-}M\text{-}1}$.

Introduction

Extended spectrum cephalosporins (ESC) are used in both human and veterinary medicine. Resistance to ESC may lead to therapy failure and is therefore of great concern [1]. Resistance to ESC in Enterobacteriaceae is mainly caused by production of extended spectrum ß-lactamases (ESBL) or AmpC ß-lactamases that hydrolyse the ß-lactam ring of these antibiotics [2]. ESBL/AmpC genes are often carried on mobile genetic elements (plasmids) [3] which can be transferred horizontally between bacteria of either the same or different species. Classification of the ESBL/AmpC enzymes is based on either their functional characteristics [4, 5], or on their primary structure [6]. A considerable number of genes associated with these groups of enzymes is reported and this number is still increasing [2], including multiple gene variants of the same enzyme, resulting in various gene families (http://www.lahey.org/studies).

ESBL/AmpC producing bacteria have been isolated from the gastrointestinal tract of food-producing animals [7-9], companion animals [10-12] and wild-life[13, 14]. The gastrointestinal tract may act as a reservoir for the carriership of ß-lactamase producing bacteria, allowing horizontal transfer, which in turn has a zoonotic potential[15]. Exposure to ESBL/AmpC-carrying companion animals, but also food-producing animals or contaminated food products may lead to transfer of bacteria to the human population.

Faecal carriage of ESBL/AmpC-producing bacteria in cattle has been reported previously [9, 16-21]. However, cattle are kept for different purposes in livestock production. Dairy farms are predominantly closed production systems, while at veal calf farms virtually all animals originate from different farms. Also the housing facilities, farm management and the life span of the animals differ greatly between dairy cattle and veal farming. Furthermore, dairy cattle are generally less exposed to antimicrobials [22]. These differences in environment and management may lead to differences in prevalence and epidemiology of ESBL/AmpC- producing bacteria. The type of cattle is not always specified in reported studies, or the examined population was either local or relatively small. Especially data on faecal carriage of ESBL/AmpC producing bacteria in veal calves is limited. This in contrast to carriership of MRSA in veal calves [23, 24]. The aim of this study was to retrospectively determine the emergence, trends and molecular characteristics of cefotaxime resistant *Escherichia coli* in faecal samples obtained from veal calves in the Netherlands from 1997 to 2010.

Material and Methods

Sampling design and method

Faecal samples from veal calves were collected at farms by the Netherlands Food and Consumer Product Safety Authority (NVWA) from 1997 to 2010 to monitor zoonotic food borne pathogens in food-producing animals. Farms were randomly selected by the Foundation for Quality Guarantee of the Veal Sector (SKV), not taking animal age into account. On each farm one herd was sampled. A herd was defined as a group of animals of similar age and maintained in the same open space. Each faecal sample was a pooled sample of at least 100 grams, consisting of 20 to 60 fresh droppings (depending on herd size, ranging from 25 to ≥500 animals) picked from the floor equally distributed over the stable. Animal age differed

between farms and was not further specified. Samples collected from 1997 to 2005 were stored at the National Institute for Public Health and Environment (RIVM). At the RIVM, ten gram faeces was stored in a 1:1 (w/v) suspension of faeces in Tryptone Soy Broth with 30% glycerol at -80°C. From the samples collected from 2006 to 2010, one gram faeces was stored at the Central Veterinary Institute, part of Wageningen UR (CVI) in a 10% (w/v) suspension of faeces in buffered peptone water with 30% glycerol at -20°C.

Escherichia coli isolates

A tube with 1 ml Luria-Bertani broth (Beckton Dickinson), supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (LB+) was inoculated using a sterile cotton swab saturated with the faecal suspension and incubated overnight at 37°C. From these overnight cultures MacConkey agar plates (product no. 212123, Becton Dickinson) supplemented with 1 mg/L cefotaxime (MC+) were inoculated and incubated overnight at 37°C. From each MC+ plate showing growth, one typical pink colony was selected for further analysis, and confirmed as *E. coli* by a check for tryptophan hydrolysis.

Phenotypic screening and ESBL/AmpC gene identification

ESBL phenotype of all selected isolates was determined by combination disk diffusion test using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson) according to CLSI guidelines [25]. In addition a cefoxitin disk (30 µg; Beckton Dickinson) was added to detect AmpC phenotypes. All isolates classified as intermediate or resistant to cefoxitin, according to CLSI guidelines (≤ 17mm), were suspected to be AmpC producers. Subsequently, all isolates were screened by PCR and sequence analysis for the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{CMY} and mutations in the promoter region of the chromosomally encoded *ampC* gene using primers and conditions as described previously [26]. The primers and conditions for detection of bla_{OXA} group 1, 2 and 10 were also described previously [27]. All positive PCR-products were sequenced using Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems; USA) with additional primers for bla_{TEM} (TEM-Fseq: 5'-GCCAACTTACTTCTGACAACG) and bla_{CMY} (CMY-F-838: 5'-TGGCGTATTGGCGATATGTA and CMY-R-857: 5'-TACATATCGCCAATACGCCA). All sequences were analysed using Sequencher v4.9 (Gene Codes Corporation; USA) and BioNumerics v6.6 (Applied Maths; Belgium). All mutations in the promoter region of the chromosomally encoded ampC gene were identified as described by Mulvey et al [28]. All isolates for which no ESBL/AmpC gene could be determined were screened on the Check-MDR CT-101 array platform (Check-Points; The Netherlands) according to the manufacturer's protocol.

Statistical analysis

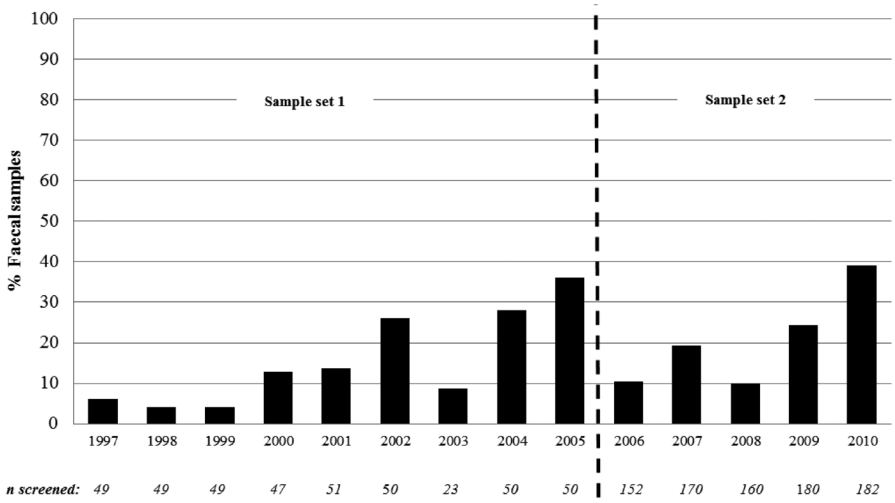
Prevalence data were analysed by a linear regression model with intercept and slope using the open source "R" statistics software.

Results

The two sample sets used included faecal samples isolated from 1997 to 2010. The prevalence of cefotaxime resistant *E. coli* showed a discontinuous trend, ranging from 4% in 1998 and 1999 to 39% in 2010 (Figure 4.1). A relatively high prevalence of 20% or more was observed in 2002, 2004, 2005, 2009 and 2010 (Figure 4.1). In contrast, a relatively low prevalence of 10%

or less was observed in 1997 to 1999, in 2003 and 2008. Based on a linear regression model, the prevalence showed an increasing tendency of 6% per year (p=0.09).

Figure 4.1: Percentage of faecal samples positive for cefotaxime reduced susceptible *E. coli*



Percentage of E. coli with non-wild type susceptibility to cefotaxime, isolated from faecal samples collected and stored at -80˚C in a 1:1 w/v dilution (sample set 1) and stored at -20˚C a 1:10 w/v dilution (sample set 2)

The diversity of ESBL/AmpC genes isolated from veal calves from 1997 to 2010 gradually increased (Table 4.1). Both the number of gene families (e.g. bla_{CTX-M}, bla_{TEM}, bla_{CMY}) as well as the diversity within the gene families increased. In all years from 1997 to 2010, promoter mutations of the chromosomal *ampC* gene were found. Using the ampC-type numbers described by Mulvey et al [28], the most commonly found *ampC* promoter mutation was *ampC*-type 3 (Table 4.1). The *ampC* production was confirmed by combination disk diffusion tests (data not shown). One isolate from 1997 harboured an *ampC*-type 34 gene. This variant is similar to *ampC*-type 3, but has one additional G→A mutation at position +30 of the attenuator region. In the years 1997, 1999, 2000, 2002, 2005, 2009 and 2010 new variants were found (Table 4.1). A new *ampC*-type from 2009 (Table 1) only had one C→G mutation at +57, outside the promoter and attenuator region. All other new variants did have mutations in the promoter and/or attenuator region (see footnote 4, Table 4.1). Of all remaining isolates 63% harboured a wild-type *ampC* (wt), 33% harboured *ampC*-type 18, and 4% harboured either *ampC*-type 5, 7 or 11. All isolates harbouring *ampC*-types wt, 5, 7, 11 or 18 also harboured plasmid mediated resistance genes and showed an ESBL phenotype.

The vast majority of ESBL genes found in veal calves from 2000 onwards belonged to the bla_{CTX-M} gene family (Table 4.1). The number of different bla_{CTX-M} variants increased gradually over the years. In 2000, only $bla_{CTX-M-1}$ was found. In 2002, $bla_{CTX-M-2}$ was first observed, followed by $bla_{CTX-M-15}$ in 2004, $bla_{CTX-M-14}$ and $bla_{CTX-M-32}$ in 2007 and $bla_{CTX-M-79}$ in 2010.

Table 4.1: ESBL/AmpC genes detected in *E. coli* isolated from faecal samples from veal calves

		Year >	1997	1998	1999	2000
		***n* fecal samples screened >**	49	49	49	47
		***n*(%) non-wt cefotaxime susceptibility >**	3(6)	2(4)	2(4)	6(13)
ESBL/AmpC genes *n*(%)[1]	**Chromosomal *ampC*[2]**	*ampC*-type 3	1(33)	2(100)	1(50)	2(34)
		ampC-type 34	1(33)			
		ampC-type new[4]	1(33)		1(50)	1(17)
	pAmpC	bla_{CMY-2}				
	ESBLs	$bla_{CTX-M-1}$				1(17)
		$bla_{CTX-M-2/97}$[5]				
		$bla_{CTX-M-14}$				
		$bla_{CTX-M-15}$				
		$bla_{CTX-M-32}$				
		$bla_{CTX-M-79}$				
		bla_{TEM-52}				1(17)
		bla_{TEM-20}				1(17)
		$bla_{SHV-12\ /\ 129}$[5]				
	Combinations	$bla_{CTX-M-15}$ + $bla_{SHV-12\ /\ 129}$[5]				
		$bla_{CTX-M-1}$ + *ampC-type 3*				
		$bla_{CTX-M-1}$ + *ampC-type new*[4]				
		bla_{TEM-52} + *ampC-type 3*				
		bla_{TEM-52} + *ampC-type new*[4]				
	Unknown[3]	unknown				

2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
51	50	23	50	50	152	170	160	180	182
7(14)	13(26)	2(9)	14(28)	18(36)	16(11)	33(20)	16(10)	44(24)	71(39)
7(100)	7(54)	2(100)	9(64)	5(28)	5(31)	10(30)	2(13)	11(25)	20(28)
	1(8)			1(6)				1(2)	1(1)
				1(6)		2(6)		1(2)	2(3)
	2(15)		1(7)	3(17)	4(25)	8(24)	5(31)	12(27)	28(39)
	1(8)		1(7)	2(11)	1(6)	4(12)	1(6)	3(7)	3(4)
						1(3)	1(6)	6(14)	7(10)
			1(7)	2(11)	2(13)	3(9)	3(19)	4(9)	3(4)
						1(3)		2(5)	2(3)
									2(3)
	2(15)		1(7)	2(11)	3(19)	2(6)	3(19)	2(5)	
								2(5)	2(3)
									1(1)
				1(6)					
				1(6)					
						1(3)			
						1(3)			
			1(7)		1(6)		1(6)		

[1] n(%); n= number of isolates; (%) = percentage of isolates with non-wild type cefotaxime susceptibility harboring this resistance gene.

[2] ampC-types are designated as described by Mulvey et al [28]

[3] No ESBL/AmpC gene was found. Only the small spectrum ß-lactamase gene bla_{OXA-1}

[4] The following promoter mutations were observed:

1997: -42 C>T, -18 G>A, -1 C>T, +30 G>A, +58 C>T
1999: -32 T>A, -18 G>A, -1 C>T, +37 G>A, +58 C>T
2000: -32 T>A, -18 G>A, -1 C>T, +24 C>A, +58 C>T
2002: -42 C>T, -18 G>A, -1 C>T, +24 C>A, +27 A>T, +32 G>A, +58 C>T
2005: -42 C>T, -18 G>A, -1 C>T, +32 G>A, +58 C>T AND -42 C>T, -18 G>A, -1 C>T, +31 C>G, +58 C>T
2007: -18 G>A, -11 C>T, -1 C>T, +58 C>T
2009: +57 C>G
2010: -42 C>T, -18 G>A, -1 C>T, +35 C>G, +58 C>T

[5] Based on the primers used in this study, no destinction could be made between these two variants.

Table 4.2: Small spectrum ß-lactamase genes detected in *E. coli* with reduced susceptibility to cefotaxime

	Year >	1997	1998	1999	2000	2001
	***n* fecal samples screened >**	49	49	49	47	51
	***n*(%) non-wt cefotaxime susceptibility >**	3(6)	2(4)	2(4)	6(13)	7(14)
ß-lactamase genes *n*(%)[1]	bla_{TEM-1}	2 (67)			3(50)	1(14)
	bla_{TEM-30}		2(100)	1(50)		
	bla_{TEM-40}					
	bla_{TEM-79}					
	$bla_{TEM-135}$					
	$bla_{TEM-176}$					
	bla_{OXA-1}					

2002	2003	2004	2005	2006	2007	2008	2009	2010
50	23	50	50	152	170	160	180	182
13(26)	2(9)	14(28)	18(36)	16(11)	33(20)	16(10)	44(24)	71(39)
4(31)		6(43)	9(50)	8(50)	20(60)	6(38)	22(50)	35(49)
1(8)		1(7)			2(6)			1(1)
					1(3)		1(2)	
				1(6)				
							1(2)	
								1(1)
		2(14)[2]		2(13)[2]	2(6)	4(25)[2]	3(7)	1(1)

[1] n(%); n= number of isolates; (%) = percentage of isolates with non-wild type cefotaxime susceptibility harboring this resistance gene.
[2] One of these isolates was designated "unknown" as depicted in Table 4.1

The prevalence of bla_{CTX-M} genes also increased strongly, especially of $bla_{CTX-M-1}$. In 2010 64% of all isolates with non-wild type cefotaxime susceptibility carried genes of the bla_{CTX-M} gene family, of which $bla_{CTX-M-1}$ was most abundant (39% of all non-wild type isolates). The prevalence of ESBL gene families other than bla_{CTX-M} was relatively low. From the bla_{TEM} gene family, bla_{TEM-52} was found in low numbers in all years from 2000, except for 2001, 2003 and 2010. bla_{TEM-20} was found once in 2000. Furthermore, $bla_{SHV-12\ /\ 129}$ was observed in 2009 and 2010, both with a prevalence of 5% and 4%, respectively. The plasmid mediated AmpC gene (pAmpC) bla_{CMY-2} was found in 2005, 2007, 2009 and 2010 with a low prevalence of ≤6%.

Several isolates with multiple ESBL/AmpC genes were detected. From a faecal sample from 2010 an isolate harbouring both $bla_{CTX-M-15}$ and $bla_{SHV-12/129}$ was found. In samples from 2007 two isolates were found harbouring bla_{TEM-52} with *ampC*-type 3 or a new *ampC*-type. In samples from 2005 two isolates harbouring a combination of $bla_{CTX-M-1}$ with *ampC*-type 3 or a new *ampC*-type were found.

In three isolates no ESBL/AmpC resistance gene could be determined. In these isolates, only the small spectrum ß-lactamase bla_{OXA-1} was identified. Additional testing using the Check-MDR CT101 array (Check-Points, The Netherlands) also gave a negative result. These isolates were therefore designated "unknown" (Table 4.1). Furthermore, one strain from 2009 harboured a new *ampC*-type, however this new type was very similar to the wild-type K-12 reference strain only differing by a C→G mutation at position +57. This strain also harboured bla_{OXA-1} and showed an ESBL phenotype, with resistance to cefotaxime only (21 mm diameter and 26 mm with clavulanic acid) and not to ceftazidime.

In addition to the ESBL/AmpC genes described above, there was a relatively high prevalence of narrow-spectrum ß-lactamase genes (Table 4.2). These ß-lactamase genes were both present in isolates harbouring either ESBL type genes or *ampC* promoter mutations, not exclusively associated with a specific ESBL/AmpC gene. The predominant ß-lactamase was bla_{TEM-1}. This variant was found throughout the whole study period. bla_{TEM-30} was also found throughout the whole study period, although with a very low prevalence. The variants bla_{TEM-40}, bla_{TEM-79}, $bla_{TEM-135}$ and $bla_{TEM-179}$ were found incidentally in the period 2006 to 2010 (Table 4.2). Furthermore, several isolates harboured multiple bla_{TEM} genes. In one case it was a combination of bla_{TEM-20} with bla_{TEM-1}, in all other cases it was a combination of either bla_{TEM-52} with bla_{TEM-1} or two variants of bla_{TEM-1}. Furthermore bla_{OXA-1} was first observed in 2004 and remained present with a relatively low prevalence (Table 4.2).

Discussion

This study showed an increase in prevalence and molecular diversity of genes encoding cefotaxime resistance in *E. coli* from veal calves over a fourteen-year period. The subset of faecal samples from 2006-2010 used in this study comprised the same samples as used for routine surveillance on antimicrobial resistance in food-producing animals in the Netherlands. However, unlike the present study, the surveillance data did not show an increasing trend of reduced susceptibility to cefotaxime in this period [29]. This is most likely due to the difference in isolation method of the *E. coli*. In the routine surveillance, from each faecal sample one *E.*

coli colony was randomly selected from non-selective MacConkey agar. In this way the chance to pick an ESBL/AmpC producing *E. coli* is depended of the prevalence of these isolates in the sample. The higher the prevalence, the bigger the chance an ESBL/AmpC producer will be selected from the plate. In the present study we used a selective enrichment broth followed by inoculation on a MacConkey agar plate, both supplemented with 1 mg/L cefotaxime. Using selective enrichment increases the chance to find an ESBL/AmpC producing *E. coli*, since it does not depend on one randomly selected colony from an agar plate. This may implicate there is an increasing trend, as shown in this study, but with relatively low numbers of ESBL/AmpC producing *E. coli* in each sample.

Furthermore, since the two sets of faecal samples were stored under different conditions, we cannot fully exclude a possible bias in the prevalence results. Faecal samples stored at CVI were five times more diluted compared to the samples stored at the RIVM. A higher dilution may reduce sensitivity of isolating ESBL/AmpC producing *E. coli*.

To our knowledge, similar studies on faecal carriage covering a large number of years have not been performed in other countries. In contrast to other studies we used an enrichment supplemented with cefotaxime to increase the sensitivity of the isolation method. Using different sampling methods and different populations (e.g. dairy cows, veal calves, a mixed population or a population not further specified as cattle) makes comparing prevalence data rather difficult, since different kinds of cattle are kept in different housing conditions, with different farm management, receive different nutrition and have a different lifespan. These factors may influence the gut flora. Despite these differences, some data on faecal carriage of ESBL/AmpC producing *E. coli* is available. The best comparable study was performed in Switzerland where 63 calves (maximum of two per farm) were included in the study and sampled at slaughter in 2009, 2010 and 2011. In this study 25% of the faecal samples were positive for bla_{CTX-M} producing *E. coli*, using EE broth enrichment and subsequently Brilliance ESBL agar[16]. This is similar to the prevalence we found in 2009. In contrast, the prevalence in other cattle ((young) cows, fattening bulls and bullocks) in this Swiss study was 1.6%, confirming the possible differences in prevalence between different kinds of cattle. Furthermore, in the UK, in 2007/2008 7% of farms (mixed cattle population) that submitted faecal samples for diagnostic purposes were positive for bla_{CTX-M} producing *E. coli* [20]. These samples were cultured on selective plates, but did not include a selective enrichment broth. Furthermore, several studies from the UK have shown within-farm prevalence of faecal carriage of bla_{CTX-M} producing *E. coli*, ranging from 41% (based on the lowest of three farms. Non-selective enrichment and subsequently CHROM-agar CTX medium were used) to 93% (based on one farm. Sample directly streaked onto CHROMagar ECC) [9, 19]. However, in our study each isolate represented one farm, so nothing can be concluded towards within farm prevalence. In Poland no ESBL/AmpC-producing *E. coli* were found in 2009 (no enrichment; agar supplemented with 2 mg/L cefotaxime; cattle not further specified) [21]. In France, in a study in the Burgundy region performed in 2009, a prevalence of 5% was demonstrated (no enrichment; ESBL screening agar supplemented with 4 mg/L cefotaxime or 4 mg/L ceftazidime; mixed population of healthy adults and diseased calves). In a small study (n=16) from Japan in 2007 31% of the faecal samples from beef cattle showed growth on Chromocult Coliform Agar supplemented with 1 mg/L cefotaxime, without pre-enrichment [18].

Mutations in the promoter region of the chromosomal *ampC* were found in all years from 1997 to 2010 (Table 4.1). The *ampC*-type 3, as well as *ampC*-type 34 and all new *ampC* types except the one from 2009 all harboured mutations at multiple positions (-42, -32, -18, -11, -1, +17 to +37, or +58) that have been previously described to cause a 'strong' promoter. Especially mutations at positions -42 and -32 are thought to have a large effect on promoter strength [30-33]. Our data show that from all mutations in the chromosomal *ampC*, *ampC*-type 3 was most abundant. This has also been shown in samples from diseased animals in Spain in the period of 1997 to 2001 [34]. Furthermore, a Canadian study, introducing the *ampC*-type numbers, described samples of human origin with similar mutations, of which *ampC*-type 3 was also most abundant [28]. The second most abundant non-wild type variant in our study was *ampC*-type 18. This type 18 promoter only differs from *ampC*-type 3 by lacking the mutation at position -42, but all isolates harbouring *ampC*-type 18 showed an ESBL phenotype, including being susceptible to cefoxitin. In contrast, cefoxitin resistance due to *ampC*-type 18 has been described by others [35]. Therefore it is likely that also other factors play a role in reducing susceptibility, not only the presence of *ampC*-type 18.

Next to the presence of chromosomal *ampC* promoter variations, this study showed an increasing diversity in plasmid mediated ESBL/AmpC gene families like $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{CMY} as well as an increasing number of gene variants within a gene family (Table 4.1). The first ESBL genes in this study were found in 2000. These were $bla_{\text{CTX-M 1}}$, $bla_{\text{TEM-52}}$, $bla_{\text{TEM-20}}$. To our knowledge in other countries $bla_{\text{CTX-M 1}}$ was not found in cattle until 2003 [36, 37]. Furthermore, $bla_{\text{TEM-52}}$ was not reported in cattle until 2004, isolated from Danish cattle meat products imported from Germany [38] and $bla_{\text{TEM-20}}$ has been reported in cattle meat from Tunisia in 2007 [39].
Both $bla_{\text{TEM-20}}$ and $bla_{\text{TEM-52}}$ were also observed in Dutch poultry in 2001/2002 [40] and 2006 [7]. The prevalence of $bla_{\text{CTX-M 1}}$ increased over the years, in contrast to $bla_{\text{TEM-52}}$, which' prevalence remained relatively low, and $bla_{\text{TEM-20}}$, which was found only in 2000. In contrast to the low prevalence of TEM ESBLs, TEM ß-lactamases, especially $bla_{\text{TEM-1}}$, were highly dispersed (Table 4.2). From 2007 to 2010 the vast majority of ESBLs in our study were $bla_{\text{CTX-M 1}}$, followed by either $bla_{\text{CTX-M 15}}$ in 2008 or $bla_{\text{CTX-M 14}}$ in 2009 and 2010. The high prevalence of these three $bla_{\text{CTX-M}}$ gene variants is in agreement to what is found in cattle in the rest of Europe, as has been summarized by Ewers *et* al [8]. In the present study the prevalence of other ESBL/AmpC genes, $bla_{\text{CTX-M 2/97}}$, $bla_{\text{CTX-M 32}}$, $bla_{\text{CTX-M 79}}$, $bla_{\text{SHV-12/129}}$ and $bla_{\text{CMY-2}}$, was relatively low. This is also in agreement to what is found elsewhere in Europe [8].

The three isolates from 2004, 2006 and 2008, designated "unknown", all showed values close to the cut-off values in the combination disk diffusion assays, as defined by CLSI [25]. This reduced susceptibility may be the result of reduced permeability or an increased efflux of cefotaxime, possibly in combination with $bla_{\text{OXA-1}}$ [41, 42]

Based on the results of this study, no conclusions can be drawn towards the origin of ESBL/AmpC producing *E. coli* in veal calves, and the cause of the observed increase in gene diversity. The structure of the industry resembles an inversed pyramid, in which calves are collected at young age from dairy farms from many different European countries (approximately 50% of the animals are imported). They are collected at distribution centres from where new herds are supplied to fattening farms in the Netherlands. At the farms, stables are cleaned, but not

disinfected in between rounds. This structure may contribute to the relatively high diversity of ESBL genes found. The fact that another study also showed a high diversity in MRSA spa types in veal calves may support this view [43].

In summary, in the years 1997 to 2010 both the prevalence and diversity of different ESBL/AmpC producing *E. coli* in veal calves increased. The proportion of isolates with a mutation in the promoter region of the chromosomal *ampC* remained more or less the same in the whole study period. The prevalence and diversity of different ESBL/AmpC genes increased gradually from 2000 to 2010. The fact that in 2010 39% of the faecal samples, each representing one farm, harboured *E. coli* with reduced susceptible to cefotaxime, indicates that veal calves are a reservoir of ESBL/AmpC producing bacteria. Further research is required to assess the transmission between the animals in relation to the health risks for the animals themselves, and to assess the relative importance of the food transmission route towards humans.

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Chapter 5

Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC producing *Escherichia coli* isolated from veal calves at slaughter

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Abstract

Objectives: The presence of ESBL/AmpC producing *E. coli* in cattle has been reported previously, however information on veal calves is limited. This study describes the prevalence and molecular characteristics of ESBL/AmpC producing *E. coli* in veal calves at slaughter.
Methods: Faecal samples from 100 herds, 10 individual animals per herd, were screened for cefotaxime resistant *E. coli*. Molecular characterization of ESBL/AmpC genes and plasmids was performed on one isolate per herd by microarray, PCR and sequence analysis.
Results: 66% of the herds were positive for ESBL/AmpC producing *E. coli*. Within-herd prevalence varied from zero to 90%. 83% of ESBL/AmpC producing *E. coli* carried $bla_{CTX\text{-}M}$ genes, of which $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ were most prevalent. The dominant plasmids were IncI1 and IncF-type plasmids.
Conclusions: A relatively high prevalence of various $bla_{CTX\text{-}M}$ producing *E. coli* was found in veal calves at slaughter. The genes were mainly located on IncI1 and IncF plasmids.

Introduction

The development of resistance to extended spectrum cephalosporins (ESC) has evolved rapidly world-wide in both clinical settings as well as in the community. Resistance to ESC is mainly caused by various extended spectrum ß-lactamases (ESBL) or AmpC ß-lactamases (AmpC) that are often found in *Enterobacteriaceae* and hydrolyse the ß-lactam ring of these antibiotics [1]. These enzymes are encoded by genes that are frequently located on mobile genetic elements (plasmids) [2] which have the ability to transfer horizontally within and between different bacterial species. Novel ESBL/AmpC genes or gene variants are reported on a regular basis (http://www.lahey.org/studies) and are classified based on either their functional characteristics [3] or primary structure [4].

Food-producing animals have been suggested as the primary reservoir of zoonotic foodborne pathogens, including antimicrobial resistant bacteria [5]. Faecal carriage of ESBL/AmpC producing *E. coli* in cattle has been reported previously [6-10]. In addition, a recent study showed that in the period from 2005 to 2010, resistance to ESC in veal calves was mainly caused by plasmid mediated beta-lactamases, in contrast to the years before 2005 (Hordijk *et al*, submitted for publication). Furthermore, beef, chicken and pig meat products have also been found positive for ESBL/AmpC producing bacteria [11], which may constitute a transmission route to humans. However, these studies are difficult to compare, because isolation methods and sample sizes vary greatly, and the type of cattle studied is not always specified. For instance dairy farming is very different from veal calves by means of housing, life expectation and exposure to antibiotics. These factors may influence the prevalence and genetic characteristics of antimicrobial resistance genes in isolates from these animals.

The aim of this study was to determine the between-herd, and within-herd prevalence of plasmid mediated ESBL/AmpC-producing *E. coli* in veal calves at slaughter. Moreover the ESBL/AmpC genes and plasmids on which they are located were identified to determine their molecular characteristics in relation to those found in other food-producing animals and humans.

Material and Methods

Sampling design and isolation of Escherichia coli

From January to December 2011, faecal samples from 10 individual randomly selected veal calves from 100 slaughter batches, originating each from different herds (1000 faecal samples in total) were collected at slaughter houses in The Netherlands. All faecal samples were individually screened for ESBL/AmpC-producing *E. coli*. A cotton swab was saturated in the faecal sample and placed in a tube with 1 ml Luria-Bertani broth (Beckton Dickinson) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (LB+). LB+ tubes were incubated aerobically at 37˚ C for 20 ± 2 hours. Subsequently, the overnight LB+ culture was inoculated on a MacConkey agar plate (product no. 212123, Becton Dickinson), supplemented with 1 mg/L cefotaxime (MC+). All MC+ plates were incubated aerobically at 37˚ C for 20 ± 2 hours. From each MC+ plate showing growth, one colony typical for *E. coli* was selected, subcultured on blood agar, and the next day suspended in 1 ml of buffered peptone water

supplemented with 30% glycerol and stored at -80°C, pending analysis. All selected *E. coli* were inoculated in 5 ml Gersbach medium and incubated at 37° C for 20 ± 2 hours for *E. coli* confirmation. One droplet of Kovac's Indole reagent (Merck, Germany) was added to the Gersbach culture to check for tryptophan reduction. All indole-positive isolates were considered *E. coli*. From all isolates showing growth on MacConkey, one randomly picked isolate was selected per herd for antimicrobial susceptibility testing phenotypical and molecular characterization.

Antimicrobial susceptibility testing

All selected isolates (n=66) were tested for susceptibility to antimicrobials by broth micro-dilution according to ISO standard 20776-1:2006 using microtitre trays with a custom made dehydrated panel of antibiotics (Sensititre, Trek Diagnostic Systems, Basingstoke, UK). The following antibiotics were included: ampicillin, cefotaxime, ceftazidime, tetracycline, sulfamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin and colistin. All results were interpreted using cut-off values defined for epidemiological purposes as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://mic.eucast.org/Eucast2/). For sulfamethoxazole, the clinical break point defined by the Clinical and Laboratory Standards Institute (CLSI) was used [12].

Characterization of ESBL/AmpC genes and plasmids

All selected isolates were screened for ESBL/AmpC genes using the tube based amr-ve-05 microarray (Alere, Tilburg, The Netherlands). Beta-lactamase gene families identified by this microarray were subsequently characterized by PCR and sequence analysis as described previously [13]. For sequence analysis the following additional primers were used: TEM-Fseq: 5'-GCCAACTTACTTCTGACAACG, CMY-F-838: 5'-TGGCGTATTGGCGATATGTA and CMY-R-857: 5'-TACATATCGCCAATACGCCA. Primers and conditions used to identify and sequence the modified aminoglycoside resistance gene *aac(6')Ib-cr* were described previously [14]. Plasmids were isolated using a modified miniprep method as follows. One colony of interest was inoculated in 3 ml LB broth and incubated overnight. Subsequently 1.5 ml of the culture was transferred and spinned down at 14000 rpm for 5 minutes. The pellet was suspended in 60 µl TEG buffer (25 mM Tris/Hcl; 10 mM EDTA; 50 mM glucose). Then, 120 µl NaOH/SDS (0.2M/1%) was added and placed on ice for 5 minutes. Subsequently, 90 µl NaOH (3 M) was added and placed on ice for 5 minutes. Suspension was spinned down at 14000 rpm for 5 minutes. Supernatant was transferred and 270 µl LiCl (5 M) was added. After 10 minutes, suspension was spinned down at 14000 rpm for 5 minutes. Supernatant was transferred and 1 ml of EtOH (96%) was added. Suspension was spinned down at 14000 rpm for 10 minutes and pellet was resuspended in 200 µl EtOH (70%). Suspension was spinned down at 13000 rpm for 10 minutes and the pellet was resuspended in 20 µl H_2O. Plasmids were transformed into Electromax DH10B cells by electroporation (Invitrogen, USA) by mixing 2 µl of plasmid DNA suspension with 20 µl competent cells. Cells were electroporated using the following conditions: 1.25 kV, 200 ohm, 25 µFar. Transformants were subsequently plated on LB agar plates supplemented with 1 mg/L cefotaxime. PCR-based replicon typing (PBRT) was conducted on the transformants to identify the incompatibility class of the plasmid inside the transformant [15]. Plasmid MLST (pMLST) [16] and replicon sequence typing (RST) were used to further characterize IncI1 and IncF plasmids, respectively [17]. Because this study focussed

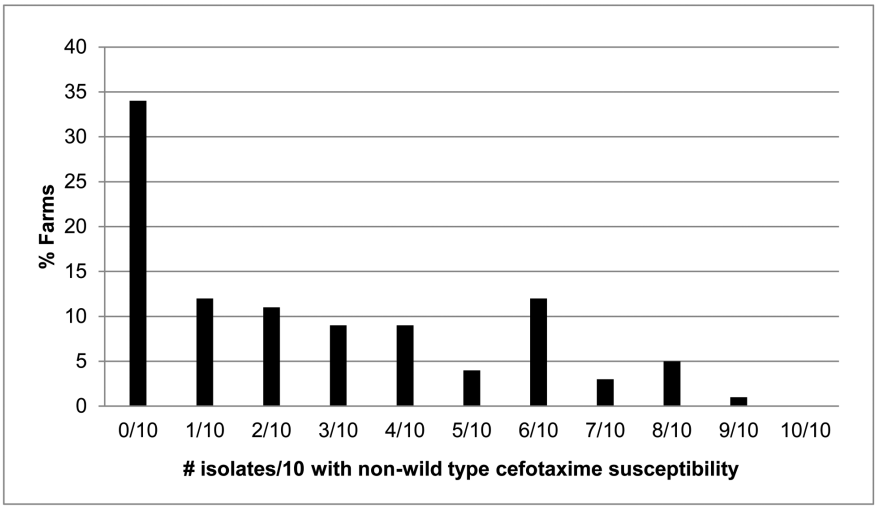
at plasmid mediated genes, if the microarray results were negative, another ESBL/AmpC suspected isolate from the same herd was included as a replacement.

Results

Prevalence of non-wild type cefotaxime susceptible E. coli

From the 100 herds that were screened for the presence of *E. coli* with non-wild type susceptibility at slaughter, 66% were found positive for *E. coli* with non-wild type susceptibility to cefotaxime. From all individual animals (n = 1000), 26% were found positive. The within-herd prevalence of *E. coli* with non-wild type susceptibility varied greatly, ranging from zero to 90%. However, in the majority of the positive farms, less than five out of ten faecal samples harboured cefotaxime non wild-type *E. coli* (Figure 5.1)

Figure 5.1: Within-farm prevalence of *E. coli* with non-wild type cefotaxime susceptibility



Ten faecal samples were obtained per farm at slaughter. The number of faecal samples harbouring ESBL/AmpC-producing E. coli is shown on the X-axis. The percentage of farms is depicted on the Y-axis.

Of all selected isolates with a non-wild type susceptibility to cefotaxime, 83% carried genes belonging to the $bla_{\text{CTX-M}}$ gene family (Table 5.1). The predominant genes within this $bla_{\text{CTX-M}}$ group, were $bla_{\text{CTX-M-1}}$ (45.5%), $bla_{\text{CTX-M-14}}$ (16.4%), and $bla_{\text{CTX-M-15}}$ (21.8%). One of the isolates harbouring $bla_{\text{CTX-M-1}}$ also harboured $bla_{\text{TEM-52c}}$. The remaining $bla_{\text{CTX-M}}$ variants were $bla_{\text{CTX-M-2/97}}$ (5.5%), $bla_{\text{CTX-M-3}}$ (3.6%) and $bla_{\text{CTX-M-32}}$ (7.2%). From 11 isolates (17%) that did not harbour $bla_{\text{CTX-M}}$, three harboured $bla_{\text{TEM-52c}}$ (4.5%), and one $bla_{\text{CMY-2}}$ (1.5%). In 6 isolates (9%), only mutations in the promoter region of the chromosomal *ampC* were detected. Finally, in one isolate (1.5%) neither plasmid mediated ESBL/AmpC genes nor mutations in the promoter region of the chromosomal *ampC* were detected. This isolate only harboured the ß-lactamases $bla_{\text{TEM-1c}}$ and $bla_{\text{OXA-1}}$.

Table 5.1: *E. coli* carrying ESBL/AmpC genes with their corresponding plasmids and resistance profile

		Plasmid	
ESBL	**Strain**	**Replicon**	**incI1 pMLST / incF RST [1, 2]**
$bla_{CTX\text{-}M\text{-}01}$	OT-ESBL-0199	Inc nt	(97 kb)[5]
	OT-ESBL-0294	Inc nt	(97 kb)[5]
	OT-ESBL-0285	IncB/O	
	OT-ESBL-0547	IncB/O	
	OT-ESBL-0589	IncB/O	
	OT-ESBL-0054	IncF	F2; A-; B-
	OT-ESBL-0198	IncF	F2; A-; B-
	OT-ESBL-0591	IncF	F2; A-; B-
	OT-ESBL-0600	IncF	F17*; A-; B-
	OT-ESBL-0382	IncF	F13#; A-; B20#
	OT-ESBL-0519	IncF	F35#; A-; B-
	OT-ESBL-0565	IncF	F35#; A-; B_
	OT-ESBL-0328	IncI1	ST3; CC 3
	OT-ESBL-0359	IncI1	ST3; CC 3
	OT-ESBL-0406	IncI1	ST58; CC 58
	OT-ESBL-0018	IncI1	ST58; CC 58
	OT-ESBL-0062	IncI1	new; 1 2 8# 3 3
	OT-ESBL-0441	IncI1	new; 1 5* 17 1 7
	OT-ESBL-0450	IncI1	new; 1 5^ 16 1 7
	OT-ESBL-0477	IncI1	new; 1 9# 17 1 7
	OT-ESBL-0546	IncK	
	OT-ESBL-0262	IncN	
	OT-ESBL-0361	IncN	
	OT-ESBL-0434	IncN	
$bla_{CTX\text{-}M\text{-}01}$ + $bla_{TEM\text{-}52c}$	OT-ESBL-0414	IncI1	ST58; CC 58
		unknown	(145 kb)[5]
$bla_{CTX\text{-}M\text{-}02/97}$ [6]	OT-ESBL-0301	IncF	F1; A6; B26#
	OT-ESBL-0310	IncHI1	
	OT-ESBL-0514	IncP, IncHI2	
$bla_{CTX\text{-}M\text{-}03}$	OT-ESBL-0437	IncB/O	
	OT-ESBL-0567	IncF	F35#; A-; B-
$bla_{CTX\text{-}M\text{-}14}$	OT-ESBL-0403	IncF	F2; A-; B-
	OT-ESBL-0021	IncI1	ST80
	OT-ESBL-0336	IncI1	ST80
	OT-ESBL-0337	IncI1	ST80
	OT-ESBL-0058	IncK	
	OT-ESBL-0291	IncK	

ß-lactamase	Non-wild type susceptibility[3]
bla_{TEM-1a}, bla_{OXA-1}	Amp-Ctx-Caz-Tet-Chl-Str-Kan
	Amp-Ctx-Caz-Smx-Tmp-Gen
	Amp-Ctx-Caz-Tet-Smx-Tmp-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Gen-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Chl-Str
	Amp-Ctx-Caz-Tet-Smx-Ffn-Chl-Gen-Str
	Amp-Ctx-Caz-Tet- Gen-Str
	Amp-Ctx-Caz-Tet-Ffn-Chl-Str
	Amp-Ctx-Caz-Tet-Gen
	Amp-Ctx-Caz-Tet-Smx-Str-Kan
	Amp-Ctx-Caz-Tet
	Amp-Ctx-Caz-Tet
	Amp-Ctx-Caz-Smx-tmp
	Amp-Ctx-Caz-Tet-Smx-Tmp-Str
	Amp-Ctx-Caz-Tet-Smx-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Str
	Amp-Ctx-Caz-Tet-Cip-Nal-Chl-Str
	Amp-Ctx-Caz-Tet-Str
	Amp-Ctx-Caz-Tet-Smx-Cip-Nal-Chl-Str
	Amp-Ctx-Caz
	Amp-Ctx-Caz-Tet-Smx-Cip-Nal-Chl-Str-Col
bla_{OXA-1}	Amp-Ctx-Caz-Tet-Smx-Tmp-cip-Nal-Ffn-Chl-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Ffn-Chl-Str-Kan
	Amp-Ctx-Caz
	Amp-Ctx-Caz-Tet-Smx-Tmp-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-cip-Nal-Ffn-Chl-Gen-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Ffn-Chl-Gen-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Ffn-Chl-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Gen-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-cip-Nal-Ffn-Chl-Gen-Str-Kan-Col
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Str
	Amp-Ctx-Caz-Tet-Smx-Cip-Nal-Ffn-Chl-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Cip-Nal-Ffn-Chl-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-tmp-Str
	Amp-Ctx-Caz-Tet-Smx-Tmp-Str

(Continued)

ESBL	Strain	Plasmid	
		Replicon	IncI1 pMLST / IncF RST [1, 2]
bla$_{CTX-M-14}$	OT-ESBL-0380	IncK	
continued	OT-ESBL-0405	IncK	
	OT-ESBL-0590	IncK	
bla$_{CTX-M-15}$	OT-ESBL-0327	Inc nt	(110 kb)[5]
	OT-ESBL-0502	Inc nt	(40 kb)[5]
	OT-ESBL-0028	IncF	F2; A-; B-
	OT-ESBL-0031	IncF	F31; A4; B1
	OT-ESBL-0156	IncF	F31; A4; B1
	OT-ESBL-0221	IncF	F31; A4; B1
	OT-ESBL-0161	IncF	F31; A4; B1
	OT-ESBL-0563	IncF	F31; A-; B1
	OT-ESBL-0534	IncF	F46; A-; B20
	OT-ESBL-0443	IncHI2	
	OT-ESBL-0256	IncI1	ST31; CC 31
	OT-ESBL-0549	IncI1	ST31; CC 31
bla$_{CTX-M-32}$	OT-ESBL-0163	Inc nt	(40 kb)[5]
	OT-ESBL-0173	Inc nt	(40 kb)[5]
	OT-ESBL-0449	Inc nt	(40 kb)[5]
	OT-ESBL-0473	Inc nt	(40 kb)[5]
bla$_{TEM-52c}$	OT-ESBL-0192	IncI1	ST36; CC 5
	OT-ESBL-0364	IncI1	ST36; CC 5
	OT-ESBL-0392	IncI1	ST10; CC 5
bla$_{CMY-2}$	OT-ESBL-0357	IncK	
ampC-type-3	OT-ESBL-0281	-	
	OT-ESBL-0386	-	
	OT-ESBL-0453	-	
	OT-ESBL-0599	-	
	OT-ESBL-0601	-	
ampC-type-11var	OT-ESBL-0543	-	
Unknown	OT-ESBL-0261	-	

ß-lactamase	Non-wild type susceptibility[3]
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-cip-Nal-Ffn-Chl-Gen-Str-Kan
	Amp-Ctx-Caz-Tet-Gen
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Chl-Gen-Str
	Amp-Ctx-Caz
qnrS[4] (other plasmid)	Amp-Ctx-Caz-Cip
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Str
bla_{OXA-1}, (aac6'-Ib-cr)[4]	Amp-Ctx-Caz-Tet-Cip-Nal-Kan
bla_{OXA-1}, (aac6'-Ib-cr)[4]	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Gen-Str-Kan
bla_{OXA-1}, (aac6'-Ib-cr)[4]	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Gen-Str-Kan
bla_{TEM-1b}, bla_{OXA-1}, (aac6'-Ib-cr)[4]	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Gen-Str-Kan
bla_{OXA-1}, (aac6'-Ib-cr)[4]	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Gen-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Gen-Str-Col
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Gen-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Gen-Str-Kan
	Amp-Ctx-Caz-Cip-Nal
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Chl-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Chl-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Ffn-Chl-Str-Kan
	Amp-Ctx-Caz-Tet-Smx-Tmp-Cip-Nal-Chl-Str-Kan
	Amp-Ctx-Caz
	Amp-Ctx-Caz-Smx-tmp-Cip-Nal-Chl-Str
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Tmp-Chl-Str
	Amp-Ctx-Caz-Tet-Str
	Amp-Ctx-Caz-Tet-Smx-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Ffn-Chl-Str
	Amp-Ctx-Caz
bla_{TEM-1a}	Amp-Ctx-Caz-Tet-Smx-Tmp-Str-Kan
bla_{TEM-1b}	Amp-Ctx-Caz-Tet-Smx-Ffn-Chl-Str
bla_{TEM-1a}	Amp-Ctx-Caz-Tet-Smx-Tmp-Str
bla_{TEM-1c}, bla_{OXA-1}, *ampc* WT	Amp-Ctx-Caz-Tet-Smx-Tmp-cip-Nal-Ffn-Chl-Str-Col

[1] The following deviations were found in IncI1 pMLST types: ESBL-0062: trbA8#: nucleotide 371 T>C; ESBL-0441: ardA5: 59 G>A, 137 A>T, 271 C>G, 331 C>T; ESBL-0450: ardA5^: 199 G>A, 271 C>G, 331 C>T; ESBL-0477: ardA9#: 199 G>A/C*

[2] The following deviations were found in IncF RST types: ESBL-0301: FIB-26#: 251 G>T; ESBL-0382: FII-13#: 77 A>G and FIB-20#: 194 G>A, 203 T>C; ESBL-0519 / 0565: FII-35#: 22 A>T, 38+39>CG insertion.

[3] Amp = ampicillin, Ctx = cefotaxime, Caz = ceftazidime, Nal = nalidixic acid, Cip = ciprofloxacin, Ffn = florfenicol, Chl = chloramphenicol, Tet = tetracycline, Smx = sulfamethoxazole, Tmp = trimethoprim, Str = streptomycin, Kan = kanamycin, Gen = gentamicin), Col = colistin.

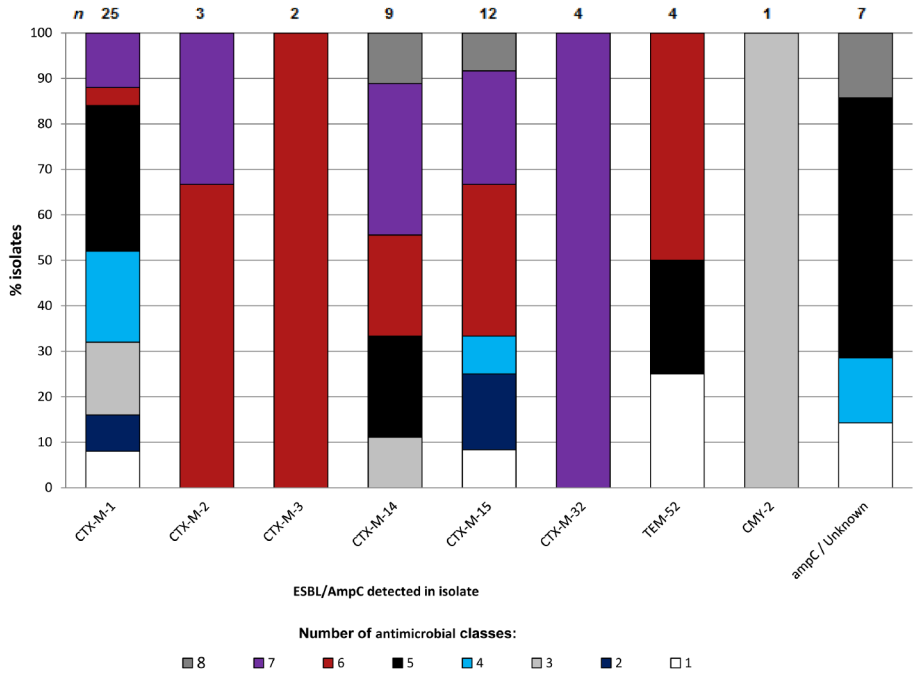
[4] qnrS and aac(6')Ib-cr are not ß-lactamase genes, but cause reduced susceptibility to quinolones. aac(6')Ib-cr also causes reduced susceptibility to aminoglycosides.

[5] Plasmid size in kilo bases (kb) of non-typeble plasmids.

[6] Based on the primers used in this study, $bla_{CTX-M-2}$ and $bla_{CTX-M-97}$ cannot be distinguished

The predominant plasmid types among all isolates harbouring ESBL/AmpC genes were IncI1 and IncF, both present in 26% of the isolates (Table 5.1). The highest diversity of plasmids was observed among $bla_{CTX\text{-}M\text{-}1}$ positive isolates, which was also the most abundant ESBL variant. In 9 isolates, $bla_{CTX\text{-}M\text{-}1}$ was located on IncI1 with six different IncI1 pMLST types (Table 5.1). From these six different pMLST types, four types were not yet reported in the pMLST database (http://www.pubmlst.org/plasmid, last accessed: 22 Oct. 2012). Furthermore, in 7 isolates $bla_{CTX\text{-}M\text{-}1}$ was located on IncF plasmids with four different IncF RST profiles. From these four different profiles, two were not yet reported in the pMLST database. The remaining $bla_{CTX\text{-}M\text{-}1}$ genes were located on IncB/O (three isolates), IncN (three isolates) and IncK (one isolate). In two isolates the plasmid was not typeble. The second largest group of ESBL genes, $bla_{CTX\text{-}M\text{-}15}$, was predominantly carried on IncF plasmids, of which four different RST types were found among seven isolates (Table 5.1). From these seven isolates, five also harboured $bla_{OXA\text{-}1}$ and *aac(6')Ib-cr*. The remaining $bla_{CTX\text{-}M\text{-}15}$ genes were located on IncI1 (two isolates), IncHI2 (one isolate) and two on not typeble plasmids. The $bla_{CTX\text{-}M\text{-}14}$ genes were mainly carried on IncK plasmids (Table 5.1), but also on IncI1 of which all three plasmids had the same pMLST sequence type (ST80) and one on IncF (F2; A-; B-)). From the isolates harbouring $bla_{CTX\text{-}M\text{-}2/97}$ or $bla_{CTX\text{-}M\text{-}3}$, all genes were located on different plasmids (Table 5.1). All plasmids carrying $bla_{CTX\text{-}M\text{-}32}$, were not typeble. Furthermore, four isolates harboured $bla_{TEM\text{-}52c}$, of which three were carried on IncI1 plasmids with two different pMLST types (ST10 and ST36), both

Figure 5.2: Multi-resistance of ESBL/AmpC producing *E. coli*.



The following antimicrobial classes were screened: ß-lactams (ampicillin, cefotaxime, ceftazidime), quinolones (nalidixic acid, ciprofloxacin), phenicols (forfenicol, chloramphenicol), tetracyclins (tetracycline), sulfonamides (sulfamethoxazole), trimethoprim, aminoglycosides (streptomycin, kanamycin, gentamicin), polypeptides (colistin)

belonging to the same clonal complex (CC5) (Table 5.1). From the fourth $bla_{TEM-52c}$, which was found in an isolate also harbouring $bla_{CTX-M-1}$, transformation of the plasmid failed, so no replicon type was established. Finally, in the single isolate harbouring an *ampC* gene, bla_{CMY-2} was located on an IncK plasmid.

Furthermore, 86% of the ESBL/AmpC producing *E. coli* was multi-drug resistant (showing non-wild type susceptibility to three or more antimicrobial classes, including ß-lactams). To determine if there were any ESBL/AmpC genes more commonly found in multi-drug resistant *E. coli* than others, the number of antimicrobial classes to which non-wild type susceptibilities were observed is depicted in Figure 5.2. All isolates carrying $bla_{CTX-M-2/97}$, $bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M-32}$ or bla_{CMY-2} showed a non-wild type susceptibility to three or more antimicrobial classes. The lowest prevalence of multi-drug resistance was observed in isolates harbouring $bla_{CTX-M-15}$ and $bla_{TEM-52c}$, however, still 75% of these isolates were multi-drug resistant (Figure 5.2). The most abundant group of isolates, positive for $bla_{CTX-M-1}$, showed the highest diversity in multi-drug resistance patterns, ranging in resistance from one to seven classes, with the highest proportion of 5 classes. The highest level of multi-drug resistance was observed in isolates carrying $bla_{CTX-M-14}$, $bla_{CTX-M-15}$ and mutations in the chromosomal *ampC* promoter region, with up to 11%, 8% and 14% of the isolates being non-wild type susceptible to 8 antimicrobial classes (Figure 5.2).

Discussion

This study showed that 66% of the slaughter batches were positive for faecal carriage of ESBL/AmpC producing *E. coli* among veal calves. In several countries studies have been performed on faecal carriage of ESBL/AmpC producing *E. coli* in cattle at slaughter. Prevalence varied greatly. In Poland no ESBL/AmpC producing *E. coli* were observed [18], in contrast to France (5.8%) [10], Switzerland (16%) [19], Hong Kong (3.1%) [20] and Japan (31.3%) [21]. However, comparing these prevalence data should be performed with care, since not all studies have used the same selection methods (*e.g.* the use of enrichment broth and/or different selective media), which may result in different screening sensitivities. All studies mentioned above were performed on individual animals. In this study 66% of the herds were positive for ESBL/AmpC producing *E. coli*. To put this into perspective, from the 1000 examined individual animals, 26% were found positive (10 animals per herd were sampled). In 68% of the positive herds, five or less animals out of ten were found carrier for *E. coli* with a non-wild type susceptibility to cefotaxime.

The vast majority of plasmid mediated ESBL/AmpC genes in the present study belonged to the bla_{CTX-M} gene family, of which $bla_{CTX-M-1}$ was most abundant, followed by $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ (Table 5.1). These findings are in line with the observed trend in the last five years of a retrospective study performed on faecal samples from veal calves collected at farms from 1997 to 2010 (Hordijk *et al*, submitted for publication). Studies from other countries, focussing on ESBLs in general or bla_{CTX-M} genes in specific, confirm the relatively high abundance of $bla_{CTX-M-1}$, $bla_{CTX-M-14}$ and/or $bla_{CTX-M-15}$ genes in either healthy or sick cattle [6, 8, 10, 22]. In contrast, in the United States, bla_{CMY-2} is the predominant gene found in cattle and bla_{TEM}

ESBL genes were found at a low level [23, 24]. Furthermore, a study from Hong Kong reported that *bla*$_{CTX-M-13}$ was the only ESBL observed in cattle at slaughter [20] and *bla*$_{CTX-M-2}$ was reported to be predominant in cattle in Japan [25]. This shows that there are geographical differences in the prevalence if resistance genes.
This study showed that ESBL/AmpC genes found in veal calves were predominantly located on IncI1 and IncF plasmids. These plasmids belong to the most commonly reported plasmid families in *Enterobacteriaceae* [26]. Interestingly, a relatively high proportion of IncI1 and IncF plasmid subtypes (mainly associated with *bla*$_{CTX-M-1}$) were not yet reported in the pMLST database (Table 5.1). IncI1 sequence types (ST) in combination with *bla*$_{CTX-M-1}$ have been reported either commonly (CC3, ST7) or occasionally (ST58) [26-28]. To our knowledge, the combination of *bla*$_{CTX-M-1}$ with IncF type plasmids has not been reported previously. The IncF RST-type found in this study (F2;A-;B-) has frequently been associated with *bla*$_{CTX-M-15}$ [26, 29]. Furthermore, *bla*$_{CTX-M-1}$ in was also found in combination with IncB/O plasmids. This has also been reported in isolates from humans [30] and horses [11]. The *bla*$_{CTX-M-1}$ associated with IncN was previously observed in human isolates [30, 31], poultry [32], and in both porcine isolates and their farm workers, indicating transmission may occur between food-producing animals and their care takers [33].

In this study, *bla*$_{CTX-M-15}$ was mainly located on IncF plasmids, a combination that has been reported frequently [26, 31, 34, 35]. Also the combination of *bla*$_{CTX-M-15}$ with *bla*$_{OXA-1}$ and *aac6'-Ib-cr* (the latter causing reduced susceptibility to aminoglycosides and fluoroquinolones), has been reported previously [36]. This combination was observed in the predominant IncF type found in this study carrying *bla*$_{CTX-M-15}$ (F31;A4;B1). Furthermore, all but one *bla*$_{CTX-M-15}$ carrying IncF plasmids and the IncI1 plasmid from this study have also been isolated from cattle in France [29]. The *bla*$_{CTX-M-14}$ genes in our study were mainly located on IncK plasmids. This has also been reported in calves in the UK [37], as well as in isolates from humans and turkey [38, 39]. None of the plasmids harbouring *bla*$_{CTX-M-32}$ was typeble, making it difficult to draw any conclusions towards genetic characteristics. The combination of *bla*$_{TEM-52}$ with IncI1 ST36 has been reported in cattle in France [40], but both ST36 and ST10 in combination with *bla*$_{TEM-52}$ have also been reported in humans and poultry isolates [30] and are the most commonly found types [26]. This shows that various combinations of ESBL/AmpC genes and plasmids are widely distributed in isolates of both animal and human origin. Based on the data presented in this study we cannot determine whether the overlapping genes and plasmids reside in *E. coli* that are host specific (animal or human) or that they proliferate well in both human and animal hosts.

In six herds, only promoter mutations of the chromosomal *ampC* gene were detected (Table 5.1) and no isolates were found harbouring plasmid mediated ESBL/Ampc genes within the same herd. Since this study was focussed on the dissemination of plasmid mediated ESBL/AmpC genes, this prevalence is likely to be an underestimate of the actual number of herds positive for chromosomal *ampC* genes with promoter variations leading to reduced susceptibility to cefotaxime.

The fact that there is a large scale international trafficking of live calves from many different dairy farms to Dutch veal calf farms may contribute to the high dissemination observed in this study. This may also explain why the high diversity in gene/plasmid combinations compared to Dutch poultry, in which *bla*$_{CTX-M-1}$ is highly prevalent and commonly associated with IncI1 [28].

Conclusion

A relatively high percentage of slaughter batches (66%) were found positive for *E. coli* with a non-wild type susceptibility to cefotaxime. Within these batches 26% of the individual animals were positive. The within-herd prevalence varied greatly from zero to 90% positive. In the majority of herds positive for ESBL/AmpC producing *E. coli,* the within-farm prevalence was below 50%. Furthermore, plasmid mediated resistance to cefotaxime was predominantly caused by enzymes encoded by the $bla_{CTX\text{-}M}$ gene family. Many gene-plasmid combinations found in this study have also been found in cattle in other countries, indicating that there is a non-local dissemination of resistance determinants. The fact that the prevalence of ESBL/AmpC producing *E. coli* at slaughter was relatively high, and often combined with multi-drug resistance, indicates there is a large reservoir of micro-organisms that may pose a risk to human health.

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Chapter 6

Within-farm dynamics of ESBL/AmpC producing *Escherichia coli* in veal calves: A longitudinal approach

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Abstract

Three veal calf farms maintaining an all-in-all-out system were screened for prevalence and molecular characteristics of ESBL/AmpC producing *E. coli* during the first ten weeks of a production cycle. Faecal samples from all calves within one compartment (109-150 per farm) were taken upon arrival on the farm (T0), and after 3, 6, 8 and 10 weeks (T3 - T10). At T0 the prevalence of ESBL/AmpC producing *E. coli* ranged from 18 to 26%. These were predominantly isolates carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ genes, located on various plasmids and *E. coli* MLST types. Farm 1 was negative for ESBL/AmpC producing *E. coli* after T0. Farm 2 showed an increase up to 37% at T3 which subsequently gradually decreased to 0% at T10. The presence from T3 to T10 on farm 2 was mainly caused by a clonal spread of a multi resistant *E. coli* ST 57 harbouring $bla_{\text{CTX-M-14}}$ on an IncF F2;A-;B- plasmid in. Farm 3 showed a gradual decrease in prevalence to 1.4% at T10, with a relative increase of the identical clonal variant as shown for farm 2. A second clonal variant found in farm 3 was a multi resistant *E. coli* ST 10 harbouring $bla_{\text{CTX-M-14}}$ on an IncK plasmid. In summary, prevalence of ESBL/AmpCs decreased in time. Diversity of genes, plasmids and *E. coli* was high on all farms at T0. A clonal spread was observed on farm 2 and 3, illustrative of the complex dynamics likely associated with the use of antimicrobials.

Introduction

The increasing prevalence of resistance to Extended Spectrum Cephalosporins (ESC) is of growing concern and is reported worldwide in both human and animal clinical settings, but also carriership in the community and in food-producing animals [1, 2]. Resistance to ESC in *Enterobacteriaceae* is mainly caused by Extended Spectrum ß-Lactamases (ESBL) or AmpC type ß-lactamases, which enzymatically degrade these antibiotics [3]. Genes encoding these enzymes are often located on plasmids [4], which can be transferred horizontally within and between different bacterial species. So far, many different ESBL/AmpC genes have been reported, and the number of novel gene variants is still expanding (http://www.lahey.org/studies).

Food-producing animals have been indicated as an important reservoir for ESBL/AmpC producing bacteria [5]. Faecal carriage of ESBL/AmpC producing *E. coli* food producing animals has been reported previously [6-10], and a partial genetic relatedness of genes, plasmids or host bacteria from food-producing animals and humans was described [6, 11, 12]. Evidence of direct transfer was lacking. Faecal carriage in cattle has been reported previously [9, 13-18]. However, cattle production is very diverse and includes dairy cattle and veal calves, which are kept in farms under different circumstances. In the Netherlands, dairy farms are generally closed production systems, whereas veal calf farms often maintain an all-in-all-out system in which animals originate from different farms, and are imported from several European countries. Furthermore, dairy cattle and veal calves have a different life span, a different diet and are kept in different housing facilities. Finally, dairy cattle are generally exposed to considerably lower amounts of antimicrobials than veal calves [19]. Several studies on dairy farms have shown that calves at young age rapidly acquire antibiotic resistant *Escherichia coli* [20], which are often multi resistant [21]. These studies also showed an age-related decline in resistance levels of various antimicrobial resistant *E. coli* [20, 22]. Recent studies have shown that from 2005 to 2010, resistance to ESC in veal calves is mainly caused by various bla_{CTX-M} enzymes [23]. Furthermore, 66% of animal herds screened at slaughter were positive for ESBL/AmpC producing *E. coli*, and within herd prevalence showed an average of 26% [24, 25]. These studies provide a clear picture on how prevalence of ESBL/AmpC genes developed over the years, and the genetic diversity that was present in animals at slaughter. Nonetheless, little is known about short term dynamics within a farm and a possible relation between prevalence of ESBL/AmpC producing bacteria and antimicrobial use. In veal calf farming, young animals are more at risk to acquire an infectious disease due to assembly of new herds. Furthermore, young calves generally receive more antimicrobials than older animals.

The purpose of this study was to determine the within-farm dynamics in prevalence and molecular characteristics of ESBL/AmpC producing *E. coli*, isolated from individual veal calves during the first ten weeks upon arrival at the fattening farm. The animals were approximately two weeks of age when they arrived at the farm. Furthermore, antimicrobial treatments were registered and could be associated with prevalence of ESBL/AmpC producing *E. coli*.

Material and Methods

Study design

This study was run parallel to a study designed to determine the dynamics of MRSA carriage in veal calves in a longitudinal approach [26]. Three Dutch white veal calf farms were included in the study and sampled over a ten week time period, starting October 2009. Farms were selected according to the following requirements: 1 The farm should maintain an all-in all-out system; 2 No professional activities concerning other food-producing animals were allowed; 3 All calves must originate from Dutch dairy farms to exclude differences between countries; 4 Concerning the original design for MRSA, the farm must have been positive for the presence of MRSA in the previous round (based on dust samples). All calves included in the study were housed in separated closed compartments within the stables. On farm 1, two compartments containing a total of 109 calves were included. On farm 2, one compartment containing 150 calves was included, and on farm 3, two compartments containing a total of 140 calves. Each compartment contained multiple pens. During the first six weeks all animals were housed in individual boxes within a pen. One day after T6, all calves were released from their individual boxes and relocated in pens with an average of six animals. The calves were regrouped in the pens within the compartments based on their weight and/or feed intake. All animals in these compartments were sampled individually five times, starting at the moment the calves arrived at the farm (T0), and subsequently after three weeks (T3), six weeks (T6), eight weeks (T8) and ten weeks (T10). The study population did not change during this ten week period. Calves that were relocated to stables outside the study compartments were excluded from the study. Calves from outside a study compartment were not allowed in. However, on farm 1 three calves were introduced in a study compartment at a later stage (two at T6, one at T10). These were not found positive for carrying MRSA upon arrival (based on nose and rectal swab), but were not tested for ESBL/AmpC carriage upon arrival. Antimicrobial use during the period of study was registered.

E. coli isolation

At each sampling moment rectal swabs were taken from each individual calf using a sterile cotton-wool swab (Cultiplast®). The swabs were processed the same day by suspending the faeces in 0.5 ml buffered peptone water with 30% glycerol, which was stored at -80° C. ESBL/AmpC producing *E. coli* were isolated by inoculating 10 µl of the faecal suspension on a MacConkey agar plate (product no. 212123, Becton Dickinson) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (MC+; direct plating), as well as in 1 ml Luria-Bertani (Becton Dickinson) selective enrichment broth supplemented with 1 mg/L cefotaxime (LB+; enrichment). After overnight aerobic incubation at 37°C, if MC+ plates showed growth, three typical red colonies were selected and each isolate was pure-cultured on sheep blood agar plates (Biotrading, The Netherlands). After overnight enrichment also all LB+ samples were inoculated on MC+ plates and after overnight incubation from MC+ plates showing growth one typical red colony was selected and pure-cultured on sheep blood agar plates. After *E. coli* confirmation by checking for tryptophan hydrolysis the isolates were stored at -80°C as suspensions in buffered peptone water with 30% glycerol, pending molecular analysis.

Gene characterization

All *E. coli* isolates that were selected after direct plating or after enrichment were screened by PCR for bla_{CTX-M} (including group specific PCRs for bla_{CTX-M} group 1, 2, 8, 9), bla_{TEM} and bla_{SHV} as described previously [27]. Presence of plasmid mediated AmpC beta-lactmase genes (including bla_{CMY}, bla_{ACC} and bla_{FOX}) was screened by PCR as described by Pérez-Pérez and Hanson [28]. Presence of bla_{OXA} group 1 and 2 was screened as described by Voets *et al.* [29]. For the promoter region of chromosomal *ampC* primers were used as decribed by Caroff *et al* [30]. Designation of chromosomal *ampC* subtypes was conducted as described by Mulvey *et al.* [31]. Since various bla_{CTX-M} genes that belong to group 1 are described to occur frequently in cattle, in all isolates where bla_{CTX-M} group 1 genes were detected, these genes were sequenced using Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems; USA). Subsequently a selection of isolates was made for further molecular characterization by calculating the square root of the number of isolates harbouring a specific bla_{CTX-M} group 1 gene or gene family groups (e.g. bla_{CTX-M} group 2, bla_{CMY}, etc) at each sampling moment. To calculate the number of bla_{TEM} genes to be sequenced, only the isolates solely positive for bla_{TEM} and no other ESBL/AmpC genes were taken into account. This was based on previous results that multiple ESBL/AmpC genes in one cell are rare in veal calves, and that the majority of bla_{TEM} genes that occur in veal calf isolates in addition to any ESBL or AmpC-genes are the narrow spectrum bla_{TEM-1} genes [23]. The calculations were based on one isolate per calf, unless in a sample from one animal different genes were found. All ESBL/AmpC genes in isolates selected for molecular characterization were sequenced using the sequence kit described above and primers as described previously [27] with additional primers for bla_{TEM} (TEM-Fseq: 5'-GCCAACTTACTTCTGACAACG) and bla_{CMY} (CMY-F-838: 5'-TGGCGTATTGGCGATATGTA and CMY-R-857: 5'-TACATATCGCCAATACGCCA). Sequences were analysed using Sequencher v4.9 software (Gene Codes Corporation; USA).

Plasmid typing and E. coli MLST

From the selected *E. coli* isolates for molecular characterization, ESBL/AmpC carrying plasmids were isolated and electroporated to competent Electromax DH10B cells (Invitrogen, USA). In the transformants the plasmids were identified by PCR-based Replicon Typing (PBRT) as described previously [32-34]. Isolates harbouring $bla_{CTX-M-14}$ located on IncF plasmids were further analysed by IncF Replicon Sequence Typing (RST) [35]. *E. coli* was characterized by Multi Locus Sequence Typing (MLST) as described by Wirth *et al.* [36], and analyzed using BioNumerics v6.6 software (Applied-Maths, Belgium). The sequences obtained were compared to sequences on the MLST reference website (http://mlst.ucc.ie/).

Susceptibility testing

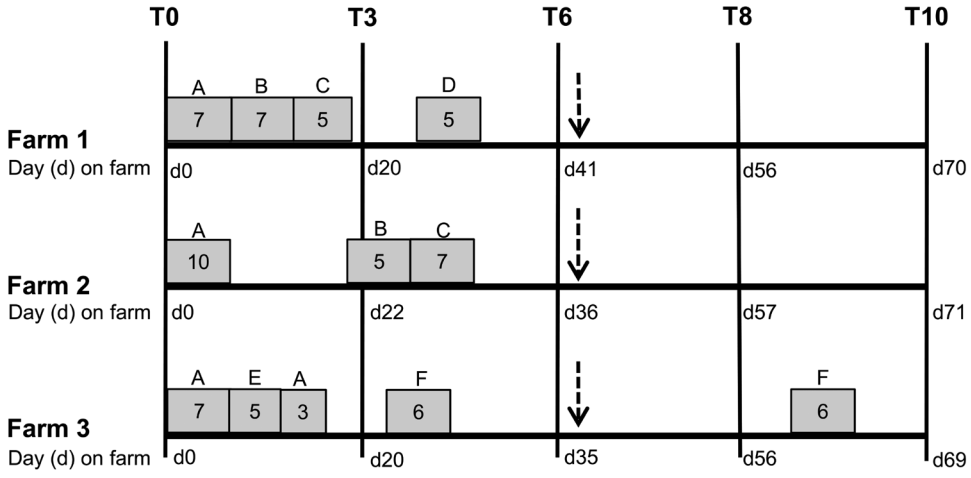
All isolates included in the molecular characterization and the corresponding transformants were tested for antimicrobial susceptibility by broth micro-dilution according to ISO20776-1:2006, using microtiter trays with a custom made dehydrated panel of antibiotics (Sensititre, Trek Diagnostic Systems, Basingstoke UK). The following antibiotics were included: ampicillin, cefotaxime, ceftazidime, tetracycline, sulfamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin and colistin. All results were interpreted using epidemiological cut-off values defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://mic.eucast.org/Eucast2/). For sulfamethoxazole, the MIC-breakpoint used was defined by CLSI [37].

Statistical analysis
Prevalence between farms was compared by Chi-square statistics as calculated with Microsoft Excel-based tool Episheet [38, 39].

Results

Animal characteristics and antimicrobial usage
At T0, all calves were approximately two weeks of age. The included calves on farm 1 and 3 were bulls. The included calves on farm 2 were heifers. On all farms the stables were cleaned, but not disinfected before the calves entered the stable. Multiple antimicrobial group treatments were administered on all farms (Figure 6.1). Within this study period, most treatments were administered within the first 30 days after arrival on the farm. On all farms, oxytetracycline and colistin were orally administered upon arrival on the farm for prophylaxis of bacterial diseases. On farms 1 and 3, these were administered for 7 days, on farm 2 for 10 days. All further treatments differed in type of antimicrobial and/or timing for each farm (Figure 6.1). Possible exposure to antibiotics before this study period was not known.

Figure 6.1: Sampling moments and antimicrobial treatments per farm

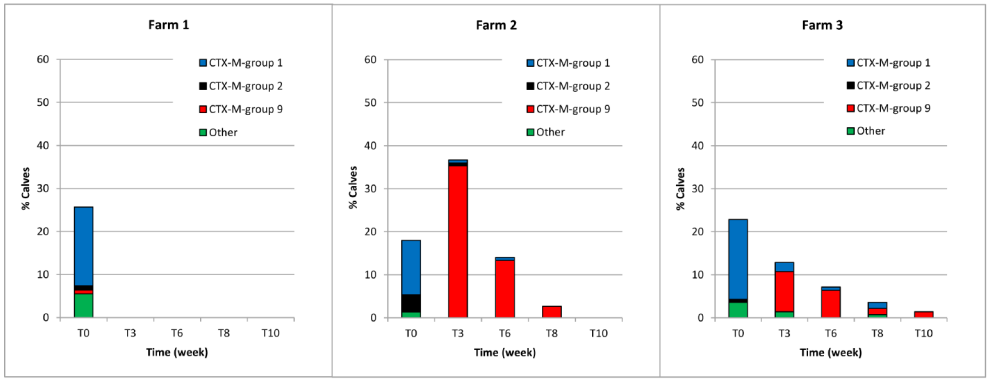


Group treatment (duration (days) inserted in box)
A: Group treatment Tetracycline (oxytetracycline) and Polymyxin E (colistin)
B: Group treatment Trimethoprim Sulfa (trimethoprim-sulfamethoxazole)
C: Group treatment Macrolide (tilmicosin)
D: Group treatment Tetracycline (doxycycline)
E: Group treatment Quinolones (enrofloxacin) and Polymyxin E (colistin)
F: Group treatment Tetracycline (doxycycline) and Macrolide (tylosin)
Dashed arrow: Calves released from individual pens (1 day after T6)
This Figure was modified from Graveland et al. [26]

ESBL/AmpC carriage

The prevalence of animals positive for ESBL/AmpC producing *E. coli* after arrival on the farms (T0) ranged from 18% to 26% (Figure 6.2), which was not significantly different between the farms (Table 6.1). After T0 all three farms showed a different trend in prevalence. At farm 1 no animals harbouring ESBL/AmpC producing *E. coli* were detected at week 3 to 10 (T3-T10). At farm 2 the prevalence first increased to 37% at T3, and then gradually decreased to 0% at T10. Finally, at farm 3 the prevalence gradually decreased after arrival on the farm to 1.4% at T10. Farm 2 and 3 only significantly differed in prevalence at T3 (Table 6.1).

Figure 6.2: Prevalence and resistance genes of ESBL/AmpC producing *Escherichia coli*



The number of calves sampled on Farm 1: n=109; Farm 2: n=150; Farm 3: n=140

Table 6.1: Differences in occurrence of *E. coli* reduced susceptible to cefotaxime expressed as prevalence ratio, with confidence intervals and p-values

Farm	week	RR	95% CI	*p* value
1 vs 2	T0	1.38	0,85 - 2,23	0.20
2 vs 3	T0	0.78	0,49 - 1,25	0.30
1 vs 3	T0	1.08	0,68 - 1,70	0.75
2 vs 3	T3	2.85	1,77 - 4,61	0.00*
2 vs 3	T6	1.78	0,89 - 3,56	0.10
2 vs 3	T8	0.75	0,20 - 2,72	0.66
2 vs 3	T10		*not calculated***	

** significant*

*** Number of positive animals on farm 2 was 0*

Chi-square was calculated using the Microsoft Excel-based tool Episheet [38, 39]

Molecular characterization
The presence of different gene families varied greatly in time (Figure 6.2). At T0 all farms showed more or less the same distribution of mainly bla_{CTX-M} genes, of which bla_{CTX-M} group 1 was present in 70 to 81% of all ESBL/AmpC producing *E. coli*. In contrast, at T3, 96% of the *E. coli* isolates from farm 2 and 72% from farm 3 harboured bla_{CTX-M} group 9 genes (Figure 6.2). These genes remained predominant until T10. On all farms, beta-lactamase genes other than bla_{CTX-M} were found only incidentally.

From all isolates, a selection was made for gene sequence analysis, plasmid identification and *E. coli* typing. From farm 1, 13 out of 27 isolates were selected for gene sequence analysis, plasmid identification and *E. coli* typing. From farm 2, 36 out of 107 were selected, and from farm 3, 31 out of 69 were selected. At T0 at all farms a variety of ESBL/ampC genes were detected in combination with plasmid replicon types and *E. coli* sequence types (ST). At farm 1 the bla_{CTX-M} genes belonging to group 1 were $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$. $bla_{CTX-M-1}$ was found on IncI1, IncF and IncN plasmids, all in *E. coli* with different ST's (Table 6.2). In contrast, $bla_{CTX-M-15}$ was found only on IncF plasmids. Two isolates harbouring $bla_{CTX-M-15}$ on IncF plasmids belonged to ST 361, implicating a clonal relatedness. Furthermore, both parent strains and transformants of these two isolates showed non-wild type susceptibility to the same antibiotics (Table 6.2). $bla_{CTX-M-2}$, $bla_{CTX-M-14}$ and $bla_{CMY-2/61}$ were all located on IncI1 type plasmids in *E. coli* with different ST's. $bla_{CMY-2/61}$ was detected in one isolate on farm 1 only.

At farm 2, the genes that belong to bla_{CTX-M} group 1 found at T0 were $bla_{CTX-M-1}$, $bla_{CTX-M-15}$ and $bla_{CTX-M-32}$ (Table 6.3). Furthermore, at T0 $bla_{CTX-M-2}$ was found on various plasmids in *E. coli* with different ST's. The predominant plasmid types were IncB/O, harbouring $bla_{CTX-M-1}$ and $bla_{CTX-M-32}$, and IncF, harbouring $bla_{CTX-M-2}$ and $bla_{CTX-M-15}$. Also IncI1 (carrying $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$) and IncHI2 (carrying $bla_{CTX-M-2}$) were detected. One plasmid carrying $bla_{CTX-M-32}$ was non-typeble. All genes were present in *E. coli* with different ST's, except for two $bla_{CTX-M-15}$ isolates. These two genes were located on IncF plasmids in an *E. coli* ST 410. Both transformants containing the $bla_{CTX-M-15}$ gene showed similar non-wild type susceptibilities, while the donor isolates showed some differences (Table 6.3).

In farm 3 all isolates showed different gene, plasmid, *E. coli* combinations (Table 6.4). $bla_{CTX-M-1}$ was found on IncN, IncB/O, IncF and one non-typeble plasmid. $bla_{CTX-M-2}$ was located on an IncN plasmid and $bla_{CTX-M-15}$ was found on IncI1 and IncF plasmids. *E. coli* isolates that harboured mutations in the chromosomal *ampC* promoter region and no plasmid mediated ESBL/AmpC genes, were only observed at T0 (Table 6.2 and 6.3). These isolates harbouring *ampC* promoter mutations were also positive for the narrow spectrum ß-lactamase bla_{TEM-1}.

In contrast to the diversity that was observed at T0 on all farms, farm 1 was negative for ESBL/AmpC producing *E. coli* after T0, and farm 2 and 3 showed only little diversity at all sampling moments after T0. Despite the low diversity, a relatively high prevalence of $bla_{CTX-M-14}$ was found in both farm 2 and 3. This $bla_{CTX-M-14}$ gene belongs to the bla_{CTX-M} group 9 shown in Figure 6.2 and was not detected at T0 on farm 2 and 3, and was the only group 9 gene variant detected in this study. On farm 2, all $bla_{CTX-M-14}$ genes were located on IncF plasmids. They were all identified in *E. coli* ST 57 and located on indistinguishable F2;A-;B- plasmids. Furthermore, all isolates harbouring $bla_{CTX-M-14}$ on this IncF RST-type showed next to ß-lactam antibiotics

a non-wild type susceptibility to seven other classes of antimicrobials, being quinolones (ciprofloxacin and nalidixic acid), phenicols (florfenicol and chloramphenicol), trimethoprim, sulfonamides (sulfamethoxazole), tetracycline, aminoglycosides (gentamicin, kanamycin and streptomycin) and polypeptides (colistin) (Table 6.3). However the transformants of these isolates, only harbouring the $bla_{CTX\text{-}M\text{-}14}$ carrying IncF plasmid, only showed non-wild type susceptibility to ß-lactam antibiotics, indicating that no other resistance genes were located on this plasmid. On farm 3 $bla_{CTX\text{-}M\text{-}14}$ was associated with two different *E. coli* ST's. The first was identical to the one found on farm 2, a combination of $bla_{CTX\text{-}M\text{-}14}$ on a F2:A-;B- plasmid in an *E. coli* ST 57, with non-wild type susceptibility to the same antibiotics (Table 6.4). The second was $bla_{CTX\text{-}M\text{-}14}$ on an IncK plasmid in a *E. coli* ST 10, also showing non-wild type susceptibility to the same antibiotics as shown for the ST 57 isolates (Table 6.4). Also the transformants only harbouring $bla_{CTX\text{-}M\text{-}14}$ on IncK plasmids, only showed non-wild type susceptibility to ß-lactam antibiotics.

Finally, a low prevalence of $bla_{CTX\text{-}M\text{-}1}$ was detected on farm 2 and 3 at T3 and T6 (Table 6.3 and 6.4). On farm 3 a low prevalence of $bla_{CTX\text{-}M\text{-}15}$ was detected at T6 and T8, and $bla_{TEM\text{-}52c}$ at T3 and T8 (Table 6.4). Some of these low prevalent genes at farm 3 were clonally related and showed a similar combination of ESBL gene, with plasmid and *E. coli* ST. These clonal variants were not detected at T0.

Discussion

This study showed that upon arrival on the farm (T0), all three farms had comparable prevalences of calves positive for ESBL/AmpC producing *E. coli* of approximately 20 % (Table 6.1 and Figure 6.2). In general, Dutch veal calves are collected from many different dairy farms. Approximately 50% are derived from domestic dairy farms and 50% from other European countries. These animals are brought together in collection and distribution centers and are subsequently transported to veal farms, as were investigated in this study. This structure of the veal calf industry, this might explain the diversity in resistance genes in association with different plasmids and *E. coli* found at T0 on all farms. The fact that all calves included in this study came from Dutch dairy farms may explain the relative homogeneity between farms in prevalence at T0. However, to show whether any differences in prevalence and molecular characteristics between countries exists, is subject for further study. Until now this type of studies has not been performed so data for comparison are not available yet. After T0 all farms showed a different trend in prevalence. Farm 1 became negative. Farm 2 and 3 differed in prevalence on T3 (Table 6.1). Despite the different trends, clonally related variants were observed on two out of three farms at T3 and later, suggesting a source on the farms. These variants were possibly introduced in the past, since they were not observed on T0, or they were present below detection level at T0.

The data provided in this study showed that $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ genes were most abundant at the beginning of the production cycle. Previous data showed that these genes were also most prevalent at the end of the production cycle, at slaughter [24].

Table 6.2: Molecular characterization of ESBL/Ampc producing *E. coli* on farm 1

Farm	Wk	Gene	Isolate	Plasmid
1	**T0**	$bla_{CTX\text{-}M\text{-}1}$	004T0	Frep
			047T0	I1
			076T0	FIA; N
			106T0	N
		$bla_{CTX\text{-}M\text{-}2/97}$[5]	059T0	I1
		$bla_{CTX\text{-}M\text{-}14}$	003T0	I1
		$bla_{CTX\text{-}M\text{-}15}$	001T0	FIA
			069T0	Frep; FIA
			108T0	Frep; FIA
		$bla_{CMY\text{-}2}$	066T0	I1
		ampC-4[6]	029T0	-
		ampC-18[6]	048T0	-
		ampC-3[6]	053T0	-

E. coli MLST[1]		
ST	**CC**	**Non-wild type susceptibility[2, 3]**
New[4]	-	***Amp-Ctx-Caz***-Chl-Smx-Tet-***Gen***-Kan
88	23	***Amp-Ctx-Caz***-Cip-Nal-Tmp-Smx-Tet-Kan
744	-	***Amp-Ctx-Caz***-Cip-Nal-Tmp-Smx-Tet-Kan-Str
973	-	***Amp-Ctx-Caz***-Smx-Tet-Gen-Kan-Str
New[4]	-	***Amp-Ctx-Caz***-Tmp-Smx
952	-	***Amp-Ctx-Caz***-Cip-Nal-***Chl-Ffn-Tmp-Smx-Tet***-Str
648	-	***Amp-Ctx-Caz***-Cip-Nal-***Tmp-Smx-Tet-Gen-Kan***-Str
361	-	***Amp-Ctx-Caz***-Cip-Nal-***Chl***-Tmp-Smx-***Tet-Gen-Kan***-Str
361	-	***Amp-Ctx-Caz***-Cip-Nal-***Chl***-Tmp-Smx-***Tet-Gen-Kan***-Str
1480	-	***Amp-Ctx-Caz***-Nal-Ffn-Tmp-Smx-Tet-Kan-Str
973	-	Amp-Ctx-Chl-Ffn-Tmp-Smx-Tet-Kan-Str
58	155	Amp-Ctx-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Str
58	155	Amp-Ctx-Caz

[1] ST = sequence type; CC = clonal complex

[2] The following abbreviations were used: Amp = ampicillin; Ctx = cefotaxime; Caz = ceftazidime; Cip = ciprofloxacin; Nal = Nalidixic Acid; Chl = Chloramphenicol; Ffn = florfenicol; Tmp = Trimethoprim; Smx = sulfamethoxazole; Tet = tetracycline; Gen = gentamicin; Kan = kanamycin; Str = streptomycin; Col = colistin

[3] Non-wild type susceptibility was detected for all antibiotics listed. Antibiotics listed in italic bold face indicate to which antibiotics the corresponding transformants showed non-wild type susceptibility. EUCAST epidemiological cut-off values were used.

[4] This sequence type was not listed on http://mlst.ucc.ie/

[5] Based on the primers used in this study, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}97}$ cannot be distinguished

[6] Chromosomal ampC type as previously described [31]. These isolates also harboured $bla_{TEM\text{-}1}$.

Table 6.3: Molecular characterization of ESBL/Ampc producing *E. coli* on farm 2

Farm	Wk	Gene	Isolate	Plasmid
2	**T0**	$bla_{CTX\text{-}M\text{-}1}$	143T0	B/O
			189T0	B/O
			253T0	I1
			258T0	B/O; P
		$bla_{CTX\text{-}M\text{-}2/97}$[4]	134T0	Frep
			188T0	Frep
			254T0	HI2
		$bla_{CTX\text{-}M\text{-}15}$	198T0	I1
			221T0	Frep; FIA
			236T0	Frep; FIA
		$bla_{CTX\text{-}M\text{-}32}$	230T0	*(nt)*[6]
			228T0	B/O; P
		ampC-3[5]	202T0	-
2	**T3**	$bla_{CTX\text{-}M\text{-}1}$	150T3	B/O
			162T3	Frep
		$bla_{CTX\text{-}M\text{-}14}$	110T3	F2;A-;B-
			126T3	F2;A-;B-
			153T3	F2;A-;B-
			172T3	F2;A-;B-
			189T3	F2;A-;B-
			207T3	F2;A-;B-
			233T3	F2;A-;B-
			141T3	F2;A-;B-
			195T3	F2;A-;B-
2	**T6**	$bla_{CTX\text{-}M\text{-}1}$	166T6	B/O
		$bla_{CTX\text{-}M\text{-}14}$	117T6	F2;A-;B-
			133T6	F2;A-;B-
			159T6	F2;A-;B-
			207T6	F2;A-;B-
			246T6	F2;A-;B-
			128T6	F2;A-;B-
			217T6	F2;A-;B-
2	**T8**	$bla_{CTX\text{-}M\text{-}14}$	158T8	F2;A-;B-
			172T8	F2;A-;B-
			137T8	F2;A-;B-
			225T8	F2;A-;B-

***E. coli* MLST[1]**		
ST	**CC**	**Non-wild type susceptibility[2, 3]**
117	-	***Amp-Ctx-Caz***-Tmp-Smx-Tet-Gen-Kan-Str
88	23	***Amp-Ctx-Caz***-Tmp-Tet-Kan-Col
58	155	***Amp-Ctx-Caz***-Tmp-***Smx***
10	10	***Amp-Ctx-Caz-Tet***
362	-	***Amp-Ctx-Caz***-Chl-Tmp-Smx-Tet-Kan-Str
69	69	***Amp-Ctx-Caz***-Cip-Nal-Chl-Tmp-Smx-Tet-Gen-Kan-Str
34	10	***Amp-Ctx-Caz***-Chl-***Tmp-Smx-Tet***-Str
58	155	***Amp-Ctx-Caz***-Tmp-Smx-Tet-Kan-Str
410	23	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-***Tet-Kan***
410	23	***Amp-Ctx-Caz***-Cip-Nal-***Tet-Kan***
224	-	***Amp-Ctx-Caz***-Cip-Nal-***Chl-Tmp-Smx-Tet-Kan***-Str
10	10	***Amp-Ctx-Caz-Tet***
448	448	Amp-Ctx-Caz-Cip-Nal-Smx-Tet-Str
648	-	***Amp-Ctx-Caz***-Tmp-Smx-Tet-Gen-Kan-Str-Col
648	-	***Amp-Ctx-Caz***-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
162	469	***Amp-Ctx-Caz***-Cip-Nal-Chl-Tmp-Smx-Tet-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str-Col

[1] ST = sequence type; CC = clonal complex
[2] The following abbreviations were used: Amp = ampicillin; Ctx = cefotaxime; Caz = ceftazidime; Cip = ciprofloxacin; Nal = Nalidixic Acid; Chl = Chloramphenicol; Ffn = florfenicol; Tmp = Trimethoprim; Smx = sulfamethoxazole; Tet = tetracycline; Gen = gentamicin; Kan = kanamycin; Str = streptomycin; Col = colistin
[2] Non-wild type susceptibility was detected for all antibiotics listed. Antibiotics listed in italic bold face indicate to which antibiotics the corresponding transformants showed non-wild type susceptibility. EUCAST epidemiological cut-off values were used.
[4] Based on the primers used in this study, $bla_{CTX-M-2}$ and $bla_{CTX-M-97}$ cannot be distinguished
[5] Chromosomal ampC type as previously described [31]. This isolate also harboured bla_{TEM-1}.
[6] This plasmid was not typable with PBRT

6

Table 6.4: Molecular characterization of ESBL/Ampc producing *E. coli* on farm 3

Farm	Wk	Gene	Isolate	Plasmid
3	**T0**	$bla_{CTX\text{-}M\text{-}1}$	263T0	B/O
			268T0	*(nt)*[4]
			288T0	N
			317T0	B/O
			332T0	N
			396T0	Frep
		$bla_{CTX\text{-}M\text{-}2/97}$[5]	361T0	N
		$bla_{CTX\text{-}M\text{-}15}$	373T0	I1
			396T0	Frep
3	**T3**	$bla_{CTX\text{-}M\text{-}1}$	353T3	N
			386T3	Frep
		$bla_{CTX\text{-}M\text{-}14}$	268T3	K
			279T3	K
			298T3	K
			324T3	F2;A-;B-
			303T3	F2;A-;B-
		$bla_{TEM\text{-}52c}$	365T3	I1
			379T3	I1
3	**T6**	$bla_{CTX\text{-}M\text{-}1}$	370T6	N
		$bla_{CTX\text{-}M\text{-}14}$	274T6	K
			318T6	F2;A-;B-
			329T6	F2;A-;B-
			299T6	F2;A-;B-
		$bla_{CTX\text{-}M\text{-}15}$	371T6	I1
3	**T8**	$bla_{CTX\text{-}M\text{-}14}$	329T8	F2;A-;B-
			292T8	F2;A-;B-
		$bla_{CTX\text{-}M\text{-}15}$	373T8	I1
			395T8	I1
		$bla_{TEM\text{-}52c}$	376T8	I1
3	**T10**	$bla_{CTX\text{-}M\text{-}14}$	292T10	F2;A-;B-
			298T10	F2;A-;B-

E. coli MLST[1]		
ST	**CC**	**Non-wild type susceptibility[2, 3]**
117	-	***Amp-Ctx-Caz***-Cip-Nal-Smx-Tet-Kan-Str
224	-	***Amp-Ctx-Caz***-Cip-Nal-***Chl-Tmp-Smx-Tet***-Str
617	10	***Amp-Ctx-Caz***-Cip-Nal-Smx-Kan-Str
117	-	***Amp-Ctx-Caz***-Chl-FfnTmp-Smx-Tet-Kan-Str
88	23	***Amp-Ctx-Caz***-Smx-Tet-Str
448	448	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-***Tmp***-Smx-Tet-Gen-Kan-Str
1291	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Gen-Kan-Str
88	23	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Col-Str
410	23	***Amp-Ctx-Caz***-Cip-Nal-***Tmp-Smx-Tet-Kan***
354	354	***Amp-Ctx-Caz***-Cip-Nal-Tmp-Smx-Tet-Col-Str
117	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Tmp-Smx-Tet-Col-Gen-Kan-Str
10	10	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
10	10	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
10	10	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
1011	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
1011	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
354	354	***Amp-Ctx-Caz***-Cip-Nal-Tmp-Smx-Tet-Col-Str
10	10	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
59	59	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
59	59	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Str
997	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Tmp-Smx-Tet-Str
1011	-	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str
57	350	***Amp-Ctx-Caz***-Cip-Nal-Chl-Ffn-Tmp-Smx-Tet-Col-Gen-Kan-Str

[1] ST = sequence type; CC = clonal complex

[2] The following abbreviations were used: Amp = ampicillin; Ctx = cefotaxime; Caz = ceftazidime; Cip = ciprofloxacin; Nal = Nalidixic Acid; Chl = Chloramphenicol; Ffn = florfenicol; Tmp = Trimethoprim; Smx = sulfamethoxazole; Tet = tetracycline; Gen = gentamicin; Kan = kanamycin; Str = streptomycin; Col = colistin

[3] Non-wild type susceptibility was detected for all antibiotics listed. Antibiotics listed in italic bold face indicate to which antibiotics the corresponding transformants showed non-wild type susceptibility. EUCAST epidemiological cut-off values were used.

[4] This plasmid was not typable with PBRT

[5] Based on the primers used in this study, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}97}$ cannot be distinguished

On farm 2 and 3, at T3 and subsequent sampling moments, the majority of ESBL/AmpC producing *E. coli* harboured $bla_{CTX\text{-}M\text{-}14}$ (Table 6.3 and 6.4). In these isolates, the combination of the $bla_{CTX\text{-}M\text{-}14}$ gene, located on an IncF F2;A-;B- plasmid, in *E. coli* ST 57, and a similar pattern of non-wild type susceptibility for the whole panel of antimicrobials tested, clearly indicates a clonal spread on these veal calf production farms (Table 6.3). The combination of $bla_{CTX\text{-}M\text{-}14}$ on this specific IncF plasmid has been reported before in China [40, 41] and Korea [42] in *E. coli* of animal origin (including cattle) as well as in human faecal samples, human urinary tract infections (UTI) and human blood culture samples. Also in the United States, $bla_{CTX\text{-}M\text{-}14}$ was associated with IncF plasmids isolated from dairy cattle, showing a clonal spread within herds, based on PFGE analysis [43]. However, no *E. coli* MLST data were available from these studies. The first $bla_{CTX\text{-}M\text{-}14}$ harbouring variant found on farm 3, was located on a similar F2;A-;B- plasmid, also in *E. coli* ST 57, showing the same non-wild type susceptibilities as described for farm 2 (Table 6.4). The second $bla_{CTX\text{-}M\text{-}14}$ harbouring variant on farm 3 was located on an IncK plasmid, in *E. coli* ST 10 (Table 6.4). This variant also showed non-wild type susceptibility to the whole panel of antimicrobials tested, similar to the IncF variant. In the United Kingdom (UK) $bla_{CTX\text{-}M\text{-}14}$ genes were also found on IncK plasmids in a longitudinal field study on a dairy farm [16]. This study in the UK showed that IncK-type conjugative plasmids with the same RFLP-pattern circulated among many different *E. coli* strains. Moreover, in the UK, the combination of $bla_{CTX\text{-}M\text{-}14}$ on IncK plasmids was not only observed in cattle, but also in human and turkey *E. coli* isolates [44]. Furthermore, in *E. coli* isolates of human origin in Spain, the spread of $bla_{CTX\text{-}M\text{-}14}$ was mainly driven by IncK plasmids [45, 46]. Interestingly, in Spain $bla_{CTX\text{-}M\text{-}14}$ was not only associated with IncK plasmids, but also with *E. coli* ST 10 [46, 47], as was found in our study. Apparently the combination of $bla_{CTX\text{-}M\text{-}14}$ on IncK plasmids is widespread in Europe in both humans and animals, and was found in various *E. coli* ST's, of which ST10 was reported frequently.

Except for the two $bla_{CTX\text{-}M\text{-}14}$ variants, all ESBL positive isolates showed a very diverse range of ST's (Tables 6.2 – 6.4). This is consistent with other studies in which samples of both human and animal origin were analyzed [1, 48, 49]. Interestingly, the highly prevalent and multi resistant *E. coli* ST 57, harbouring $bla_{CTX\text{-}M\text{-}14}$ and isolated from farm 2 and 3, is not commonly reported as an ESBL carrying sequence type, whereas the other highly prevalent and also multi resistant *E. coli* ST 10, among other sequence types belonging to clonal complex 10, has been reported more commonly as a carrier for different ESBL genes, both in animal and human isolates [1, 48, 49]. The worldwide reported uropathogenic *E. coli* ST 131, commonly associated with $bla_{CTX\text{-}M\text{-}15}$, but also $bla_{CTX\text{-}M\text{-}14}$, was not found in our study.

All isolates selected for molecular characterization were also screened for susceptibility to eight classes of antimicrobials. From this selection at T0 91% of the isolates showed a non-wild type susceptibility for three or more classes and were therefore designated multi-drug resistant. Interestingly, only at T0 60% or less of the transformants harbouring an ESBL carrying plasmid also showed non-wild type susceptibility for other antibiotics than ß-lactams, indicating their resistance genes were located on the same plasmid. In the remaining isolates at T0 and all isolates at T3 to T10 from all farms, the ESBL carrying plasmid did not harbour any other resistance genes encoding for resistance to the panel of antibiotics used in this study. This was observed previously in $bla_{CTX\text{-}M\text{-}14}$ carrying isolates in the UK [16, 50]. This indicates that a horizontal transfer of the ESBL carrying plasmid does not necessarily lead to co-transfer of multi-drug resistance to the recipient cell.

A likely explanation for the sudden shift in most prevalent ESBL genes found between T0 and T3 and later at farm 2 and 3, might be the group treatments with oxytetracycline and colistin upon arrival at the farm (Figure 6.1). At T0 the $bla_{CTX\text{-}M\text{-}14}$ carrying isolates were not detected on both farms, indicating they were either present below detection level, or they were present in the stable and colonized the calves after the sampling moment at T0. At T3, the calves were still housed in individual boxes and the $bla_{CTX\text{-}M\text{-}14}$ carrying isolates were distributed all over the stable. Since these $bla_{CTX\text{-}M\text{-}14}$ carrying isolates were also resistant to tetracycline and colistin, the group treatment with these drugs may have facilitated its proliferation in the gut. However, after T3 the prevalence decreased on both farms, when more group treatments were administered, using antibiotics to which these $bla_{CTX\text{-}M\text{-}14}$ carrying isolates possessed non-wild type susceptibility (Figure 6.1 and Tables 6.3 and 6.4). If the group treatments with oxytetracycline and/or colistin were the driving force behind this increase of $bla_{CTX\text{-}M\text{-}14}$ carrying isolates at T3, a second increase was to be expected after the subsequent treatments. Several studies at dairy farms have also shown there is an inverse relationship between prevalence of antibiotic resistant bacteria in the gut flora and animal age in young calves, with or without selection pressure of antimicrobial treatment [20, 22, 51]. All studies suggest a 'higher fitness' of the resistant strains caused this effect. Another factor that may contribute to selection pressure is waste milk fed to calves, that may contain antimicrobial residues. Waste milk is unfit for human consumption, and comprises colostrum, milk from cows with mastitis, or milk from cows treated with antimicrobial or non-antimicrobial medicines that may leave a residue in the milk [52]. A survey in England and Wales on antimicrobial usage and waste milk feeding practices on dairy farm showed that on 83% of the included farms, waste milk was fed to calves. The proportion of the diet consisting of waste milk was either 0-25%, or 76-100% on most farms. This was equally distributed [52]. In over 90% of the cases, waste milk contained either colostrum from freshly calved cows and heifers that have received dry cow antimicrobials, or milk from lactating cows undergoing antimicrobial treatment. Cefquinome and ceftiofur were among the most commonly used antimicrobials on the farms included in the survey. To what extent waste milk may contribute to the proliferation of resistant bacteria is not clear, but the survey in England and Wales showed that it is a possible source of antimicrobial exposure. Further study is needed to really understand what causes this increase and subsequent decrease in prevalence of these multi resistant strains shown in this study. Furthermore, it is noteworthy that from the three farms that were studied in this paper, two (unrelated) farms were positive for *E. coli* with a non-wild type susceptibility to colistin. Reports on colistin resistance in *E. coli* are scarce [53-56]. Studies that performed additional susceptibility testing showed that the vast majority of the colistin resistant isolates were multi resistant [54, 56], as was shown in this study.

In summary, this study showed that the within-farm prevalence of calves positive for *E. coli* producing different ESBL/AmpC genes may vary greatly over a relatively short time span and under antimicrobial pressure even decrease to undetectable levels. The changes in prevalence during the first six weeks occurred while the animals were housed in individual pens, limiting contact between animals, indicating other driving forces may influence the spread and (re) colonization of ESBL/AmpC carrying *E. coli*. The fact that clonally related multi resistant $bla_{CTX\text{-}M\text{-}14}$ producing *E. coli* variant were found on two unrelated farms suggests circulation in the veal calf production system, possibly introduced to the farms in the past. These isolates were similar to those previously described in both animals and humans.

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Chapter 7

General Discussion

Goal of this thesis

Prior to this thesis the availability of data on quinolone resistance and ESBL/AmpC carriage in commensal bacteria in veal calves was limited. The majority of reports provided information on dairy cattle, beef cattle, or cattle not further specified. Large differences exist in these different branches of cattle industry. The majority of veal calf farms produce white veal and maintain an all-in, all-out production system, in contrast to dairy farms which are generally closed production systems. In general, young cattle receive more antimicrobial treatments compared to older cattle. Since white veal calves are brought to slaughter before eight months of age, and rose veal calves before twelve months of age, relative to their age they have been exposed to antimicrobials more frequently than dairy cattle. In addition white veal calves receives mainly milk replacer as nutrition, supplemented with a little fibre rich feed. Therefore they never become fully functional ruminants before slaughter. These differences in life span, nutrition and exposure to antimicrobials may affect the occurrence and the epidemiology of antimicrobial resistance.

The studies described in this thesis were designed to assess the prevalence and molecular characteristics of resistance determinants in the commensal gut flora, leading to quinolone resistance and resistance to 3rd/4th generation cephalosporins. Both classes of antimicrobials are classified by WHO as being critically important for human medicine [1]. Furthermore, association of quinolone and 3rd/4th generation cephalosporins resistance with other classes of antimicrobials, within a single bacterial clone, was determined.

(Multi) Resistance in veal calves

Isolates with a reduced susceptibility to ciprofloxacin (**chapter 2**) or cefotaxime (**chapters 5** and **6**) were also screened for susceptibility to other classes of antimicrobials by broth microdilution, as described in these chapters. Based on the editorial on how to interpret and describe susceptibility test results by Schwarz *et al*, in this thesis multi-drug resistance was defined as bacteria showing a reduced susceptibility to three or more classes of antimicrobials [2]. Data from the Monitoring Programme of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands [3] showed that from all randomly picked *E. coli* screened in this surveillance, between 40% and 60% of the isolates from veal calves from 2005 to 2011 were multi-drug resistant. For the strains selected by reduced susceptibility to quinolones, or by selective culturing for 3rd/4th generation cephalosporin resistant isolates, the proportion of multi-drug resistance was much higher. All isolates with a reduced susceptibility to ciprofloxacin were multi-drug resistant (Figure 2.1; **chapter 2**), and of all *E. coli* carrying ESBL/AmpC genes, isolated at slaughter, 86% was multi-drug resistant (Figure 5.2; **chapter 5**). Furthermore, in the longitudinal study, from all isolates obtained upon arrival at the farm and included in the molecular characterization, 91% was multi-drug resistant. At later sampling moments, a high level of multi-drug resistance was observed, but this was due to a clonal spread (Table 6.2 to 6.4; **chapter 6**). Even though *E. coli* with reduced susceptibility to ciprofloxacin and cefotaxime were highly associated with multi-drug resistance, the majority of resistance genes to other antimicrobials were not genetically linked to quinolone resistance determinants or ESLB/AmpC genes detected in these isolates. The reduced susceptibility to

ciprofloxacin described in **chapter 2** was caused by chromosomal mutations in the Quinolone Resistance Determining Region (QRDR) of the chromosomal DNA, and were therefore not likely to be linked to other resistance genes generally located on plasmids. As described in **chapter 6,** when transferring plasmids carrying ESBL/AmpC genes to a susceptible *E. coli*, less than 30% of plasmids at T0 co-transferred resistance to three or more antimicrobial classes. Based on the results described above, no conclusions can be drawn as to why *E. coli* harbouring quinolone resistance determinants or ESBL/AmpC genes are more multi-drug resistant compared to randomly selected *E. coli*, since these were shown not to be coupled to other resistance genes. Horizontal transfer of resistance genes and subsequent selection pressure due to antimicrobial treatment could facilitate such an accumulation of resistance genes. However, this hypothesis still does not explain the higher association of multi-drug resistance with quinolone resistance or ESBL/AmpC genes.

Transmission of resistance genes

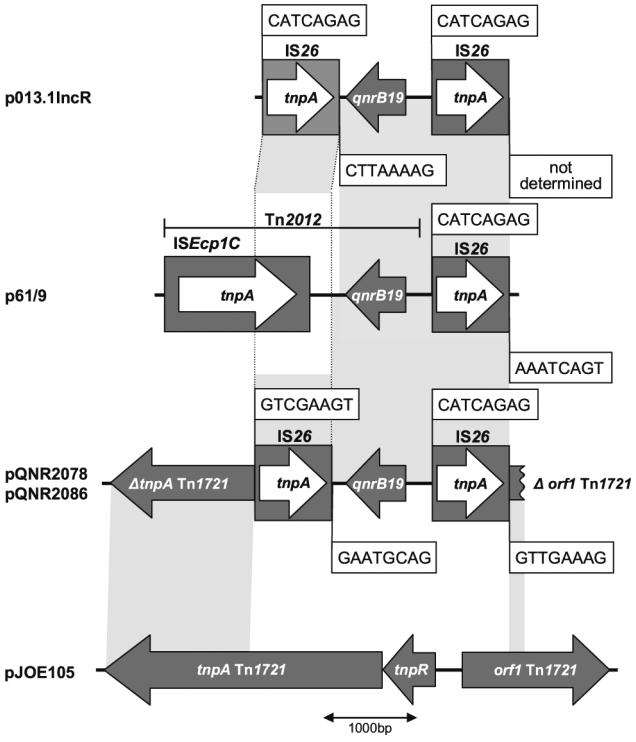
Quinolone resistance genes

As described in **chapter 2**, quinolone resistance in veal calves is mainly caused by mutations in the QRDR of the chromosomal topoisomerase genes. A key role was played by mutations in the *gyrA* gene, which were present in all isolates with a reduced susceptibility to quinolones (Table 2.2; **chapter 2**). Additional mutations in the *parC* and *parE* gene were also observed. Nonetheless, their contribution to a decrease in quinolone susceptibility was less compared to a second mutation in *gyrA* (Table 2.2; **chapter 2**). These topoisomerase genes code for enzymes involved in the winding and unwinding of DNA during DNA replication [4]. Since these topoisomerase genes are located chromosomally, transmission of resistance caused by mutations in these genes is limited to cell division (clonal spread). In contrast, plasmid mediated resistance genes have the potential to be transferred both horizontally, from cell to cell, as well as through clonal spread.

Plasmid mediated quinolone resistance was only observed in veal calves at a very low prevalence. The first *qnr* gene found in veal calves was described in **chapter 3**. This *qnr* gene was identified as *qnrB19* and analyzed in further detail by primer walking analysis. Furthermore, a *qnrS* gene was found in an *E. coli* also harbouring $bla_{\text{CTX-M-15}}$, and the modified aminoglycoside resistance gene *aac(6')-Ib-cr* [5] conferring reduced susceptibility to ciprofloxacin was detected in four additional *E. coli* isolates also harbouring $bla_{\text{CTX-M-15}}$. Both *qnrS* and *aac(6')-Ib-cr* were isolated from faecal samples at slaughter (**chapter 5**). In the last five years, the *qnrB19* gene has been reported frequently in various bacteria of both animal and human origin [6-20]. In **chapter 3** it was shown that the flanking regions of the *qnrB19* gene shared similar structures as compared to other studies [6, 7]. Despite the similarities, several transposons and/or insertion sequences (mobile genes involved in transfer of resistance genes within the bacterial chromosomal and plasmid DNA) were interrupted by other insertion sequences or (partial) transposons. This indicates that multiple transposition events have occurred (Figure 3.1; chapter 3). The observation of similarities in flanking mobile elements on various plasmids was confirmed by Schink *et al* [21]. In their study they showed that the *qnrB19* gene was also bracketed by two IS26 insertion sequences. However, compared to our results, they found an additional 96

base pairs in the spacer region between *qnrB19* and the downstream located IS26 (Figure 7.1). This indicates that in both studies the formation of *qnrB19* flanked by two IS26 elements occurred independently. Comparative studies as described in **chapter 3** and Schink *et al* [21], but also Tran *et al* [20] showed that not only plasmids, but also transposable genetic elements play an important role in the dissemination of the *qnrB19* gene. Furthermore, in literature other *qnr* variants, *aac(6')-Ib-cr* and *oqxAB* were also shown to be associated with IS26 [22-25]. Interestingly, even though genetic tools (plasmids and insertion sequences) facilitating a possible horizontal spread were present, the prevalence of PMQR genes in veal calves is very low. This was also shown for food-producing animals (cattle, pigs, fowls and turkeys) in an international collaborative study on plasmid mediated quinolone resistance in *Salmonella* enterica and *E. coli* [15]. Fluoroquinolones have been used in food-producing animals since the late 1980s, but the use of these antimicrobials has not massively selected for PMQR genes. This may be due to the fact that administration of fluoroquinolones results in concentrations in the intestinal tract, higher than the MIC for PMQR-positive isolates (lacking mutations in the QRDR region) [15]. Nonetheless, this does not rule out co-selection in case of multi-drug resistance while other antibiotics are administered.

Figure 7.1: Schematic representation of the *qnrB19* gene with flanking regions



Comparison made by Schink et al [21] of the qnrB19 gene and flanking regions, found on different plasmids. Permission to use this illustration was granted by Oxford University Press

Extended Spectrum ß-Lactamase (ESBL) / AmpC genes
In this thesis, genetic elements, other than plasmids, involved in transmission of ESBL/AmpC genes were not determined. Other studies have shown that various mechanisms play a role in the acquisition and/or transfer of ESBL/AmpC genes [26]. Insertion sequences IS*26*, IS*CR1* or IS*Ecp1* are reported frequently in association with a variety of ESBL/AmpC genes [27-31]. Also transposons have been described to facilitate horizontal transfer of ESBL/AmpC genes [26, 31, 32]. The presence of partial sequences of mobile elements or other genes in the flanking regions of ESBL/AmpC genes, interrupted by other mobile elements, indicate again that multiple transposition events have occurred, similar to what was described for the *qnrB19* gene in this thesis (**chapter 3**).

Mobile elements such as insertion sequences and transposons may partially explain the diversity of plasmids carrying ESBL/AmpC genes, as described in **chapters 5** and **6**. In these chapters, the ESBL/AmpC genes were located on plasmids belonging to various incompatibility groups. Analyzing these plasmids into further detail by plasmid Multi Locus Sequence Typing (pMLST) for IncI1 [33], or Replicon Sequence Typing (RST) for IncF [34], showed that also within these different incompatibility groups a large variation exists (Table 5.1; **chapter 5**).

Transmission of resistant micro-organisms in the production chain
Next to the presence of insertion sequences and transposons, the geographical distribution of animals may play a role in the transmission of ESBL producing bacteria, and thus its accompanying plasmids carrying ESBL/AmpC genes. In the Netherlands veal calves are collected from many different dairy farms, and approximately 50% of the animals is imported from many different European countries, of which Germany, Poland and Belgium/ Luxembourg are most important. Even though the effect of transportation of these animals on the distribution and transmission of ESBLs has not been studied, it may contribute as was shown for human travel [35, 36].

Persistence of ESBL/AmpC producing *E. coli* within animals

In the longitudinal study on three farms it was shown that upon arrival at the farm, approximately 20% of the veal calves were positive for ESBL/AmpC producing *E. coli* (Figure 6.2; **Chapter 6**). At subsequent sampling moments, after three (T3) to ten (T10) weeks, all farms showed a different trend in prevalence. Prevalence at farm 1 was 0% from T3 onwards. Prevalence at farm 2 first increased to 37% at T3 and subsequently decreased gradually to 0% at T10. At farm 3 the prevalence decreased gradually after T0 to 1.4% at T10. As can be seen in Figure 6.2, the different ESBL/AmpC genes found at T0 were no longer detected (farm 1), or detected at a very low prevalence (farm 2 and 3) after T0. The sudden increase of $bla_{\text{CTX-M-14}}$ at T3 also decreased dramatically afterwards. Furthermore, the calves from farm 2 and 3 were not continuously found positive during the ten-week study period. The animals positive for the different $bla_{\text{CTX-M}}$ genes varied between the sampling moments. In the vast majority of animals, $bla_{\text{CTX-M}}$ genes were not detected for more than 2 sampling moments at a row. Both the fact that the number of ESBL/AmpC positive *E. coli* declined during the study period on all three farms, and that the majority of calves was not found positive for more than 2 sampling moments at a row, suggests that the ESBL/AmpC producing *E. coli* decreased in concentration

over time. However, as shown in Figure 6.1, during this decrease, antimicrobial selection pressure was present after T3, and at farm 3 also after T8. Still the prevalence decreased. In addition, in this longitudinal study no samples were taken at the end of the production round. Therefore we do not know whether these three farms would have fit the 34% of herds found negative for ESBL/AmpC producing *E. coli* at slaughter, or that at a later moment the prevalence of ESBL/AmpC producing *E. coli* would have (re)emerged above detection level.

In contrast to the low persistence that was observed in the longitudinal study described in this thesis, an *in vitro* study performed in the United Kingdom (UK), using plasmid pCT harbouring a $bla_{CTX\text{-}M\text{-}14}$ gene, showed a high persistence both with and without antimicrobial selection pressure [37]. This plasmid had an IncK replicon type, as was shown for one of the $bla_{CTX\text{-}M\text{-}14}$ harbouring clones on farm 3 (**chapter 6**). It was obtained from a dairy farm in the United Kingdom where $bla_{CTX\text{-}M\text{-}14}$ producing *E. coli* were isolated from both animals and the farm environment in a longitudinal study over a seven month period [38]. It is unclear whether the animals on the dairy farm were colonized with a high persistence, or that (re)colonization was a continuous process with the farm environment as a source.

Furthermore, the decrease in ESBL prevalence during the first ten weeks on the farm showed an inverse relationship with age. It has been shown for ampicillin and nalidixic acid resistant *E. coli* in dairy calves, that colonization within few days after birth increased rapidly, and subsequently declined consistently with increasing age [39, 40]. Even though quinolone resistance described in this thesis (**chapter 2** and **3**) was not studied over time or in specific age groups, an age related effect on quinolone resistance may also apply for veal calves. To identify factors or parameters that may contribute to a possible age related effect on colonization of resistant *E. coli* in veal calves requires further research. How this possible age related decline in resistance correlates with the prevalence and diversity of ESBL/AmpC genes found at slaughter remains unclear. Hoyle *et al* also found an association of carriage of resistant bacteria with housing status [39], but, since housing of calves at a dairy farm is different from a veal calf farm, it is unclear whether this might play a role on veal calf farms. Other factors that might play a role are nourishment, the establishment of the gut flora in young animals, and antimicrobial treatments, even though the latter was shown not to be the only factor.

Molecular diversity in ESBL/AmpC genes over years

As described in **chapter 4** the prevalence of *E. coli* harbouring mutations in the promoter region of the chromosomally encoded *ampC* gene in veal calves is relatively stable during the studied period from 1997 to 2010. In contrast, ESBL/AmpC genes generally associated with plasmids were not detected until 2000. In 2000, a low prevalence of $bla_{CTX\text{-}M}$ and bla_{TEM} genes was observed. Subsequently both prevalence and diversity of the different ESBL/AmpC gene variants, but mainly $bla_{CTX\text{-}M}$, increased over time (figure 4.1; **chapter 4**). Comparative data on veal calf farms is limited, however, despite geographical differences, the increasing prevalence and diversity of ESBL/AmpC gene variants is consistent to what is reported on faecal carriage and/or clinical infections in humans, livestock and companion animals [41-44]. A review by Carattoli nicely illustrates the pace with which this development occurred, especially in animals. In 2008, Carattoli concluded that ESBL genes were not frequently reported, but

that recent findings at the time could rapidly change this perspective [45]. In addition, new ESBL/AmpC gene variants are reported on a regular basis (www.lahey.org/Studies/), leading to adjustments and refinements of classification schemes [46-48]. An important factor in the rapid dissemination of resistance determinants may be the trade and transportation of livestock. Even though each sector dealing with food-producing animals (e.g. broilers, pigs, cattle) has its own unique logistic system, dependent on the type of animal and organization of the industry, a high rate of exchange is facilitated because many animals are transported to many different countries.

Reducing prevalence of resistant *E. coli*

After 2005 the majority of ESBL/AmpC genes in *E. coli* isolated from veal calves are plasmid mediated (**chapter 4** to **6**). Although the prevalence is low, plasmid mediated quinolone resistance genes are also present in veal calves (**chapter 3** and **5**). The fact that these resistance genes are commonly associated with various insertion sequences and transposons, as described above (transmission of resistance), indicates there is a high potential of horizontal spread. The flanking regions described for the *qnrB19* gene (**chapter 3**) [6, 7, 21] support this view. This potential horizontal spread and the fact that over the years the diversity in ESBL gene variants increased (**chapter 4**), shows the genetic background of resistance becomes increasingly complex. Furthermore, the epidemiology of antimicrobial resistance is largely determined by the interaction between gene, plasmid and the bacterial host. If plasmids harbouring resistance genes also harbour addiction factors [49], it will be more difficult for the host bacteria to lose this plasmid, and thus the resistance genes, even if the selective advantage through antibiotic usage would disappear.

As shown in chapter 2, 5 and 6, the majority of *E. coli* with reduced susceptibility to ciprofloxacin and cefotaxime are multi-drug resistant (Table 2.1, Table 5.2 and Table 6.2 to 6.4). The more resistance determinants are present in the host bacteria, the higher the survival chance will be when antimicrobials are being administered. This selection pressure does not only count for plasmid-mediated resistance, but also for chromosomally located resistance determinants, as was the case for most quinolone resistant *E. coli*. Therefore, reducing selection pressure is likely to reduce the selection of resistant bacteria. The above-mentioned criteria all plead for the fact that the prevalence of antimicrobial resistance will be hard to reduce. However, as was shown in the longitudinal study on farm 2 and 3, the prevalence of the multi resistant $bla_{\text{CTX-M-14}}$ carrying *E. coli* variants declined to levels below or near detection limits within ten weeks (Figure 6.2; **chapter 6**). Figure 6.1 showed that during these weeks an antimicrobial selection pressure was still present, indicating other factors played a role in this decline. Identifying these factors may help to actively reduce antimicrobial resistance.

Furthermore, multi-drug resistant $bla_{\text{CTX-M-14}}$ carrying *E. coli* variants on farm 2 and 3 were not observed upon arrival at the farm. All animals were housed individually, limiting transmission through contact, while the prevalence of these variants increased suddenly. This suggests that these *E. coli* variants may have been already present at the farm, and that the calves were colonized after they arrived. Therefore, adequate cleaning and disinfection of the stable between production cycles may also help to reduce resistance by preventing (re)colonization

of the animals from the farm environment. Whether it is feasible to disinfect a stable in such a way that this (re)colonization with ESBL/AmpC producing *E. coli* from the farm environment can be prevented could be subject for further research. This is currently being studied.

Colistin resistance

As illustrated in Figure 6.1 (**chapter 6**) Polymixin E (colistin) has been a commonly used antimicrobial agent for prophylactic and metaphylactic purposes in veal calves in the Netherlands. In the past, and when the longitudinal study was conducted still, it has been administered frequently as a so-called start treatment in combination with oxytetracycline to prevent gastro-intestinal diseases during the start of the fattening period. Recently this approach is no longer allowed, and now solely oxytetracycline is often administered for treatment purposes. Colistin is also commonly used in both dairy cattle and pig herds [50, 51]. Due to the growing occurrence of multi-drug resistant Gram-negative bacterial infections, colistin is also reintroduced as a last resort drug in human medicine [52-54]. However, prevalence of resistance to colistin remains low. In the Netherlands colistin resistant *E. coli* and *Salmonella enterica* in food-producing animals, including veal calves, have been reported incidentally [55]. This is similar to what was found in other countries in bacteria of both human and animal origin [56-59], although one study in Japan reported a relatively high incidence of colistin resistant *E. coli* from clinical samples of cattle and pigs [60]. Even though colistin resistance seems rare, its prudent use remains essential. As shown from faecal samples at slaughter, two isolates were found with a reduced susceptibility to colistin (Table 5.1; **chapter 5**), indicating that prevalence of *E. coli* with a reduced susceptibility to colistin was relatively low. Nonetheless, as described in **chapter 6**, due to the clonal spread of one *E. coli* variant at farm 2 and two *E. coli* variants at farm 3, the within-farm prevalence may increase dramatically in relatively short period. This clonal spread may have been triggered by the preventive administration of colistin. Colistin binds to the lipopolysaccharide (LPS) in the bacterial cell wall through ionic bonds with the phosphoryl groups of lipid A, solubilising the membrane (similar as a detergent), and allowing permeabilization of both outer and inner membranes [61]. So far, the exact mechanism leading to colistin resistance is not known. However, several mechanisms have been suggested to be involved.[61-65]

Public health risks

The presence of ESBL/AmpC producing bacteria and quinolone resistance is frequently reported in human medicine. It is not only related to hospital settings, but carriership is also widespread in the community, often associated with urinary tract infections [44, 66-69]. A study on faecal carriage of ESBL/AmpC producing *E. coli* in four Dutch hospitals showed that 4.9% of the admitted patients were carrier [70]. Prevalence of quinolone resistance is highly variable in different groups of patients. In *E. coli* from urine samples from community patients (sent to laboratory by General Practitioners) 5% was resistant to ciprofloxacin. From urology departments 25% was resistant to ciprofloxacin [71]. To what extent public health is threatened by the occurrence of quinolone resistance determinants or ESBL/AmpC producing *E. coli* in food-producing animals is not known. In perspective of this thesis, also the possible attribution from veal calves is unknown. It is hard to assess a degree of exposure of the general

human population and a possible subsequent transmission of resistant bacteria. It is likely that people with a high occupational exposure are at higher risk for transmission of resistant bacteria, however, this was not assessed in this thesis. Furthermore, at slaughter, 26% of the individual animals were positive for ESBL/AmpC producing *E. coli*. The meat products were not included in this study. 90% of these meat products are exported, reducing the domestic exposure to a large extent. The export is mainly to southern European countries, meaning that if these meat products were to be positive for ESBL/AmpC producing bacteria, mostly people from other countries will consume these products. As shown in **chapter 5** and **6**, similarities between isolates from veal calves and humans at the level of genes, plasmids and *E. coli* sequence types were shown. Also, the mutations found in the QRDR region have been commonly reported in both humans and animals. Nonetheless, these genetic similarities do not provide any information on epidemiological associations and transmission between veal calves and humans, because humans directly linked to these veal calves were not part of this study. For Methicillin Resistant *Staphylococcus aureus* (MRSA), a direct link between veal calves and farm workers has been shown [72-74]. Furthermore, studies performed on broiler farms [75] and pig farms [76] have shown similarities in ESBL/AmpC genes and plasmids between farm animals and farm workers, suggesting transmission has occurred. In addition, a likely horizontal transfer of non-ESBL carrying plasmids between cattle and farm workers and veterinarians was also shown [77]. How these transmissions between food-producing animals and field workers correlate to the general human population remains unclear.

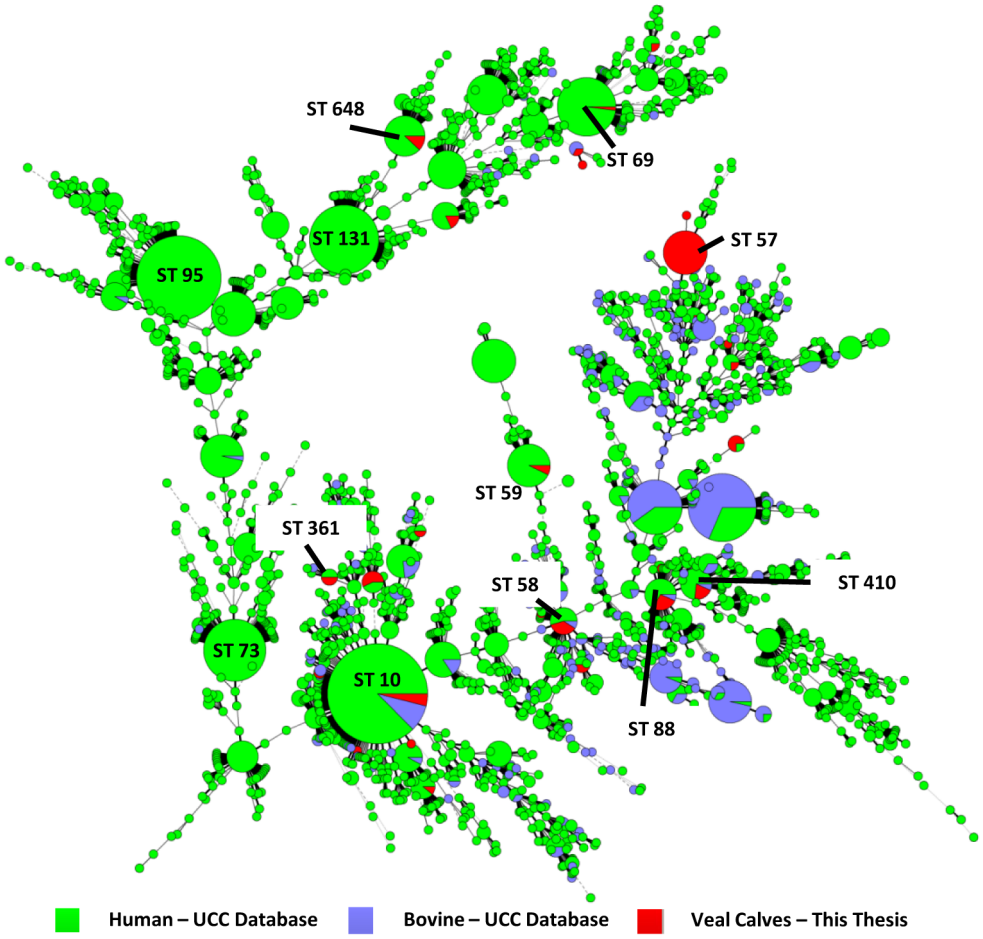
Quinolone resistance

As shown in **chapter 2**, quinolone resistance in veal calves is mainly caused by chromosomal mutations in the QRDR region. Therefore a potential dispersion is limited to clonal spread. Nonetheless, also plasmid mediated quinolone resistance genes (PMQR) have been found (**chapters 3** and **5**). These PMQR resistance genes cause a mild decrease in quinolone susceptibility, not exceeding the CLSI clinical breakpoint [78]. However, several studies have shown in a murine urinary tract infection model, that the presence of PMQR resistance genes or a single mutation in the *gyrA* gene, despite their low-level resistance, leads to a reduced bactericidal activity of fluoroquinolones [17, 79]. Whether these genes may lead to actual therapeutic failure is not known [80].

ESBL/AmpC

As was shown in **chapter 6**, based on Multi Locus Sequence Type (MLST), the diversity of ESBL/AmpC producing *E. coli* in veal calves was high upon arrival at the farm. To determine whether there were any similarities between *E. coli* MLST sequence types obtained from humans and veal calves, the results presented in this thesis were plotted against the different sequence types of human and bovine origin submitted to the MLST database [4] (Figure 7.2). Figure 7.2 shows the diversity of human *E. coli* MLST types submitted to the database was high, and importantly, that there is a large overlap between the sequence types from veal calves reported in this thesis and the sequence types of human origin. The number of different isolates reported, reflected by the size of the circle, is not of interest since both sets of isolates are not representative for the actual prevalence in both populations. In contrast, the *E. coli* isolated from veal calves only show little overlap with submitted ST's of bovine origin (Figure 7.2). Even though the diversity of ST's described in **chapter 6** is relatively high, the limited overlap with bovine ST's submitted to the MLST database, might be caused by the small population of veal calves described in in this thesis.

Figure 7.2: *E. coli* MLST types in humans and cattle



Minimum spanning tree, calculated using BioNumerics v6.6 (Applied Maths, belgium)
Data from human and bovine E. coli were obtained from the UCC database [4].
Data from veal calves were obtained from this thesis (chapter 6)

E. coli ST131 has been described as a worldwide dispersed sequence type, mainly observed in humans [81-83], but also reported in animals [32, 83-86]. ST131 is commonly associated with $bla_{CTX\text{-}M\text{-}15}$, but was also found positive for carrying $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}10}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}35}$ or $bla_{SHV\text{-}12}$ [33, 87-89]. The $bla_{CTX\text{-}M\text{-}15}$ gene from these ST131 strains is mostly located on IncF plasmids with FII replicon type, either with or without FIA and/or FIB replicons [81]. In addition, $bla_{CTX\text{-}M\text{-}15}$ is often associated with the presence of *aac(6')-Ib-cr* gene and the narrow spectrum ß-lactamase $bla_{OXA\text{-}1}$ [81, 84, 88, 90]. **Chapter 6** shows that *E. coli* ST131 was not found in the selection of veal calves for which *E. coli* MLST types were determined. Nonetheless, $bla_{CTX\text{-}M\text{-}15}$ was commonly associated with various IncF plasmids, all positive for the FII replicon type (**chapter 5** and **6**). Furthermore, the *aac(6')-Ib-cr* and $bla_{OXA\text{-}1}$ genes were present in

40% of the $bla_{CTX\text{-}M\text{-}15}$ harbouring isolates sampled at slaughterhouses (**chapter 5**). As shown in Table 5.1, in all isolates positive for $bla_{CTX\text{-}M\text{-}15}$ as well as $bla_{OXA\text{-}1}$ and *aac(6')-Ib-cr*, the ESBL gene was located on a F31;A4;B1 plasmid. However, these plasmids were located in *E. coli* ST361 (*n* = 3) and ST410 (n = 1) respectively. These isolates were all obtained from different herds, from different farms. Therefore, the fact that this plasmid was observed in *E. coli* with different MLST types suggests horizontal plasmid transfer. In contrast, the fact that these plasmids were found in several *E. coli* ST361, all from different herds and different farms, also indicates that clonal spread might have contributed to the transmission. In addition, as shown in Figure 7.2, both ST361 and ST410 have also been reported in humans.

As described in **chapter 6**, a clonal distribution of *E. coli* sequence types ST10 and ST57 was observed in one and two farms respectively. *E. coli* ST57 is not commonly reported. In contrast, *E. coli* ST10 is frequently reported in both humans and animals [43, 91, 92]. Interestingly, in Spain, ST10 was isolated from humans, and also harboured $bla_{CTX\text{-}M\text{-}14}$ on an IncK plasmid, as was reported in this thesis [87, 93]. Despite the molecular similarities in isolates reported in Spain and our findings, these isolates were epidemiologically unrelated, and further typing would be necessary to show any potential clonal relationship.

Final thoughts

The way the veal calf industry is organized makes it rather vulnerable for the introduction and dissemination of antimicrobial resistant bacteria. Calves are collected from many different dairy farms and assembled into new herds. In theory, this creates an inverse pyramid, potentially introducing many different resistance determinants. This is reflected in the diversity in ESBL/AmpC genes, plasmids and *E. coli* detected in veal calves. Although not discussed in this thesis, the presence of (multi-drug) resistant bacteria is not only of public health concern but may also compromise animal health due to treatment failure of bacterial infections as well.

As was shown in the general introduction (**Chapter 1**), over the years a large amount of antimicrobials was used in food-producing animals in the Netherlands. As shown in the Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN) [3], but also this thesis, a high level of multi-drug resistance exists in veal calves. The Dutch authorities determined that, using 2009 as a base line, antimicrobial use in food-producing animals in the Netherlands must be decreased with 20% in 2011, and 50% in 2013. Lowering exposure will decrease selection pressure, which may subsequently prevent within-farm clonal spread as shown for farm 2 and 3, described in **chapter 6**. However, as shown in this thesis for quinolone resistance determinants and ESBL/AmpC producing *E. coli*, bacteria have various molecular mechanisms to adapt to their environment, including the acquisition of resistance determinants, which is also possible in healthy animals, without selection pressure. The accumulation of various resistance mechanisms in one bacterial cell leads to multi-drug resistance, which increases chances for bacterial survival. Furthermore, many resistance determinants are plasmid mediated. Many plasmids have acquired plasmid-addiction systems, making it virtually unable for bacteria to lose the plasmid [49]. This includes IncF-type plasmids described in this thesis. Therefore, when exposure to antimicrobials is

decreased and/or certain antibiotics are no longer used, it remains unpredictable how the resistance level will develop over time.

Finally, other factors causing or prohibiting spread of antimicrobial resistance determinants still need to be determined. Plasmid mediated ESBL/AmpC genes were shown to be highly prevalent in veal calves. In contrast, despite their similar genetic tools (association with insertion sequences and transposons) plasmid mediated quinolone resistance was present, but at a very low level. What caused this difference is still unclear.

Conclusions

The findings in this thesis show that resistance to fluoroquinolones and 3rd generation cephalosporins, which are regarded as critically import antimicrobials for human medicine, is also present in Dutch veal farming.

Fluoroquinolone resistance in veal calves was shown to be mainly caused chromosomal mutations, therefore limiting a potential spread to clonal distribution.

Prevalence of ESBL/AmpC producing *E. coli* increased over the years and showed a high genetic diversity on gene, plasmid and bacterial host level. In addition, within-farm dynamics is very diverse, and factors, other than antimicrobial selection pressure, contributing to these dynamics still need to be determined.

Resistance determinants described in this thesis have shown genetic overlap with what was reported in human isolates. However, an epidemiological relation was not apparent. Therefore no conclusions can be drawn about veal calves as a possible source for transmission to humans.

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Summary

Worldwide an increase in antimicrobial resistance has been reported. In both human and veterinary clinical settings antimicrobial resistance may lead to therapy failure. Antimicrobial resistant bacteria are not only found in clinical settings, but also in the community; humans, companion animals and food producing animals have been reported as carrier. Animals may act as a reservoir for antimicrobial resistant bacteria and may therefore have a zoonotic potential towards humans.

Both (fluoro)quinolones and Extended Spectrum Cephalosporins (ESC) are antimicrobial classes considered by the World Health Organization (WHO) to be of critical importance in both human and veterinary medicine. Increasing levels of antimicrobial resistance in general, including resistance to (fluoro)quinolones and ESC, in both humans and animals lead to public and subsequent political debate. Even though both classes of antimicrobials were not the first choice for treatment of bacterial infections in animals, both classes were often used in veterinary medicine in the Netherlands.

Within the food producing industry, cattle are kept for different purposes (e.g. dairy, meat) and under different circumstances. In the Netherlands dairy farms are generally closed production systems, whereas veal calf farms often maintain an all-in-all system. Furthermore, dairy cattle have a different life span, a different diet and are generally exposed to lower amounts of antimicrobials compared to veal calves. Data on antimicrobial resistance in dairy cattle or cattle in general has been reported. However, very little is known about antimicrobial resistance in veal calves specifically.

In this thesis the prevalence and molecular characteristics of resistance to (fluoro)quinolones and ESC in veal calves were described using *Escherichia coli* as an indicator organism.

Ciprofloxacin and nalidixic acid were used as indicator antimicrobials for quinolone resistance. In **chapter 2** the prevalence and molecular characteristics of quinolone resistance were described. From all randomly selected *E. coli,* isolated in 2007 for antimicrobial resistance surveillance purposes, 14% was reduced susceptible. All reduced susceptible isolates carried one or more mutations in the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene. Approximately 50% of the reduced susceptible isolates showed additional mutations in the QRDR of *parC* and/or *parE* genes. No plasmid mediated quinolone resistance determinants (PMQR) were found in this set of isolates. The contribution of efflux pumps in quinolone resistance was considered to be limited.

In an epidemiological study on antimicrobial resistance in veal calves performed in 2009 and 2010, parallel to the studies in this thesis, one *E. coli* isolate showed a phenotype characteristic for PMQR. This isolate, described in **chapter 3**, harboured a *qnrB19* gene located on an IncR plasmid. The flanking region of this *qnrB19* gene showed large genetic similarities compared to flanking regions of *qnrB19* genes described in other studies. The (interrupted) mobile genetic elements implicate that multiple transposition events did occur.

Cefotaxime was used as an indicator for resistance to ESC. Resistance to ESC is mainly caused by Extended Spectrum Beta Lactamases (ESBLs) or AmpC-type beta lactamases that hydrolyze beta-lactam antibiotics. The genes encoding these beta-lactamases are often located on plasmids. Also mutations in the promoter region of the chromosomal *ampC* gene may lead to derepression of transcription and reduced susceptibility to ESC. A retrospective study, described in **chapter 4**, was performed using pooled fecal samples each representing one farm, collected from 1997 to 2010. The prevalence data showed a discontinuous increasing trend of resistance to ESC ranging from 4% in 1998 to 39% in 2010. Various promoter mutations of chromosomal *ampC*-genes were found in fecal samples in all years. In 2000 $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-20}}$ and $bla_{\text{TEM-52}}$ were the first ESBL genes observed. Up to 2010 the diversity in ESBL/AmpC genes gradually increased. Most ESBL genes found belonged to the $bla_{\text{CTX-M}}$ gene family, of which $bla_{\text{CTX-M-1}}$ was most prevalent, followed by $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$. Plasmid mediated genes coding for AmpC type enzymes were found rarely.

A second study on ESC resistance, described in **chapter 5**, was performed in 100 veal calf slaughter batches. Fecal samples from ten individual animals per batch were taken. These data showed that 66% of the batches were positive for *E. coli* with reduced susceptibility to cefotaxime. Within-herd prevalence varied greatly, however, from all individual animals tested (n = 1000) 26% were found positive. ESBL gene $bla_{\text{CTX-M-1}}$ was most prevalent, followed by $bla_{\text{CTX-M-14}}$ and $_{\text{-15}}$. The ESBL genes were located on a wide variety of plasmids. $bla_{\text{CTX-M-1}}$ and $_{\text{-15}}$ were predominantly associated with different variants of IncI1 or IncF plasmids. $bla_{\text{CTX-M-14}}$ was predominantly associated with IncK or IncI1 plasmids.

In a longitudinal study, described in **chapter 6**, fecal samples from individual animals from three farms were screened for *E. coli* with reduced susceptibility to cefotaxime. All animals within a closed compartment on each farm were sampled upon arrival at the farm and after three, six, eight and ten weeks (T0 to T10). At T0 18% to 26% of the animals were positive for *E. coli* with reduced susceptible to cefotaxime. At T0 all farms showed a similar distribution of mainly $bla_{\text{CTX-M}}$ genes. After T0, no reduced susceptible *E. coli* were found on farm 1. Farm 2 and 3 showed a clonal spread of $bla_{\text{CTX-M-14}}$ on an IncF plasmid in E. coli ST57 (farm 2 + 3) and $bla_{\text{CTX-M-14}}$ on an IncK plasmid in *E. coli* ST10 (farm 3). These variants were not observed on T0. On all farms prevalence decreased to (near) 0% while antimicrobial selection pressure was present due to treatment.

The data presented in this thesis indicate that quinolone resistance in veal calves is mainly caused by mutations in the topoisomerase type II and IV genes *gyrA*, *parC* and *parE*. Plasmid mediated quinolone resistance was present on a low level, therefore horizontal transfer will be limited. Furthermore, the prevalence and diversity in ESC resistance determinants has increased from 1997 to 2010. The majority of ESC resistance genes were plasmid mediated and mainly belong to the $bla_{\text{CTX-M}}$ gene family. Within herd prevalence varies greatly between farms, but also varies greatly over time within farms. The identification of factors that contribute to these dynamics in prevalence requires further study. Furthermore, resistance determinants described in this thesis showed large similarities in the genetic make-up of genes, plasmids and *E. coli* sequence types as described by others in human isolates. However, despite the genetic similarities, based on these data, no conclusions can be drawn towards a potential transmission of antimicrobial resistant *E. coli* to the human population.

Samenvatting

Wereldwijd wordt er een toename in antimicrobiële resistentie in bacteriën waargenomen. Dit geldt zowel voor ziekteverwekkende als commensale (niet ziekteverwekkende) bacteriën, maar ook voor bacteriën uit de omgeving. In zowel de humane gezondheidszorg als in de diergeneeskunde kan antimicrobiële resistentie leiden tot een verminderd therapeutisch succes bij behandeling van patiënten. Resistente bacteriën worden niet alleen gevonden in ziekenhuizen en andere gezondheidszorginstellingen, maar ook in de algemene bevolking, zij het vaak met een lagere prevalentie. Bij zowel mensen, gezelschapsdieren als voedselproducerende dieren is aangetoond dat zij drager zijn van resistentiegenen ''verpakt'' in diverse soorten bacteriën. Omdat via de voedselketen bacteriën over kunnen gaan van dier(product) naar mens, vormen dieren een potentieel reservoir voor resistente bacteriën als bron voor de mens. Gebruik van antibiotica bij mens en dier selecteert voor resistente bacteriën. Hoewel er ook andere drijvende krachten zijn, wordt algemeen aangenomen dat het gebruik van antimicrobiële middelen de belangrijkste reden is van resistentietoename.

Zowel (fluoro)chinolonen als extended spectrum cefalosporinen (ESC) zijn klassen antibiotica die door de Wereld Gezondheids Organisatie (WHO) worden beschouwd als middelen die van cruciaal belang zijn voor de mens . De toenemende mate van antimicrobiële resistentie, inclusief resistentie tegen (fluoro)chinolonen en ESC, in zowel mensen als dieren heeft geleid tot een publieke discussie, gevolgd door een politiek debat. Beide klassen antibiotica zijn geen eerste keus middelen om bacteriële infecties bij dieren te behandelen, echter beide klassen worden in Nederland aan dieren regelmatig toegediend.

In de dierlijke produktieketen worden runderen onder verschillende omstandigheden gehouden, afhankelijk van het productiedoel waarvoor ze gehouden worden (bijv. zuivel of vlees). In Nederland wordt op melkveebedrijven voornamelijk een gesloten systeem gehanteerd, waarbij introductie van nieuwe dieren van buiten het bedrijf relatief weinig voorkomt. Dit in tegenstelling tot de vleeskalverbedrijven, waar veelal een all-in all-out systeem wordt gehanteerd waarbij steeds nieuwe dieren worden aangevoerd. Vergeleken met vleeskalveren worden melkkoeien veel ouder, krijgen ander voer en worden doorgaans minder vaak behandeld met antibiotica. Gegevens over antimicrobiële resistentie in melkvee, of runderen in het algemeen zijn reeds beschreven. Er is echter nog weinig bekend over antimicrobiële resistente bacterien die voorkomen bij vleeskalveren.

Dit proefschrift beschrijft de prevalentie en moleculaire karakterisatie van resistentiegenen tegen (fluoro)chinolonen en ESC in vleeskalveren, waarbij *Escherichia coli* gebruikt is als indicatororganisme. Deze *E. coli* komt als commensaal (niet ziekteverwekkend) voor in hoge aantallen in de darm van kalveren.

Ciprofloxacine en nalidixine zuur zijn gebruikt als indicator-antibiotica om resistentie tegen chinolonen te onderzoeken. In **hoofdstuk 2** is de prevalentie en moleculaire karakterisatie van quinolonenresistentie beschreven. Van alle *E. coli*'s die in 2007 in feces van Nederlandse vleeskalveren zijn geïsoleerd voor het monitoren van antimicrobiële resistentie, was 14% verminderd gevoelig voor (fluoro)chinolonen. Al deze verminderd gevoelige isolaten hadden één of meerdere mutaties in het Quinolone Resistance Determining Region (QRDR) van

het *gyrA*-gen. Ongeveer 50% van de verminderd gevoelige isolaten hadden daarnaast ook mutaties in het QRDR van het *parC*- en/of *parE*-gen. In deze isolaten zijn geen plasmide gemedieerde resistentie determinanten (PMQR) gevonden. De bijdragen van effluxpompen aan de resistentie tegen chinolonen was beperkt.

In een epidemiologische studie over antimicrobiële resistentie in vleeskalveren in 2009 en 2010, uitgevoerd parallel aan dit proefschrift, werd een *E. coli* isolaat gevonden met een specifiek fenotype dat kon duiden op de aanwezigheid van PMQR. Dit isolaat, beschreven in **hoofdstuk 3**, bevatte een *qnrB19*-gen gelegen op een IncR plasmide. Het flankerende DNA van dit gen toonde verschillende genetische overeenkomsten met flankerend DNA van *qnrB19*-genen beschreven in andere studies. De gevonden, al dan niet onderbroken, mobiele DNA sequenties duidden op een herhaaldelijke opname van addition DNA, waaronder dit *qnrB19*-gen.

Cefotaxim is gebuikt als indicator-antibioticum om resistentie tegen ESC te onderzoeken. Resistentie tegen ESC wordt voornamelijk veroorzaakt door Extended Spectrum Beta Lactamases (ESBLs) of AmpC-type Beta Lactamases, waarbij door een hydrolytische reactie de beta-lactam antibiotica worden afgebroken. De genen die coderen voor deze enzymen liggen in *E. coli* voornamelijk op plasmiden. Ook mutaties in het promotorregio van het chromosomaal gelegen *ampC*-gen kunnen leiden tot derepressie van gentranscriptie, wat vervolgens kan leiden tot een verminderde gevoeligheid voor ESC. In **hoofdstuk 4** is een retrospectieve studie beschreven waarin gebruik werd gemaakt van gepoolde fecesmonsters, verzameld van 1997 tot 2010. Ieder fecesmonster was representatief voor één bedrijf. Een discontinue stijgende trend was te zien in de prevalentie van ESC resistentie, variërend van 4% in 1998 tot 39% in 2010. Verschillende promotorrmutaties in het chromosomale *ampC*-gen zijn gevonden in isolaten uit alle jaren. De eerste ESBL-genen, $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-20}}$ en $bla_{\text{TEM-52}}$ werden gevonden in fecesmonsters uit 2000. Tot in 2010 nam de diversiteit in gevonden ESBL/AmpC-genen geleidelijk toe. De meerderheid van ESBL-genen behoorde tot de $bla_{\text{CTX-M}}$ genfamilie, waarvan $bla_{\text{CTX-M-1}}$ het meest prevalent was, gevolgd door $bla_{\text{CTX-M-14}}$ en $bla_{\text{CTX-M-15}}$. Plasmide gemediëerde AmpC-genen werden zeer weinig gevonden.

In een tweede onderzoek naar ESC resistentie, beschreven in **hoofdstuk 5**, zijn fecesmonsters genomen van 100 koppels in het slachthuis. Per koppel zijn 10 individuele dieren bemonsterd. Deze studie liet zien dat 66% van de bemonsterde koppels positief was voor *E. coli* met een verminderde gevoeligheid voor cefotaxim. De prevalentie binnen koppels varieerde sterk. Van alle individueel bemonsterde dieren (n = 1000) was 26% positief. Ook in deze studie kwam $bla_{\text{CTX-M-1}}$ het meest voor, gevolgd door $bla_{\text{CTX-M-14}}$ en $bla_{\text{CTX-M-15}}$. De ESBL-genen waren gelegen op zeer verschillende plasmiden. $bla_{\text{CTX-M-1}}$ en $bla_{\text{CTX-M-15}}$ lagen voornamelijk op verschillende varianten van IncI1 of IncF plasmiden. $bla_{\text{CTX-M-14}}$ lag voornamelijk op IncK of IncI1 plasmiden.

In een longitudinale studie, beschreven in **hoofdstuk 6**, zijn fecesmonsters van individuele dieren op drie verschillende bedrijven gescreend op dragerschap van *E. coli* met verminderde gevoeligheid voor cefotaxim. Op de drie bedrijven zijn alle dieren binnen een gesloten bedrijfscompartiment bemonsterd bij binnenkomst op het bedrijf, en na 3, 6, 8 en 10 weken (T0 t/m T10). Op T0 was 18% tot 26% van de dieren positief voor *E. coli* met een verminderde gevoeligheid voor cefotaxim. Op T0 vertoonden alle bedrijven een vergelijkbare verdeling

van voornamelijk bla_{CTX-M}-genen. Na T0 zijn op bedrijf 1 geen verminderd gevoelige *E. coli* meer gevonden. Op bedrijf 2 en 3 is een klonale verspreiding waargenomen van *E. coli* ST57 met daarin $bla_{CTX-M-14}$ op een IncF plasmide (bedrijf 2 + 3) en een *E. coli* ST10 met daarin $bla_{CTX-M-14}$ op een IncF plasmide (bedrijf 3). Deze varianten werden niet gevonden op T0. Op alle bedrijven daalde de prevalentie naar (bijna) 0%, terwijl er door een koppelbehandeling met antibiotica nog wel een selectiedruk was.

De data, weergegeven in dit proefschrift, laten zien dat chinolonenresistentie in vleeskalveren voornamelijk wordt veroorzaakt door mutaties in de topoisomerase type II en IV genen; *gyrA*, *parC* en *parE*. Plasmide gemedieerde chinolonenresistentie kwam op zeer lage schaal voor, wat betekent dat horizontale overdracht van resistentiegenen waarschijnlijk maar beperkt zal voorkomen. De prevalentie en diversiteit in resistentie tegen ESC nam toe tussen 1997 en 2010. De meerderheid van ESC resistentiegenen was plasmide gemedieerd en behoorde tot de bla_{CTX-M} genfamilie. Zowel de variatie in prevalentie binnen koppels, als de prevalentie op bedrijven gevolgd in de tijd, varieerde sterk. Het identificeren van factoren die bijdragen aan deze dynamiek vereist vervolgonderzoek. De verschillende resistentiedeterminanten beschreven in dit proefschrift vertoonden genetische overeenkomsten op basis van genen, plasmiden en *E. coli* sequentie typen zoals beschreven voor humane isolaten. Echter, ondanks deze overeenkomsten kunnen op basis van de in dit proefschrift beschreven data geen conclusies worden getrokken over een potentiële transmissie van antimicrobiële resistente *E. coli* naar de mens.

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>>

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Curriculum Vitae

Joost Hordijk was born on 28 October 1979, in Warnsveld, the Netherlands. In 1999 he finished high school (VWO) at the Isendoorn College in Warnsveld and started his study Biology at Utrecht University. After an internship in toxicology at the Institute for Risk Assessment Sciences (IRAS) and an internship in cell biology at the Netherlands Cancer Institute (NKI/AVL) he received his doctorandus degree in 2005.

In 2006, Joost started working at the Research and Development department of BioMerieux, the Netherlands. At BioMerieux he worked on a quantitative PCR for determination of viral load of HIV. In 2007 he started at the National Institute for Public Health and Environment (RIVM). At RIVM he worked on a research project on methicillin resistant *Staphylococcus aureus* (MRSA) in pigs.

Joost started his PhD project on molecular characterization of antimicrobial resistance in veal calves, described in this thesis, in 2008. In this period he was connected to both the Faculty of Veterinary Medicine in Utrecht and the Central Veterinary Institute, part of Wageningen UR in Lelystad. Currently he is continuing his research on molecular characterization of antimicrobial resistance in animals as post doc at the Faculty of Veterinary Medicine of Utrecht University.

List of publications

Related to this thesis:

Hordijk J, Veldman K, Dierikx C, Van Essen-Zandbergen A, Wagenaar JA, Mevius D; Prevalence and characteristics of quinolone resistance in *Escherichia coli* in veal calves; *Vet Microbiol.* 2012 Apr 23;156(1-2):136-42

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Sanderson JT, Hordijk J, Denison MS, Springsteel MF, Nantz MH, van den Berg M; Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells; *Toxicol Sci.* 2004 Nov;82(1):70-9