# Beta-lactamases in *Enterobacteriaceae* in broilers

**Cindy Maria Dierikx**

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# Beta-lactamases in *Enterobacteriaceae* in broilers

Beta-lactamasen in *Enterobacteriaceae* in vleeskuikens

*(met een samenvatting in het Nederlands)*

**Proefschrift**

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door

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geboren op 19 juli 1974 te Hoogerheide

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# Chapter 1

## General introduction

## 1.1 Background

### *1.1.1 History of antibiotic usage and resistance development in bacterial isolates derived from food-producing animals*

Already at the beginning of the use of antimicrobials in animals it became clear that these compounds not only had benefits for the health of animals, but they also seemed to enhance the growth of the animals. The first discovery of these growth promoting effects of antimicrobials was made after feeding dried mycelia *of Streptomyces aureofaciens* containing chlortetracycline residues to animals[1]. The growth promoting effect was not always observed as a direct effect, i.e. it was discovered that when a few chickens in a flock were treated with antibiotics the growth response of the treated chickens became less, but the overall growth performances increased[2, 3]. Procaine penicillin and tetracyclines fed to chickens resulted in an increase in growth of 8.5-8.8% and 10.2-12.3% respectively[4].

Although there is still debate with respect to the exact effect of antibiotic treatment on animals and the growth promotion observed[5, 6], it was clear that antibiotics had a beneficial effect. This knowledge gave rise to the use of antibiotics for growth promotion instead of the use solely for therapeutic treatment.

It was not till the early seventies that the first European regulations were issued concerning the use of antibiotics in animals in respect to the antibiotics used in humans. These regulations were developed after the recommendations of the Swann Report (1969). In this report, written by the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine chaired by Professor M Swann, it was stated that sub-therapeutical usage of antibiotics in food animal production could lead to strains resistant to antibiotics and thereby posing a hazard to human and animal health. For the use of feed-additives in animal production, the report recommended not to use antibacterial compounds which can lead to the development of resistance in bacteria to antibiotics that are therapeutically used in humans or animals. The report specifically named the following antibiotics as unsuitable for growth promotion: chlortetracycline, oxytetracycline, penicillin, tylosin and the sulphonamides.

In the late '70s publications on the transfer of resistant bacteria from animal to man by direct contact were already published[7]. However until in 1980 the animal-to-human spread of a resistant *Salmonella Heidelberg* strain was described[8] no important consequences for human health had been observed. Later, the discovery of vancomycin resistant enterococci (VRE) made everybody aware that antibiotic usage and resistance development in animal strains cannot be without serious consequences for human health[9]. For the first time a causal relation between the use of avoparcin in pigs and poultry and the occurrence of VRE in these animals was established[10], which led to a ban on the use of avoparcin as growth promoter in 1997 and an EU-wide ban on the use of four other growth promoters (spiramycin, tylosin, zink-bacitracin and virginiamycin)[11]. At that time, other growth promoters, like monensin, salinomycin, avilamycin and flavomycin were still allowed, until 2006 when all use of growth promoters was finally banned in the European Union.

VRE was followed by the discovery in 2004 of a Methicillin Resistant *Staphylococcus aureus* (MRSA) in pigs, that was transferred from pigs to men[12]. This strain differed from other MRSA strains found in humans that it showed resistance to digestion with restriction-endonuclease *Sma*I, which was at that time the standard enzyme used in Pulsed-Field Gel Electrophoresis to type MRSA strains. The discovery of this Live-stock associated MRSA (LA-MRSA) made clear that transfer of these bacteria to humans can lead to infections in humans.

MRSA infections are associated with impaired treatment, higher mortality and morbidity, and they also increase the costs of treatment of patients in hospitals as due to a prolonged stay in hospital, necessary isolation of patients and more expensive treatments[13].

This resulted in further debate on the use of antibiotics in animals, a debate that is still going on. In 2005 the first list of critically important antibiotics for human health was made by a subcommittee of the World Health Organization (WHO)[14]. In this list all bacterial compounds used in human medicine are categorized in three groups: important, highly important and critically important. This list can be used by policy makers as guidance for antimicrobial use in food production animals and to preserve the effectiveness of antibiotics for human medicine. In the second revision in 2009 quinolones, 3th and 4$^{th}$ generation cephalosporins and macrolides are among the top 3 of critically important antibiotics and therefore preferably not used in animal production. However all these three classes are important drugs to treat animal infections, therefore until now they are still used in animal production, although stricter rules will be implemented to reduce the use of these three antimicrobial classes in animals.

### *1.1.2 Development of antibiotic resistance*

According to the World Health Organization: "Antimicrobial resistance (AMR) is defined as resistance of a microorganism to an antimicrobial medicine for which it was previously sensitive. Resistant organisms (they include bacteria, viruses and some parasites) are able to withstand attack by antimicrobial medicines, such as antibiotics, antivirals, and antimalarials, so that standard treatments become ineffective and infections persist and may spread to others. AMR is a consequence of the use, particularly the misuse, of antimicrobial medicines and develops when a microorganism mutates or acquires a resistance gene."[15]. For bacteria this means that antibiotic resistance is the resistance of bacteria to an antibiotic to which it was previously sensitive. Bacteria can obtain resistance through mutations, or by the uptake of foreign DNA. Uptake of foreign DNA is possible by transformation, transduction or conjugation (Figure 1.1). Transformation is the passive uptake of free DNA derived from other bacteria that have died in the vicinity. Transduction is the uptake of DNA with help of bacteriophages that inject their DNA into the bacterial cell and conjugation is the active uptake of DNA fragments (plasmids) which contain different accessory bacterial genes. (Figure 1.1)

Antibiotic usage can select for resistant bacteria. All susceptible bacteria will die or are inhibited and the resistant ones will survive. Prescribers of antibiotics should be aware that antibiotics act not solely at the area in which the infection is situated, but also in other parts of the body. Monitoring programs for antibiotic resistance in food-producing animals use this principle by sampling the gut flora and determine the susceptibility of sentinel or indicator organisms which are representatives of Gram-positive and Gram-negative bacteria in the gut flora [16, 17]. In these indicator organisms the occurrence and trends in resistance development over time in the different food-producing animals can be demonstrated. There are many classes of antibiotics, for the scope of this thesis the focus will be on the beta-lactam class of antibiotics.

### *1.1.3 Beta-lactam antibiotics*

Beta-lactam antibiotics are widely used drugs in human and animal medicine. They are used to treat infections of Gram-positive and Gram-negative bacteria. Beta-lactam antibiotics are named after their structure, the beta-lactam ring, which forms the center and active part of the

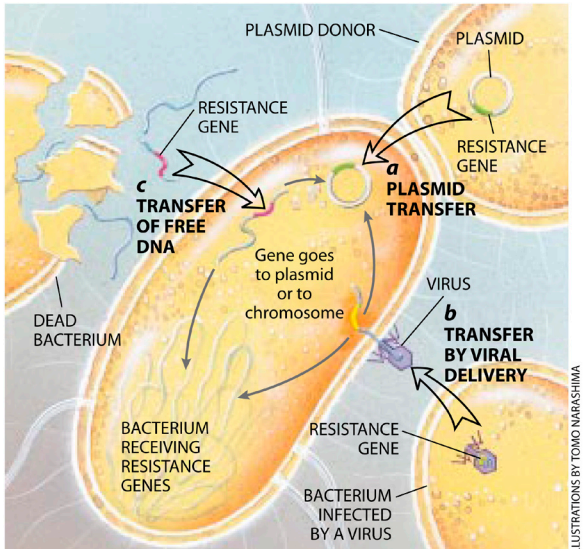



***Figure 1.1*** *Horizontal gene transfer by a. conjugation, b. transduction or c. transformation.*

drug. One can divide this large class of antibiotics in different groups based on their mode of action: penicillins, cephalosporins (1st, 2nd, 3rd, 4th generation), carbapenems, penems, monobactams and beta-lactamase inhibitors like clavulanic acid. This last group has no antibacterial activity on its own but inhibits the activity of beta-lactam degrading enzymes (beta-lactamases) and is often given in combination with other beta-lactam antibiotics.

The most famous and oldest beta-lactam antibiotic is penicillin. In 1928 Alexander Fleming, a microbiologist, discovered after unintended longer incubation of a Gram-positive culture that a fungus had grown on this bacterial plate. Around this fungus, the bacteria did not grow. He investigated the fungus and the compound it produced and made it known to the world[18]. He received the Nobel Prize for discovering penicillin. Many years passed between the discovery of *Penicillum notatum* (the fungus that produces penicillin) and the use of the drug penicillin as a medicine. It took until the second world war that penicillin could be isolated (thanks to Ernst Boris Chain and Howard Walter Florey, who received the Nobel prize together with Alexander Fleming in 1945) and produced to be used to treat the infected wounds of the soldiers[19]. This first beta-lactam antibiotic acted mainly against Gram-positive bacteria, like *Staphylococcus* spp. and *Streptococcus* spp., but showed no activity against infections with Gram-negative bacteria like *Salmonella* or *Escherichia coli (E. coli)*.

Penicillin and all later discovered beta-lactam antibiotics, interfere with the last phase of cell-wall synthesis[20]. The cell wall consists of polymeric chains built of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). These polymers have short peptide side-chains, which can be cross linked by alanine, D-glutamine or lysine with the help of Penicillin Binding Proteins (PBPs). The beta-lactam antibiotic binds to the active site of the PBPs, which will prevent the cross-linking of the cell wall (Figure 1.2).

There are several penicillin binding proteins which vary with type and species of the bacteria. Differences in affinities of individual beta-lactam agents for certain PBPs determine differences in activity found related to various cephalosporins. This partly explains the difference in affinity of some compounds for Gram-positive and Gram-negative bacteria. Generally the first discovered beta-lactam antibiotics (penicillins and first generation cephalosporins) are more active against Gram-positives than against Gram-negatives. Later generations of these drugs have a broader activity against Gram-negatives (2nd generation cephalosporins) as well as against both Gram-negative and Gram-positive bacteria (3rd generation cephalosporins, or extended spectrum cephalosporins (ESC))[20].

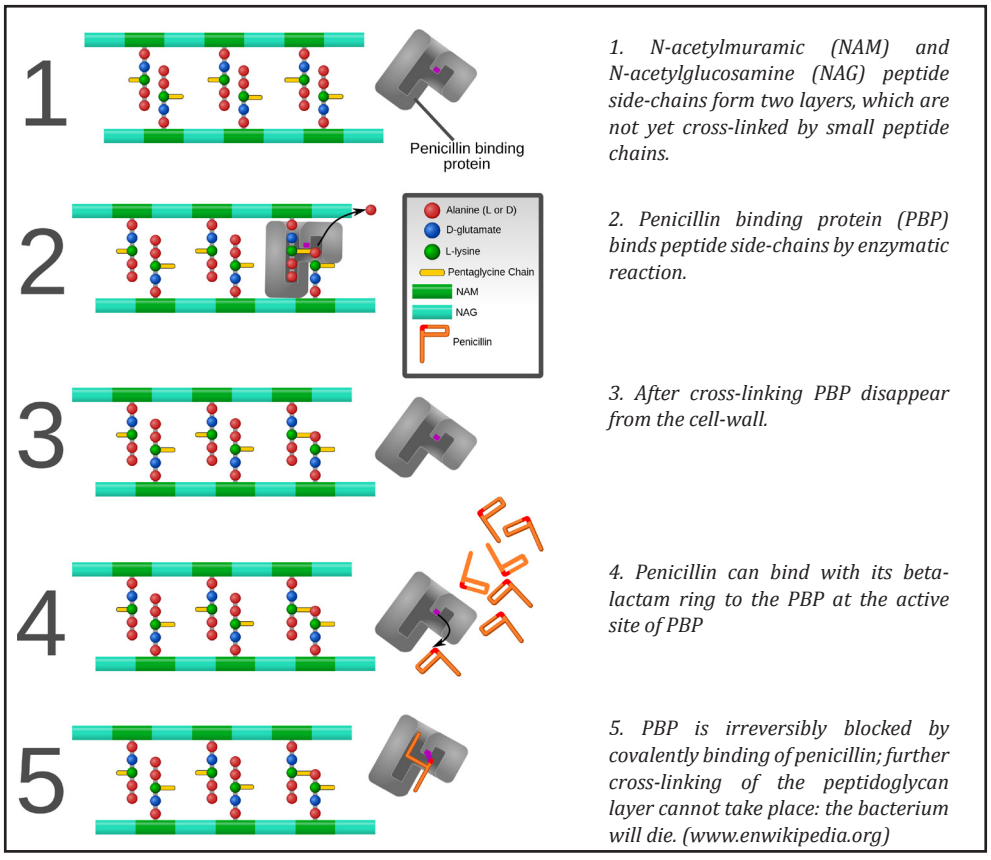



***Figure 1.2*** *Crosslinking of peptidoglycan layer and interference by penicillin.*

### *1.1.4 Resistance to beta-lactam antibiotics*

Bacteria can use one or more of four basic mechanisms for being resistant to beta-lactam antibiotics: 1) alteration of PBPs in such a way that the beta-lactam ring has less affinity to the PBPs. 2) having a cell wall that will not allow the small antibiotic molecules to penetrate the cell. 3) active efflux of the antibiotic by the cell. 4) production of enzymes that inactivate the beta-lactam antibiotic (beta-lactamases)[21].

The first mechanism is successfully adapted by Gram-positive bacteria and some Gram-negative bacteria and mostly described to occur in *Staphylococcus aureus*. If PBP2 is altered into PBP2a all beta-lactam antibiotics are unable to bind and interfere with cell wall synthesis. This PBP-alteration is characteristic for Methicillin Resistant *Staphylococcus aureus* (MRSA). This mechanism is mediated by the MecA gene which encodes for the PBP2a enzyme[21, 22].

The second resistance mechanism refers to the membrane envelop, located outside the peptidoglycan layer in Gram-negative bacteria. This serves as a barrier to certain beta-lactam antibiotics. This cell envelop consists of lipopolysaccharides and contains so called 'porins' (water-filled open channels). The antibiotic can enter the cell by diffusion through these porins, which have selective filters concerning the charge and size of the solutions, which can make a

bacterium by itself less sensitive to certain antibiotics. In addition, alterations in porin structure, or loss of porins, can lead to less influx of the antibiotic or even a blockage of the channel, leaving every hydrophilic compound outside the cell[23, 24].

The third resistance mechanism, an active efflux of beta lactam antibiotics due to the activation of multi drug efflux pumps, has been observed in for example *Klebsiella spp.*[25] and *Pseudomonas aeruginosa*[26]. Due to efflux pumps the antibiotic will immediately be actively removed from the cell when it enters through the cell membrane.

The fourth resistance mechanism, the production of degrading enzymes, is the most important resistance mechanism to beta-lactam antibiotics in Gram-negative bacteria. These enzymes, the beta lactamases, are able to hydrolyze the active site of the antibiotic, the beta-lactam ring[27]. Beta lactamases can be produced by Gram-positive as well as Gram-negative bacteria. However the broad-spectrum and extended-spectrum beta-lactamases (ESBLs) are mainly produced by Gram-negative bacteria. The genes encoding this resistance mechanism in *Enterobacteriaceae*, are mostly located on plasmids[28].

### *1.1.5 Plasmids*

Plasmids are extrachromosomal pieces of DNA which are non-essential for the growth of the bacteria in which it is located[29]. They are self-replicating circular double stranded DNA that can reside in the host cell. Although they are able to contain a lot of different genes of benefit to the host cell, they are clinically important if they contain virulence or resistance genes. Because nowadays many beta-lactamase genes are located on plasmids, they are very important in the epidemiology and spread of resistance to beta-lactam antibiotics.

Plasmids can be horizontally transferred from one bacterial host to another even between bacterial species[28]. Classification systems for plasmids appeared at the end of the 1950s and the one that is mostly used for plasmids in *Enterobacteriaceae* is based on plasmid incompatibility[30]. This means the inability of two plasmids of the same incompatibility group to reside in one cell. This incompatibility is due to the sharing of one or more elements of the plasmid replication or partitioning system[31]. Initially the identification of incompatibility groups was done by conjugation experiments. Nowadays a commonly used PCR based system has been developed targeting genes specific for different plasmids[32, 33]. This way plasmids can be divided into plasmid families, which themselves can be subdivided in sequence types by Multi Locus Sequence Typing, Double Locus Sequence Typing or Replicon Sequence Typing which has now been described for IncI1, IncN, IncHI2 and IncF plasmids[34-37]. These methods have led to an increase in publications dealing with the characterization of beta-lactamase carrying plasmids, which led to more knowledge about the epidemiology and spread of these resistant traits.

### *1.1.6 Beta-lactamases produced by* Enterobacteriaceae

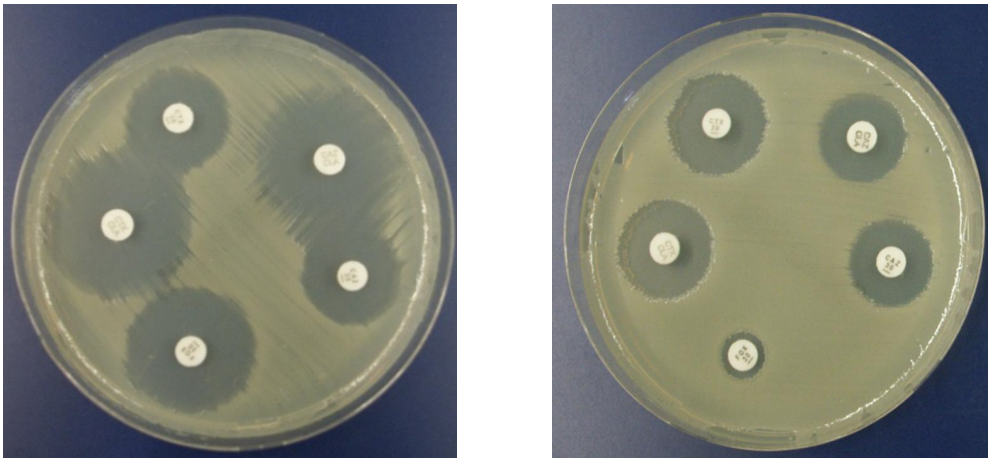
Beta-lactamases can be divided into different groups and many classification systems exist. Two of these classification systems are most widely used: that from Bush, Jacoby and Medeiros based on functional characteristics of the enzymes in relation to their molecular structure[38, 39] and the older one by Ambler[40-43] based on the structure of the beta-lactamases. Four years ago a proposal for a more simplified classification system based on clinical perspectives was made[44]. Beta-lactamases hydrolyse either small, broad or extended spectrum beta-lactam antibiotics. The discovery of the beta-lactamases has followed the development of small-, broad-

and extended spectrum beta-lactam antibiotics (or extended spectrum cephalosporins (ESC)). The two most important groups of enzymes conferring resistance to ESC are the Extended Spectrum Beta-Lactamases (ESBLs) (Bush-Jacoby-Medeiros class 2be, Ambler class A/D, Giske class $ESBL_A$) and plasmid mediated AmpC beta-lactamases (Bush-Jacob-Medeiros class 1, Ambler class C, Giske class $ESBL_{M-C}$). The focus in this thesis will be on ESBL and AmpC beta-lactamases. There are many excellent reviews describing these beta-lactamases[24, 45-49] therefore, only a short description of these two groups will be given.

- Extended Spectrum Beta-Lactamases (ESBLs)

ESBL-producing bacteria hydrolyse penicillins, cephalosporins including 3rd and 4th generation cephalosporin and monobactams. They can be inhibited by beta-lactamase inhibitors, like clavulanic acid and are not resistant to cephamycins, like cefoxitin or carbapenems (Figure 1.3A). The genes encoding these resistance mechanisms can be located on the chromosome, but are mostly located on mobile genetic elements (plasmids). They are classified in different families, like TEM, SHV, CTX-M, OXA, PER and VEB which can be found at www.lahey.org/studies. The first ESBLs found were derived from genes encoding broad-spectrum beta-lactamases like TEM-1 and SHV-1, which were already discovered in the early 1960s[50]. Due to mutations in the gene leading to amino acid substitutions in the enzymes the affinity of the beta-lactamase expanded from only penicillins and broad spectrum cephalosporins to the extended spectrum cephalosporins[46]. There are now more than 200 TEM-variants and 160 SHV-variants, most of them encoding for resistance to ESC and new variants are still appearing (www.lahey.org/studies).

Since the 1980s variants of a new ESBL family, the CTX-M-family was discovered[51,52]. Nowadays the CTX-M-family is the most reported family with respect to ESC resistance in *Enterobacteriaceae*[49]. More than 130 CTX-M variants are already described (www.lahey.org/studies). Based on sequence similarity the CTX-M family can be divided into five major groups: CTX-M-1-group, CTX-M-2-group, CTX-M-8-group, CTX-M-9 group and the CTX-M-25 group[45].



A B

***Figure 1.3*** *These images show the result of the combination disk diffusion test for two* E. coli *strains. The disks at both Mueller Hinton agar plates contain (starting above in clockwise direction) cefotaxime, ceftazidime with clavulanic acid, ceftazidime, cefoxitin and cefotaxime with clavulanic acid. Figure 3A shows the ESBL-phenotype, which displays inhibition of resistance by clavulanic acid (inhibition zones of the cephalosporins without clavulanic acid are ≥5 mm smaller than the inhibition zones around the disks with both clavulanic acid and the cephalosporin) and the inhibition zone around the cefoxitin disk is > 17 mm. Figure 3B shows the AmpC-phenotype, which displays no inhibition of resistance by clavulanic acid (inhibition zones round the cephalosporin disks with and without clavulanic acid are similar) and the inhibition zone around the cefoxitin disk is ≤ 17 mm.*

- AmpC beta lactamases

The first *ampC* beta-lactamase was detected in 1940, although at the time it was called penicillinase. It was an enzyme that destroyed penicillin, produced by *Escherichia coli*[53]. Later it was found that the *ampC*-gene in *E. coli* was the structural gene encoding for this enzyme[54]. Also other members of the *Enterobacteriaceae* family can harbour a chromosomally located *ampC*-gene. Resistance to ESC occurs when mutations in the promoter or attenuator of the gene result in overproduction of the enzyme. Nowadays, bacterial species which do not have the chromosomal *ampC*-gene also can become AmpC producers by the uptake of plasmid mediated *ampC*-genes (for example in *Salmonella*). AmpC-producing bacteria have a broader spectrum of resistance than ESBL-producing bacteria. Next to the hydrolysation of penicillins, broad spectrum cephalosporins and Extended spectrum cephalosporins of the 3rd generation cephalosporins (but not the 4th generation of cephalosporins), they can also hydrolyze cephamycins and monobactams and they are not inhibited by beta-lactamase inhibitors, like clavulanic acid (Figure 1.3B). Like ESBLs many different families of plasmid-mediated AmpC beta-lactamases have been described[48] www.lahey.org/studies), like CMY, ACC, ACT, DHA, FOX, MIR and MOX. The CMY-2 gene belonging to the CMY-family is the most common plasmid mediated *ampC*-gene found worldwide[47].

### *1.1.7 ESBL/AmpC producing* E. coli *in broilers*

Commensal *E. coli* isolates are used as indicator organisms to monitor non-wild type susceptibilities in Gram-negative bacteria in food-producing animals[17]. Prevalence of non-wild type susceptibility to the 3rd generation cephalosporin cefotaxime in commensal *E. coli* found in food-producing animals as a result of the Dutch monitoring program on antimicrobial resistance in food-producing animals is shown in Figure 1.4[16]. ESBL/AmpC producing *E. coli* are most predominant in broilers[16]. This is also seen in other European countries, although the prevalence found in most countries is lower than found in the Netherlands as shown in Figure 1.5 for non-wild type susceptibility to cefotaxime in commensal *E. coli* derived from *Gallus gallus* in different European countries[55].

The data from these monitoring programs are based on non-selective growth of indicator *E. coli* isolates, as commensal organisms from the gastro-intestinal tract. Most (but not all) research papers describing research concerning ESBL/AmpC producing isolates are based on selective methods to obtain ESBL/AmpC producing *E. coli*. The data of these research papers are summarized in excellent reviews[27, 56-58]. As broilers play an important role as reservoir of isolates displaying Extended Spectrum Cephalosporin resistance mechanisms[59] a summary of the literature dealing with ESBL/AmpC enzymes in isolates of broilers will be discussed. Publications about prevalence and characterization of ESBL/AmpC gene-carrying isolates in broiler samples are still appearing, but are sometimes difficult to compare due to differences in selection methods. The publications can be divided by source of the samples (broiler meat at supermarkets, carcases at slaughterhouses, faecal samples taken at slaughterhouses or at farms, pooled samples and individual samples) or by the method that was used (pre-enrichment versus no pre-enrichment, selective pre-enrichment versus non-selective pre-enrichment, selective agar plates versus non-selective plates). In addition, the cephalosporins used to select for ESBL/AmpCs can differ in the type of cephalosporin (cefalozin, cefotaxime, ceftazidime, ceftriaxone, ceftiofur, cefpodoxime, cefoxitin, or commercial plates with unknown selecting cephalosporins) or the concentration used (between 0.25 mg/L and 64 mg/L).

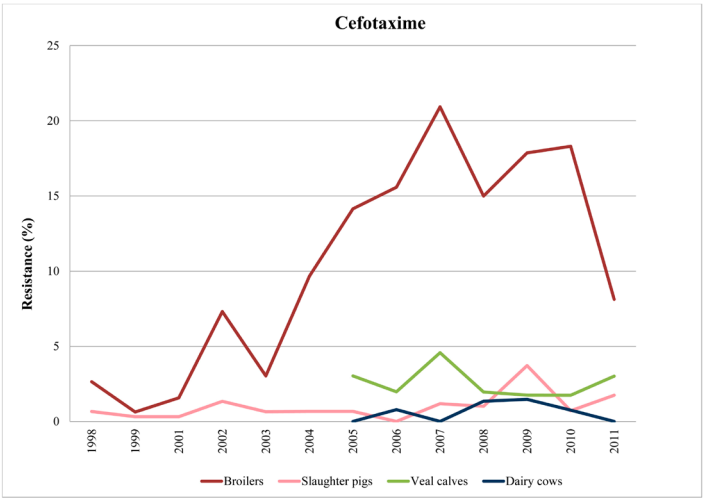



***Figure 1.4*** *Trends in cefotaxime non-wild-type susceptibility in* E. coli *from Dutch broilers, slaughter pigs, veal calves and dairy cows from 1998-2011*[16].

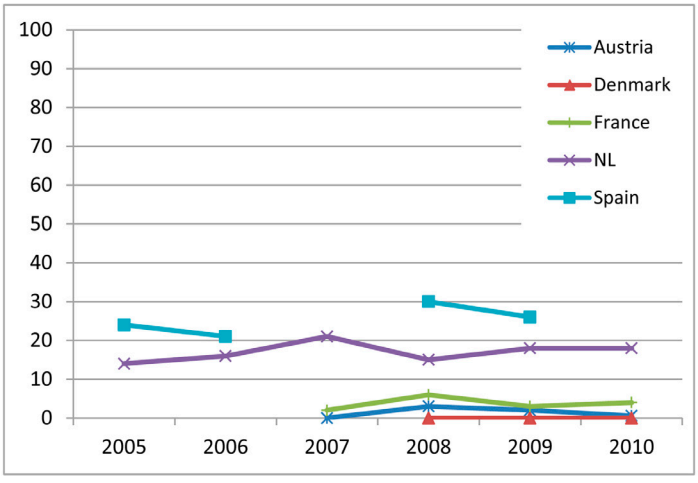



***Figure 1.5*** *Percentage of cephalosporin non-wild type susceptibility in commensal* E. coli *isolates obtained from* Gallus gallus *species in different European countries*[55].

Using selective methods, the prevalence of ESBL/AmpC producing isolates in fresh broiler meat is found to be low (3% in Danish meat (no-enrichment, selective agar containing 1 mg/L ceftriaxone), relatively high (44% in Germany (non-selective enrichment, selective commercial ESBL chromagar plate), 35-50% in Tunisia (non-selective enrichment, selective agar containing 2 mg/L cefotaxime or no-enrichment, selective agar containing 2 mg/L cefotaxime respectively) to very high (80-94%, 84-93% respectively in the Netherlands (non-selective enrichment followed by selective enrichment with vancomycin 8 mg/L and cefotaxime 0.25 mg/L or by selective agar containing 2 mg/L cefotaxime respectively) and Spain (non-selective enrichment followed by ChromID ESBL-plate containing cefpodoxime or followed by selective agar containing 1 mg/L cefotaxime or ceftazidime respectively)[60-65]. ESBL/AmpC prevalence in broiler chickens at farms using selective methods varies between 10%-100% per farm (no-enrichment, sample inoculated on commercial chromagar-CTX [66] and 27-75% per farm (no enrichment, selective agar containing 8 mg/L ceftiofur[59].

***Table 1.1*** *ESBL/AmpC genes in* E. coli *derived from faecal samples of broilers/poultry found worldwide*

| Continent | Country | Year of isolation | ESBL enzymes | pAmpC enzymes | References |
|---|---|---|---|---|---|
| Asia | China | 2000-2010 | CTX-M-3, -14, -15, -24, -27, -55, -64, -65, -102, -104, TEM -52, SHV-12 | CMY-2 | *78, 79, 82, 87* |
| | Japan | 1999-2002, 2004-2006, 2007 | CTX-M-2, -14, -15, -18, -44, SHV-2, -12 | CMY-2 | *63, 76, 77* |
| | Taiwan | 2002 | | CMY-2 | *86* |
| Europe | Spain | 2000-2001, 2003 | CTX-M-1, -9, -14, -32, TEM-52, SHV-2, -5, -12 | CMY-2 | *66, 69, 70* |
| | Portugal | 2004, 2005 | CTX-M-14, -32, TEM-52 | | *71, 81* |
| | France | 2005 | CTX-M-1 | | *75* |
| | Italy | 2007 | CTX-M-1, -32, SHV-12 | | *68* |
| | Belgium | 2007 | CTX-M-1, -2, -14, -15, TEM-52, -106 | CMY-2 | *59* |
| | Czech Republic | 2008 | CTX-M-14 | CMY-2 | *80* |
| | Denmark | 2010 | SHV-2 | CMY-2 | *72, 73* |
| | Sweden | 2010,2011 | CTX-M-1 | CMY-2 | *84, 85* |
| | UK | 2010 | CTX-M-1, -3, -15, TEM-52 | | *83* |
| | Switzerland | 2009 | CTX-M-1, TEM-52, SHV-12 | | *74* |
| Africa | Tunisia | 2011 | CTX-M-1 | CMY-2 | *88* |

Most publications on ESBL/AmpC enzymes in isolates of broilers deal with the occurrence in *E. coli* isolates but to a lesser extent also in *Salmonella* isolates[67]. There are many different ESBL-genes described that occur in *E. coli* derived from broilers and broiler meat. These publications are summarized and categorized by country in Table 1.1 and 1.2. From these data we can conclude that some genes are ubiquitous and widespread among poultry meat in Europe, Africa and North America, like $bla_{\text{CTX-M-1}}$, $\text{bla}_{\text{TEM-52,}}$ $\text{bla}_{\text{SHV-12}}$ and $bla_{\text{CMY-2}}$. Other genes are more restricted to certain geographical areas, like $bla_{\text{CTX-M-2}}$ and CTX-M- genes belonging to CTX-M-group 8, which are mainly found in isolates from broilers in South-American countries and $bla_{\text{CTX-M-24, -27, -44, -55, -64, -102, -104}}$ which are strictly found in Asia. Interestingly, studies characterizing ESBL/AmpC genes in isolates derived from poultry meat display much less diversity in the ESBL/AmpC genes found (Table 1.2) than studies in which isolates are derived from faecal samples of broilers (Table 1.1).

***Table 1.2*** *ESBL/AmpC genes in E. coli derived from meat samples of broilers/poultry found worldwide*

| Continent | Country | Year of isolation | ESBL enzymes | pAmpC enzymes | References |
|---|---|---|---|---|---|
| Asia | Japan | 2004-2006 | | CMY-2 | [63] |
| | Taiwan | 2002 | | CMY-2 | [86] |
| Europe | Denmark | 2006, 2009-2011 | CTX-M-1 | CMY-2 | [60, 72, 73, 90] |
| | Imported meat in Denmark from other European countries | 2009, 2010, 2011 | CTX-M-1, -2, TEM-20, -52, SHV-2, -12 | CMY-2 | [60, 72, 73] |
| | France | 2006 | CTX-M-1, TEM-52 | | [90] |
| | Germany | 2011 | CTX-M-1, -2, -65, TEM-52, SHV-2, -12 | | [64] |
| | Portugal | 2003, 2005 | CTX-M-1, TEM-52, SHV-2 | | [81] |
| | Denmark/Estland/ Finland/France/ Croatia/Letland/ The Netherlands/ Poland/Germany imported into Sweden | 2009-2011 | CTX-M-1, -2. -25. TE,-19. -52, SHV-12 | CMY-2 | [95] |
| | Sweden | 2010 | CTX-M-1 | CMY-2 | [95] |
| | the Netherlands | 2010 | CTX-M-1, -2, -14, -15, TEM-20, -52, SHV-2, -12 | CMY-2 | [91,94] |
| | Spain | 2010 | CTX-M-1, -32, SHV-12 | | [62] |
| Africa | Tunisia | 2006,2007 | CTX-M-1, -8, -14, SHV-5 | CMY-2 | [61, 89] |
| South America | Brazil/Argentina/ Chile imported into UK | 2008 | CTX-M-2-group, CTX-M-8-group | | [92] |
| | Brazil/Argentina/ Chile imported into Sweden | 2009-2011 | CTX-M-2,-8 | CMY-2 | [95] |
| North America | Pittsburgh | 2006-2007 | CTX-M-1-group | CMY-2 | [93] |

*1.1.8 ESBL/AmpC beta-lactamases in human isolates*

ESBL/AmpC-producing *Enterobacteriaceae* occurred mainly in hospital-associated infections in humans but have been found more and more in community-associated infections, like urinary tract infections[96]. Within Europe the prevalence of resistance to ESC in clinical isolates from humans is the highest in southern- and south eastern European countries (http://www.ecde.europe.eu/en/activities/surveillance/EARS-Net/database/Pages/database.aspx, last accessed 13 November 2012). Worldwide the prevalence is higher in Asian and South-American countries than in Europe or North-America[97]. A recent comparison of ESBL/AmpC types found in human isolates from sick and healthy people in Europe, Asia and America showed that $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ are predominant all over the world. Small differences exist between continents with $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-1}}$ being more predominant in Europe compared to Asia and America and $bla_{\text{CMY-2}}$ more in Europe and America compared to Asia[57].

*1.1.9 Plasmids carrying ESBL/AmpC genes*

Many plasmid families are associated with the carriage of a specific ESBL or AmpC gene. The IncI1 family is the most predominant plasmid family associated with ESBL/AmpC-carriage in both human and animal isolates[28]. Many ESBL/AmpC genes are found on this type of plasmids, like $bla_{\text{CMY-2, -7, -21}}$, $bla_{\text{CTX-M-1, -2, -3, -9, -14, -24}}$, $bla_{\text{SHV-12}}$, and $bla_{\textit{TEM}\text{-52}}$[28]. Combinations of ESBL/AmpC genes and plasmids found in poultry isolates are $bla_{\text{CMY-2}}$ on IncA/C and IncI1, $bla_{\text{CTX-M-2}}$ on IncHI2 and IncP, $bla_{\text{CTX-M-1}}$ on IncN [75] and IncI1, $bla_{\text{CTX-M-9}}$ on IncHI2 and $bla_{\text{SHV-12}}$ on IncI1, $bla_{\text{TEM-52}}$ on IncX1[98] and IncI1[28]. Most combinations have also been described in isolates from humans. Some plasmid types are present more often in certain countries than in others. For example IncA/C carrying $bla_{\text{CMY-2}}$ is found as an epidemic plasmid in the US[28], while IncI1 carrying $bla_{\text{CTX-M-1}}$ is dominant in Europe and IncF carrying $bla_{\text{CTX-M-15}}$ is found worldwide. Mostly the spread of ESBL/AmpC genes is mediated by the spread of plasmids and not due to a specific bacterial clone. However this is not true for $bla_{\text{CTX-M-15}}$ located on IncFII plasmids. This combination is often linked to uropathogenic *E. coli* clone O25b-H4-B2-ST131 and found (mainly in humans) worldwide[99]. Other plasmids described to be associated with ESBL/AmpC carriage are ColE (found a.o. in human and rabbit isolates), IncL/M (a.o. in human isolates), other IncF-types and IncB/O (a.o. in human and horse isolates) and IncK (a.o. in human and cattle isolates)[28].

**1.2 The Dutch broiler industry**

To better understand the work that is described in this thesis, the structure of the Dutch broiler industry will be explained. During the past decades broilers have been transformed into highly efficient meat production fabrics in which with very low costs, very fast weight gain and high production can be achieved. Next to improvement of feed and management, genetic selection is one of the most important forces in this development. Five years before the meat of broilers is on the market, the development of those pieces of meat has already started in the genetic selection and development of the pure chicken lines in the Pedigree Stock. The Pedigree stock produces the Great Grand Parent Stock (GPS) from which both new pure lines and Grand Parent stock (GPS) will be produced (Figure 1.6). For the Netherlands, this is where the broiler production chain starts. In the Netherlands, depending on the primary breeding company GPS animals are imported as one-day-old chickens from the UK to the Netherlands or the eggs that

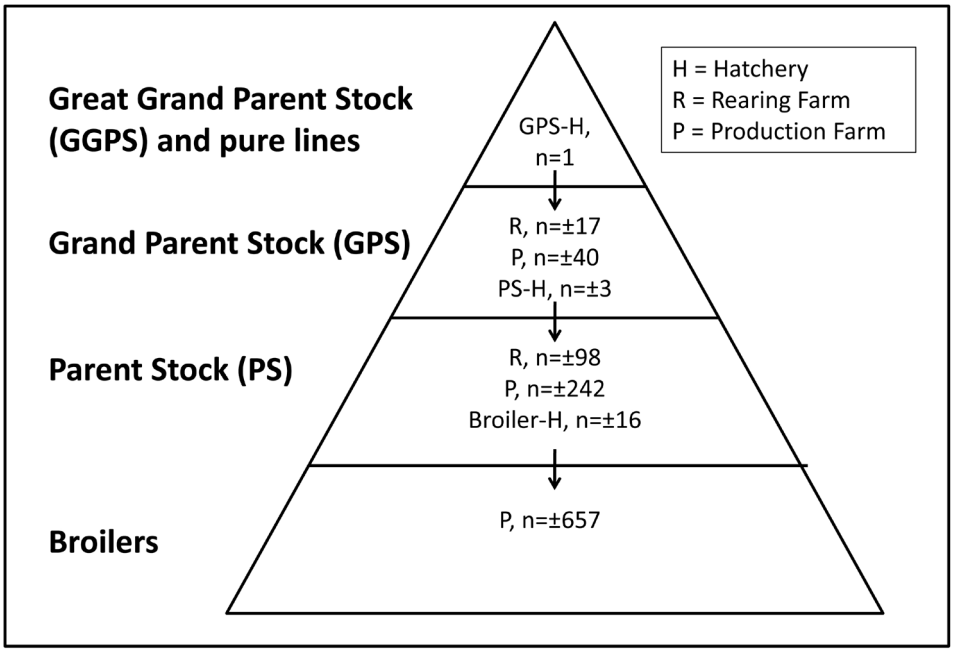



***Figure 1.6*** *The pyramidal structure of the Dutch broiler production chain is shown. Grandparent chickens are either imported as one-day old chickens or breeding eggs from abroad. The number of farms depicted in the figure are average number of farms or hatcheries present in the period 2009-2011 in the Netherlands. Data on the number of farms is derived from the Dutch Flock Information System (KIP database); data on the number of hatcheries are estimated by the primary breeding companies involved in the study described in Chapter 4.*

produce GPS animals are imported from the US to a hatchery in the Netherlands. That means that in the Netherlands there is one GGPS hatchery that produces Grand Parents (Figure 1.6). From the hatchery the chickens go to a rearing farm and after 18-20 weeks, when they are old enough to produce eggs, females and males of different breeding lines are mixed to produce eggs that will result in the Parent Stock (PS). Again a mix takes place of different parent stock lines that will result in the hybrid broiler chicken that produces meat.

At the top of the pyramid high standards of biosecurity are maintained at the farms and the hatcheries. This is because these chickens are expensive and not easy to replace when they get lost by a disease or other tragedy. At the bottom of the pyramid, especially at the broiler production farms biosecurity levels are generally much less strict and differ greatly between farms. However due to the all-in, all-out principle, in which the farms are empty between production rounds it is possible to clean and disinfect the farms thoroughly between rounds. In the Netherlands, this production period lasts for 35- 42 days at conventional farms. At the age of six weeks the broilers go to slaughter. At some farms a part of the chickens are slaughtered at about 35 days of age to give more space to the remaining chickens that stay until 42 days of age. The average slaughter weight is about 2.2 kg[100]. Since 2010, most Dutch broiler farms have more than 75.000 chickens per farm, with an average of around 69.000 animals per farm in 2011. In 2011 around 45 million broilers were produced and 785.000 tons of meat were processed in the Dutch slaughter houses. A large part is exported mainly to Germany and the UK[100].

### 1.3 ESBL research program

In 2008 a project financed by the (at that time called) Ministry of Agriculture, Nature and Food Quality, grant number WOT-01-002-003 started to investigate ESBLS in farm animals. A year later another project on the molecular characterization of ESBLs in farm animals started and was financed by the Dutch Ministry of Economic Affairs, Agriculture and Innovation, grant number WOT-01-002-003.004. This thesis contains the results of both projects.

### 1.4 Aims of this thesis

Since 2003 the results of the Dutch monitoring program on antimicrobial resistance in food-producing animals[101] showed an increase of non-wild type susceptibility to cefotaxime in commensal *E. coli* isolates derived from broilers. The prevalence of non-wild type susceptibility increased from 3% in 2003 to more than 20% in 2007. This increase was not seen in isolates derived from veal calves, slaughter pigs or dairy cows. Non-wild type susceptibility to cefotaxime is indicative for the presence of ESBL/AmpC-producing isolates in broilers. At that time it was known that ESBL/AmpC-producing isolates could cause impaired treatment in human infections and therefore it was undesirable that they were present in food-producing animals, allowing the possible transfer to humans. The finding of these isolates in broilers was regarded as worrisome. Therefore the aims of this thesis were: 1. To investigate the presence and spread of ESBL/AmpC-producing isolates in the broiler production chain, which is described in **Chapter 2, 3 and 4**. 2. To characterize the ESBL/AmpC genes, plasmids and isolates in broilers to investigate the genetic relations to ESBL-producing isolates from broilers to the ones found in humans and companion animals, described in **Chapter 6 and 7**. 3. Characterization studies of ESBL/AmpC genes give valuable information to understand the epidemiology of these strains and can be a valuable tool in outbreak situations. Unfortunately sequencing of the genes is not always feasible and cost-effective; therefore a fast and relatively cheap molecular detection method for ESBLs was developed and evaluated as described in **Chapter 5**.

*1.4.1 Outline of this thesis*

**Chapter 1**. In the general introduction an overview of the antimicrobial usage in livestock production, the development of resistance to antibiotics, most specific to beta-lactam antibiotics and the development and spread of ESBL/AmpC-producing isolates is given.

**Chapter 2.** In this Chapter the characterization of the genes responsible for non-wild type susceptibility to cefotaxime in broiler isolates is described.

**Chapter 3**. In this Chapter the prevalence of ESBL/AmpC-producing isolates at broiler farms and their spread from broilers to broiler farmers is described.

**Chapter 4**. In this Chapter ESBL/AmpC-presence in the whole Dutch production chain and the spread of ESBL-producing *E. coli* at broiler farms is described.

**Chapter 5.** In this Chapter a fast and relatively cheap molecular detection method for ESBL-genes was developed and evaluated.

**Chapter 6**. In this chapter the ESBL genes and plasmids found in broiler isolates are compared to those found in humans.

**Chapter 7.** Broilers are not the only source of ESBL/AmpC producing isolates for humans. Companion animals live in much closer contact with humans. ESBL/AmpC producing isolates and ESBL/AmpC genes in clinical isolates of pets were characterized and described in this Chapter.

**Chapter 8.** In the general discussion the results of the studies described in this thesis are discussed and future perspectives concerning antimicrobial resistance are described.

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# Chapter 2

# Increased detection of extended spectrum beta-lactamse producing *Salmonella enterica* and *Escherichia coli* isolates from poultry

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## 2.1 Abstract

To gain more information on the genetic basis of the rapid increase in the number of isolates exhibiting non-wild type Minimum Inhibitory Concentrations (MICs) for cefotaxime observed since 2003, beta-lactamase genes of 22 *S. enterica* and 22 *E. coli* isolates from broilers in 2006 showing this phenotype were characterized by miniaturized micro-array, PCR and DNA-sequencing. Presence and size of plasmids were determined by S1-digest pulsed-field gel electrophoresis and further characterized by PCR based replicon-typing. Transfer of resistance plasmids was tested by conjugation and transformation experiments. To link resistance genes and plasmid-type, Southern blot hybridization experiments were conducted.

In 42 isolates, five ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{TEM\text{-}20}$, $bla_{TEM\text{-}52}$, $bla_{SHV\text{-}2}$) different Extended Spectrum Beta-Lactamase (ESBL)-genes and two ($bla_{ACC\text{-}1}$, $bla_{CMY\text{-}2}$) AmpC-genes were present. Three of the detected ESBL-genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}52}$ and $bla_{CTX\text{-}M\text{-}2}$) were located on similar types of plasmids (IncI1 and IncHI2/P) in both *E. coli* and *Salmonella*. Two other detected ESBL- and AmpC-genes $bla_{SHV\text{-}2}$ and $bla_{CMY\text{-}2}$ respectively (on IncK plasmids), were only found in *E. coli*, whereas the AmpC-gene $bla_{ACC\text{-}1}$ (on non-typeable plasmids) and the ESBL-gene $bla_{TEM\text{-}20}$ (on IncI1 plasmids), were only detected in *Salmonella*. In two isolates, no ESBL- or AmpC-gene could be detected through these methods.

The increase in the number of *E. coli* and *S. enterica* isolates from the gastro-intestinal tract of broilers exhibiting non-wild type MICs for cefotaxime is mainly due to an increase in IncI1 plasmids containing $bla_{CTX\text{-}M\text{-}1}$. The reason for the successful spread of this plasmid type in these species is not yet understood.

## 2.2 Introduction

A rapid development of resistance to extended-spectrum cephalosporins (ESC) has been observed in *Enterobacteriaceae* worldwide. In these organisms, resistance to this group of antibiotics is predominantly based on plasmid-mediated production of enzymes that inactivate these compounds by hydrolyzing their beta-lactam ring. The most frequently detected groups of these enzymes in bacteria of animal origin are Extended-Spectrum beta-lactamases (ESBL) and AmpC-type beta-lactamases. Both are important causes of treatment failures in humans when they are produced by pathogens.

ESBL as well as AmpC-producing bacteria are frequently present in the gastro-intestinal tract of animals [1, 2] and have been isolated from swine, cattle, turkey [3], cats, dogs [4], poultry [5], wild animals [6] and horses [7]. The gastrointestinal tract of animals is seen as an important reservoir for bacteria that produce beta-lactamases, and a potential source for human pathogens to take up these resistance genes [1, 2, 8]. ESBL- and AmpC-genes are located on plasmids which enable them to spread very rapidly. Therefore plasmid characterization is an essential tool to understand the epidemiology of these genes.

Before 2003, isolates exhibiting non-wild type MICs [9] for ESC in *E. coli* from broilers was observed only incidentally in the Netherlands. This changed after 2003 with the observation of an annual increase in non-wild type MICs for cefotaxime in commensal *E. coli* from Dutch broilers. In 2006, a similar increase was also observed in *S. enterica* from various poultry sources [10].The purpose of the present study was to characterize the beta-lactamase genes and the plasmids harboring these genes in *E. coli* and *S. enterica* from broilers.

## 2.3 Materials and Methods

### *2.3.1 Bacterial isolates and susceptibility testing*

In the Dutch surveillance program on antibiotic resistance in bacteria isolated from animals, more than 500 *Escherichia coli* and 1000 *Salmonella enterica* isolates are tested each year for susceptibility to a panel of antibiotics. The Minimum Inhibitory Concentrations (MICs) of these isolates were determined for ampicillin, cefotaxime, ceftazidime, gentamicin, tetracycline, sulphamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, florfenicol and chloramphenicol by the broth micro dilution method, according to the international standard ISO 20776-1:2006.

In 2006, as part of this surveillance program, 153 *E. coli* isolates were collected throughout the year from caecal samples of healthy broilers at different slaughterhouses in the Netherlands. One *E. coli* isolate randomly picked from a MacConkey agar plate, was derived from one caecal sample taken once daily from one animal per flock. The flocks were evenly distributed over the country. In 2006, also 359 *S. enterica* isolates were obtained from various poultry sources as part of the national *Salmonella* control program in food producing animals. These isolates were sent to the National Institute of Public Health and the Environment in Bilthoven for sero-, and phage typing and to the Central Veterinary Institute in Lelystad to determine the susceptibility to a panel of antibiotics. This collection consisted of one isolate per serovar per source. From these isolates, 22 *E. coli* and 22 *S. enterica* isolates with non-wild type MICs for cefotaxime based on EUCAST epidemiological cut off values (MIC > 0.25 mg/L for *E. coli* and MIC > 0.5 mg/L for *Salmonella,* www.eucast.org) were included in the present study.

### *2.3.2 Beta-lactamase gene identification*

To determine the presence of beta-lactamase gene families in the isolates, a miniaturised microarray (Identibac AMR04, Veterinary Laboratories Agency, Weybridge, UK) was used as described previously [11]. The beta-lactamase gene families that can be detected with this array are: $bla_{SHV}$, $bla_{LEN-1}$, $bla_{TEM-1}$, $bla_{OXA-1}$, $bla_{OXA-2}$, $bla_{OXA-7}$, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$ and $bla_{OXA-9}$. The AmpC gene families that can be detected are: $bla_{DHA}$, $bla_{ACC-1}$, $bla_{ACC-2}$, $bla_{MOX}$, $bla_{CMY}$ and $bla_{FOX}$. All beta-lactamase gene families detected with the array were further characterized by PCR amplification and sequencing on crude bacterial DNA. PCR was performed as described previously[5]. All amplifications were performed in 30 cycles and, except for $bla_{CMY}$, with an annealing temperature of 55 °C. The annealing temperature of the $bla_{CMY}$-amplification was 58 °C. All amplicons were purified using the QiaQuick PCR Purification KIT (Qiagen, The Netherlands). Sequences were obtained using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA) on an Applied Biosystems HITACHI 3130 Genetic Analyzer (Applied Biosystems, UK). Sequence data were analyzed using Sequencer version 4.2 (Gene Codes Corporation, USA). The sequences obtained were compared to those registered in GenBank and found on http://www.lahey.org/studies/. Primers and product sizes used for PCR and sequencing are listed in Table 2.1.

***Table 2.1*** *Targets, primers, sequences and product sizes used for PCR and sequencing of ESBL- and AmpC-genes.*

| Targets | Primer | Nucleotide sequence 5' - 3' | Size (bps) |
|---|---|---|---|
| TEM | TEM-F[20] | GCG GAA CCC CTA TTT G | 964 |
| | TEM-R[20] | ACC AAT GCT TAA TCA GTG AG | |
| CTX-M | CTX-M-F[21] | ATG TGC AGY ACC AGT AAR GTK ATG GC | 592 |
| | CTX-M-R[21] | TGG GTR AAR TAR GTS ACC AGA AYS AGC GG | |
| CTX-M-1 | CTX-M-1-F[22] | GGT TAA AAA ATC ACT GCG TC | 863 |
| | CTX-M-1-R[22] | TTG GTG ACG ATT TTA GCC GC | |
| CTX-M-2 | CTX-M-2a-F[a] | GAT GAG ACC TTC CGT CTG GA | 397 |
| | CTX-M-2a-R[a] | CAG AAA CCG TGG GTT ACG AT | |
| SHV | SHV-F[23] | TTA TCT CCC TGT TAG CCA CC | 795 |
| | SHV-R[23] | GAT TTG CTG ATT TCG CTC GG | |
| CMY-2, CMY-4, CMY-6, CMY-7, CMY-12, CMY-13, CMY-14, CMY-18, LAT-3 | CMY-2-F[23] | ATG ATG AAA AAA TCG TTA TGC TGC | 1117 |
| | CMY-2-R[23] | GCT TTT CAA GAA TGC GCC AGG | |
| ACC-1 | ACC-1-F[5] | AGC CTC AGC AGC CGG TTA C | 818 |
| | ACC-1-R[5] | GAA GCC GTT AGT TGA TCC GG | |

[a] This study

*2.3.3 Conjugation and transformation experiments*

Conjugation experiments were conducted by broth-mating experiments in Luria-Bertani medium (LB-medium) with a rifampicin-resistant, indole-negative *E. coli* K12 strain as recipient. Transconjugants were selected on MacConkey agar containing 1 mg/L cefotaxime and 100 mg/L rifampicin.

For transformation experiments, plasmids were extracted using Qiagen Plasmid Midi

Kits according to the manufacturer's instruction (Qiagen, The Netherlands). The final DNA pellet was resuspended in 200 µl of nuclease free water. Purified plasmids were transformed using ElectroMax™DH10B™ cells (Gibco Invitrogen, USA) according to the manufacturer's instructions. Selection of transformants was performed on MacConkey agar containing 1 mg/L cefotaxime. MICs of transconjugants and transformants were determined as mentioned above.



*2.3.4 Plasmid characterization*

Plasmids were characterized by the PCR-based Replicon Typing method (PBRT) as described previously [12, 13] to detect the plasmid types: IncFIA, IncFIB, IncFIC, IncHI1, IncHI2, IncI1, IncL/M, IncN, IncP, IncW, IncT, IncA/C, IncK, IncB/O, IncX, IncY, IncF, IncFIIA, IncU, IncR and ColE. The isolates were grouped according to species, their plasmid profile and resistance phenotype. From each group, one isolate was further analyzed to prevent repetition of plasmid characterization. This resulted in a selection of 26 isolates of which plasmid sizes were determined using S1-nuclease treatment followed by PFGE as previously described [14, 15]. Agarose plugs were prepared according to the PulseNet Protocol [16] using bacterial suspensions with an absorbance at 610 nm of 1.6 – 1.7. Seakem gold 1 % agarose plugs were digested with 8 units of S1-nuclease (Amersham Biosciences, UK) for 45 minutes at 37°C. Electrophoresis was conducted in a CHEF DRIII system using 6 $Vcm^{-1}$ at 14°C for 17 hours, with pulse times ramping from 1 to 25 s using an angle of 120°. Low range PFGE markers (New England Biolabs, Schwalbach, Germany) were used as molecular markers.

*2.3.5 Hybridization experiments*

To locate the beta-lactamase genes on specific plasmids, isolates were further analyzed by Southern blot hybridization of plasmid DNA using DIG-labeled probes according to the manufacturer's instruction (Roche Diagnostics GmbH, Germany). The hybridized probes were visualized with ECF (Amersham Biosciences, UK) as substrate on the Typhoon trio variable mode Imager (Amersham Biosciences, UK).

**2.4 Results**

*2.4.1 Antibiotic resistance phenotypes*

Of 153 *E. coli* isolates obtained from Dutch poultry in 2006, 22 had non-wild type MICs for cefotaxime. In these 22 isolates, additional non-wild type MICs were found for ceftazidime (100%), sulphamethoxazole (95%), trimethoprim (86%), streptomycin (59%), nalidixic acid (55%), ciprofloxacin (50%), tetracycline (50%), chloramphenicol (32%), gentamicin (18%) and neomycin (14%).

Of 359 *Salmonella* isolates obtained from Dutch poultry in 2006, 12 *S*. Paratyphi B variant (var.) Java, 5 *S*. Infantis, 2 *S*. Braenderup, 1 *S*. Indiana, 1 *S*. Agona and 1 *S*. Saintpaul had non-wild type MICs for cefotaxime. In these 22 isolates additional non-wild type MICs were found for ceftazidime (77%), trimethoprim (76%), ciprofloxacin (38%), nalidixic acid (33%), streptomycin (62%), sulphamethoxazole (67%), tetracycline (29%) and neomycin (5%).

*2.4.2 ESBL and AmpC characterization*

Of the 44 isolates with non-wild type MICs for cefotaxime, 35 (80%) carried an ESBL-gene: $bla_{CTX\text{-}M\text{-}1}$ (11 *E. coli* and 6 *S. enterica*), $bla_{CTX\text{-}M\text{-}2}$ (1 *E. coli* and 2 *S. enterica*), $bla_{TEM\text{-}52}$ (3 *E. coli* and 7 *S. enterica*), $bla_{TEM\text{-}20}$ (4 *S. enterica*) or $bla_{SHV\text{-}2}$ (1 *E. coli*). $Bla_{CTX\text{-}M\text{-}1}$ was most frequently found in *E. coli,* while in *S. enterica* $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ were about equally frequent. Seven isolates (17%) carried an AmpC-gene: $bla_{ACC\text{-}1}$ (2 *S. enterica*) or $bla_{CMY\text{-}2}$ (5 *E. coli*) (Fig. 2.1). The narrow-spectrum beta-lactamase gene $bla_{TEM\text{-}1}$ was present in 13 (8 *E. coli* and 5 *Salmonella)* isolates and the narrow-spectrum beta-lactamase gene $bla_{TEM\text{-}135}$ [17] was present in one (*E. coli*) isolate (data not shown). $Bla_{TEM\text{-}1}$ and $bla_{TEM\text{-}135}$ were always present in combination with other beta-lactamase genes ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CMY\text{-}2}$, $bla_{SHV\text{-}2}$ or $bla_{TEM\text{-}52}$). In two isolates (1 *E. coli* and 1 *S.* Saintpaul) no ESBL- or AmpC–gene could be detected with the methods used, and these two isolates were not further analyzed and excluded from the final selection. All isolates carrying the $bla_{TEM\text{-}20}$-gene, although having a non-wild type MIC (≥ 4 mg/L) for cefotaxime, had a wild-type MIC (2 mg/L) for ceftazidime.

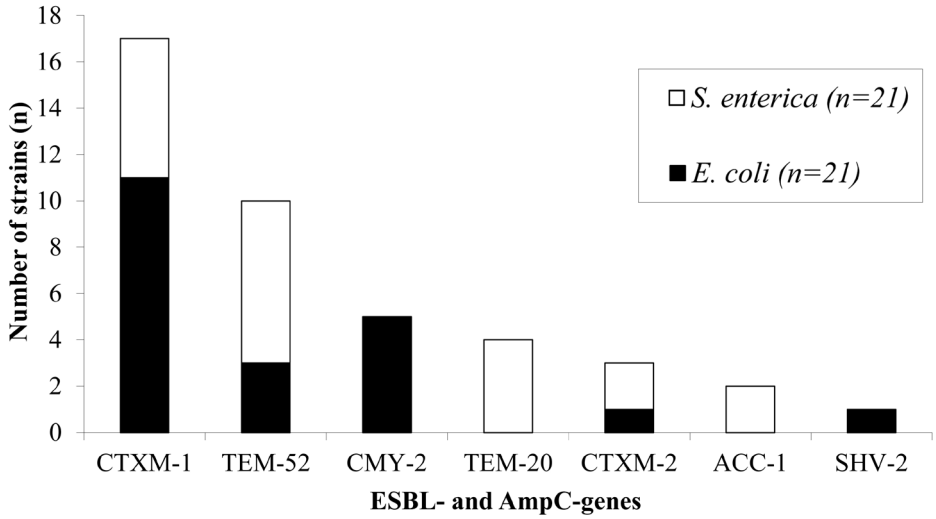


***Figure 2.1*** *ESBL and/or AmpC genes found in* S. enterica *and* E. coli *isolates with non-wild type MICs for cefotaxime, derived from Dutch broilers in 2006.*

*2.4.3 Plasmid characterization*

In all *E. coli* isolates (n=21) multiple replicons (three or more) were detected by PBRT, whereas in 10 *Salmonella* isolates (n=21), only one replicon was detected. IncI1 plasmids were predominantly present in both bacterial species (16 *E. coli*, 18 *S. enterica* isolates). Other plasmids detected in both bacterial species were ColE (n=2), IncHI1 (n=2) and multireplicon plasmid IncHI2/P (n=3). In *E. coli* IncFIB plasmids were very common (n=15), whereas none of the *Salmonella* isolates in this study carried plasmids with this replicon type. An IncN plasmid was found in one *S.* Agona isolate and IncK (n=5), IncFIC (n=2), IncF (n=14) and IncY (n=3) plasmids occurred only in *E. coli* isolates.

Table 2.2 shows the analysis of the plasmids in a subset of the isolates. S1-nuclease PFGE of 10 *E. coli* isolates confirmed that multiple plasmids (three or more) of different size were present in these isolates. In three of the *Salmonella* isolates analyzed by S1-nuclease PFGE (n=16) 3 plasmids of different sizes were found, the other isolates carried one or two plasmids.

Hybridization experiments revealed that plasmids carrying beta-lactamase genes were confined to 3 incompatibility groups: IncI1, IncK and a multireplicon plasmid IncHI2/P (Table 2.2). In 4 isolates, the ESBL-carrying plasmids were non-typeable by PBRT. Of the plasmids carrying ESBL-genes, IncI1 was predominant for both bacterial species; these IncI1 plasmids were found in association with $bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}20}$ and $bla_{TEM\text{-}52}$. Plasmids carrying $bla_{SHV\text{-}2}$ or $bla_{CMY\text{-}2}$ were all typed as IncK and plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ were all typed as multireplicon plasmids IncHI2/P. In *Salmonella* isolate S177.41 two multireplicon plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ were detected with estimated sizes of 220 kb and 340 kb. Both plasmids were identified as IncHI2/P. The largest plasmid (340 kb) was transferred by conjugation experiments (Table 2.2). This transconjugant was also positive for the IncI1 replicon by PBRT. The four non-typeable plasmids were all relatively small (the sizes varied from 25 – 35 kb). Three of the non-typable plasmids were associated with the $bla_{TEM\text{-}52}$gene and one with the $bla_{ACC\text{-}1}$gene.

Determination of MICs of transconjugants and transformants did not reveal additional plasmid-located non-beta-lactam-resistances on the plasmids carrying $bla_{TEM\text{-}52}$, whereas the transconjugants containing the plasmids carrying $bla_{TEM\text{-}20}$ showed co-resistance to sulphamethoxazole (MICs > 1024 µg/ml). In *Salmonella* all $bla_{CTX\text{-}M}$ associated plasmids carried resistances to non-beta-lactam antibiotics as well, varying from resistance to one antibiotic (sulphamethoxazole or trimethoprim) to resistance to three antibiotics (tetracycline, sulphamethoxazole and trimethoprim).

In the *E. coli* isolates, linkage of ESBL-genes to plasmids was confirmed by hybridization experiments on the donor strains. In 4 *E. coli* isolates the presence of 2 almost equally sized plasmids in the donor made the results of the hybridization experiments inconclusive. Therefore transformation experiments were performed on these isolates. The transformants showed no co-resistances to non-beta-lactam antibiotics (Table 2.2).

## 2.5 Discussion

The results obtained in this study show that non-wild type MICs for cefotaxime in *E. coli* and *S. enterica* from Dutch broilers, can mainly be explained by the presence of IncI1 plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ in both species. Although a variety of ESBL-genes and plasmids are found in both bacterial species, similar ESBL-genes and plasmid-families are those that are predominantly present (Table 2.2). This is understandable as *E. coli* and *S. enterica* isolates were derived from the same source: the gastro-intestinal tract of Dutch broilers. It is likely that interaction between these species has led to exchange of genes and plasmids.

The observed increase in prevalence of ESBL-producing bacteria in Dutch broilers began in 2003. Before 2003, ESBL-producing bacteria were found only incidentally in broilers in the Netherlands [10]. A survey in 2001-2002 on cefotaxime resistant *Salmonella* isolates from poultry, poultry products and human patients in the Netherlands showed that the ESBL-genes found in poultry were predominantly typed as $bla_{ACC\text{-}1}$ and $bla_{TEM\text{-}52}$. At that time, only one $bla_{CTX\text{-}M}$-gene ($bla_{CTX\text{-}M\text{-}2}$) was found in a broiler isolate. Other $bla_{CTX\text{-}M}$-genes, although detected in human

***Table 2.2*** *Characteristics of ESBL or AmpC-carrying plasmids in* E. coli *(n=10) and* S. enterica *(n=16) isolates from Dutch broilers in 2006.*

| **ESBL- or AmpC-gene in donor** | **Strains** | **Species** | **Plasmid types in donor** |
|---|---|---|---|
| $bla_{CTX\text{-}M\text{-}1}$ | E38.16[b] | *E. coli* | I1, FIB, F |
| | E38.27 | *E. coli* | I1, HI1 |
| | E38.52[b] | *E. coli* | I1, FIB, F |
| | S162.03 | *S.* Parathyphi B var. Java | I1 |
| | S175.77 | *S*. Infantis | I1 |
| | S186.27 | *S*. Agona | I1, N |
| | S186.74 | *S.* Parathyphi B var. Java | I1, HI1 |
| | S187.46 | *S*. Infantis | I1 |
| $bla_{CTX\text{-}M\text{-}2}$ | E39.29[b] | *E. coli* | FIC, HI2/P |
| | S168.68 | *S.* Parathyphi B var. Java | I1, HI2/P |
| | S177.41 | *S.* Parathyphi B var. Java | I1, HI2/P |
| $bla_{TEM\text{-}52}$ | E38.34 | *E. coli* | I1, FIB |
| | E38.45[b] | *E. coli* | FIB, F |
| | E39.76[b] | *E. coli* | I1, F |
| | S162.19 | *S*. Infantis | I1 |
| | S166.01 | *S.* Parathyphi B var. Java | I1 |
| | S166.22 | *S.* Parathyphi B var. Java | ColE, I1 |
| | S173.44 | *S*. Infantis | I1 |
| | S178.57 | *S.* Parathyphi B var. Java | I1, HI2/P |
| | S184.71 | *S.* Indiana | N.t. |
| $bla_{TEM\text{-}20}$ | S171.70 | *S.* Parathyphi B var. Java | I1 |
| | S180.34 | *S.* Parathyphi B var. Java | I1 |
| $bla_{SHV\text{-}2}$ | E39.44 | *E. coli* | FIB, Y, K |
| $bla_{ACC\text{-}1}$ | S177.63 | *S.* Braenderup | N.t. |
| $bla_{CMY\text{-}2}$ | E38.35 | *E. coli* | I1, FIB, Y, P, F, K |
| | E39.62 | *E. coli* | FIB, K |

| Non-beta-lactam resistance in donor[a] | Plasmid type linked to ESBL-gene | Plasmid size | Non-beta-lactam resistance transferred[a] |
|---|---|---|---|
| TET-SXL-TMP-STR | I1 | 100 | N.a. |
| TET-SXL-TMP-STR-CHL | I1 | 88 | None |
| SXL-TMP-CIP-NAL-STR | I1 | 100 | N.a. |
| SXL-TMP-CIP-STR | I1 | 97 | SXL |
| SXL-TMP-STR | I1 | 100 | SXL-TMP |
| TET-SXL-TMP-STR | I1 | 110 | TET-SXL |
| NEO-TET-SXL-TMP-STR | I1 | 97 | SXL |
| SXL-TMP | I1 | 100 | SXL-TMP |
| TET-SXL-TMP-STR-CIP-NAL | HI2/P | 244 | N.a. |
| TET-SXL-TMP-STR-CIP-NAL | HI2/P | 242 | TMP |
| TET-SXL-TMP-STR-CIP-NAL | HI2/P | 340 | TET-SXL-TMP |
| TET-SXL-TMP-STR-CIP-NAL-CHL | I1 | 97 | None |
| TET-SXL-TMP-STR-CIP-NAL | N.t. | 35 | N.a. |
| GEN-SXL-TMP-STR-CIP-NAL-CHL | I1 | 90 | N.a. |
| None | I1 | 82 | None |
| TMP-CIP-NAL-STR | I1 | 82 | None |
| TMP-CIP-NAL-STR | I1 | 82 | None |
| None | I1 | 90 | None |
| TET-SXL-TMP-STR | N.t. | 35 | None |
| None | N.t. | 25 | None |
| SXL-TMP-CIP-NAL-STR | I1 | 100 | SXL |
| SXL-TMP-STR | I1 | 100 | SXL |
| NEO-TET-SXL-TMP-STR-CIP-NAL-CHL | K | 155 | None |
| None | N.t. | 35 | None |
| GEN-NEO-TET-SXL-TMP-STR-CIP-NAL-CHL | K | 88 | None |
| None | K | 88 | None |

N.t.= non-typeable by PCR based Replicon Typing

N.a. = not applicable, see also footnote [b]

[a]GEN=Gentamicin, NEO=Neomycin, TET=Tetracycline, SXL=Sulphamethoxazole, TMP=Trimethoprim, CIP=Ciprofloxacin, NAL=Nalidixic Acid, CHL=Chloramphenicol, STR=Streptomycin

[b]This strain was not conjugated or transferred, localization of the ESBL-gene was demonstrated by Southern blot experiment using ESBL-gene and plasmid replicon probes.

isolates ($bla_{CTX\text{-}M\text{-}28}$ and $bla_{CTX\text{-}M\text{-}3}$), were not present in broilers [5]. Our results show that in 4-5 years time not only an increase of ESBL-producing bacteria has taken place, but also a shift in ESBL-genes towards CTX-M-genes.

The complexity of plasmid evolution is demonstrated by the finding of multireplicon plasmids in *Salmonella* Paratyphi B var. Java isolate S177.41. This isolate harbours three plasmids with estimated sizes of 100, 220 and 340 kb and was positive for IncI1, IncHI2 and IncP replicon types. The two largest plasmids in S177.41 both carry $bla_{CTX\text{-}M\text{-}2}$ and hybridized both with IncHI2 and IncP probes. By conjugation only the 340 kb IncHI2/P multireplicon plasmid was transferred. PBRT of the transconjugant revealed that this plasmid was also positive for IncI1. This suggests that the 340 kb IncHI2/P plasmid in S117.41 and its transconjugant was the result of co-integration of a 100 kb IncI1 plasmid and a 220 kb HI2/P plasmid.

Co-resistance to non-beta-lactam antibiotics in ESBL-producing *Enterobacteriaceae* is commonly described [18, 19]. Although most of the isolates in our study show multi drug resistance to more than two antibiotics, transfer of additional resistance genes was only detected for sulfamethoxazole, tetracycline and trimethoprim. Since these drugs are frequently used in broiler production, co-selection through usage of these drugs may have played a role in the selection for ESBL-producing isolates.

Mainly three plasmid types (IncI1, IncHI2/P and IncK) play a role in the increased dissemination of a variety of ESBL and AmpC- resistance genes in *E. coli* and *Salmonella* isolates of Dutch poultry. The occurrence of similar ESBL-genes on IncI1 and IncHI2/P plasmids present in both bacterial species indicates that transmission between these species occurs. Which determinants have led to the rapid increase in ESBL-producers in the gastro-intestinal tract of broilers and in the evolution of ESBL- and AmpC-genes is still unknown. More detailed molecular characterization studies on the plasmids might help to understand more of this matter. Future research should also focus on determining the prevalence of ESBL- or AmpC-producing bacteria on farms. By comparing farms, risk factors can be analyzed, which will help to understand the role of antibiotic usage, management and hygiene measures in the prevalence and evolution of bacteria carrying these resistance plasmids. Hopefully this knowledge will lead to a reduction in multi-resistant bacteria isolated from the poultry industry.

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# Chapter 3

# Extended spectrum beta-lactamase- and AmpC beta-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers

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### 3.1 Abstract

The aim of this study was to establish the prevalence of extended-spectrum beta-lactamase (ESBL)- and AmpC beta-lactamase-producing *Escherichia coli* at Dutch broiler farms and in farmers and to compare ESBL/AmpC-producing isolates from farmers and their animals.

Twenty-five to 41 cloacal swabs collected from broilers at each of 26 farms and 18 faecal samples from 18 broiler farmers were analysed for determination of the presence of ESBL/AmpC-producing *E. coli*. ESBL/AmpC genes were characterised by microarray, PCR and sequencing. Plasmids were characterised by transformation and PCR-based replicon typing. Subtyping of plasmids was done by plasmid multilocus sequence typing or restriction fragment length polymorphism (RFLP). *E. coli* genotypes were determined by multilocus sequence typing.

Birds from all farms were positive for ESBL/AmpC-producing *E. coli,* and on 22/26 farms the within-farm prevalence was ≥ 80%. Six of 18 farmers carried isolates containing ESBL/AmpC-genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CMY\text{-}2}$ and/or $bla_{SHV\text{-}12}$, which were also present in the samples of their animals. In five of these isolates, the genes were located on identical plasmid families (incI1 (n=3), incK (n=1), or incN (n=1)) and in isolates from two farmers the genes were carried on identical plasmid subtypes (incI1 ST12 and incN ST1, where ST stands for sequence type), as in the isolates of their animals.

This study shows a high prevalence of broilers carrying ESBL/AmpC-producing *E. coli* at Dutch broiler farms and a high prevalence of ESBL/AmpC-producing *E. coli* in farmers. This is undesirable due to the risk this potentially poses to human health. Future research should focus on identification of the source of these isolates in the broiler production chain to make interventions resulting in reduction of these isolates possible.

### 3.2 Introduction

Since 2003 an increase in cefotaxime-resistant *Escherichia coli* and *Salmonella enterica* isolates has been observed in Dutch broilers[1]. The observed increase in cefotaxime resistance was concluded from the results of testing isolates from samples routinely taken at slaughterhouses throughout the Netherlands, in which each isolate represents one flock of broilers. From these samples, one *E. coli*-like colony was randomly selected and tested for susceptibility to a custom-made panel of antibiotics that included the extended-spectrum cephalosporins (ESCs) cefotaxime and ceftazidime[1]. However, this method probably underestimates the number of farms affected by ESC resistance, because it does not include selective methods to look for specific resistance mechanisms. Nevertheless, it is very useful to monitor resistance over time and to compare resistance levels between countries[2].

The increase in cefotaxime resistance among *E. coli* isolates from broilers in the Netherlands increased from 3% in 2003 to 15-20% in 2006-07 (in contrast to the low prevalence found in isolates derived from slaughter pigs, veal calves and dairy cows), and this was reason for concern[3]. Cefotaxime resistance is indicative of the presence of isolates producing extended-spectrum beta-lactamases (ESBLs) and/or plasmid-mediated AmpC beta-lactamases, which can hydrolyse beta-lactam antibiotics such as penicillins and ESCs. The genes encoding these enzymes are mostly located on mobile genetic elements (plasmids), which can easily be transferred horizontally to other bacteria (and to other bacterial species)[4]. ESC- resistant isolates are often multidrug resistant and are considered a potential threat to animal and human health [5, 6]. Although not much is known about dose-effect relations, ESBL- and/or AmpC-producing bacteria

present in food-producing animals can act as a reservoir for the presence of ESC resistance in human pathogens[5, 7]. To determine the prevalence of ESBL/AmpC producers in samples of food-producing animals at the farm level, selective methods are preferable.

Determination of the prevalence at the slaughterhouse level of ESBL and AmpC producers among isolates from broilers has been performed in Denmark (2011), Sweden (2011), Portugal (2004) and France (2005)[8-12]. The prevalence in Denmark and Sweden was found to be 27% and 34%, respectively, and in Portugal and France the prevalence was 10%-42% and 29% respectively. The data from these latter two countries could be underestimates, since no pre-enrichment step was used in these studies.

Detection of ESBL/AmpC-producing isolates at broiler flock level using selective methods has been conducted in Belgium, Spain, Taiwan, Tunisia and Japan.[13-17] All studies were based on data derived from direct culture on selective agar plates without a selective pre-enrichment step. It was not always possible to calculate the prevalence at flock level either because only a few farms were sampled (prevalence on one farm in Taiwan was 6,3%)[15] or because only a small number (≤10) of isolates of each farm were tested (in Spain, Tunisia and Japan)[14, 16, 17]. One study investigated the diversity of ESBL/AmpC-producing isolates at five broiler farms in Belgium. Although some isolates might have been missed by not using a pre-enrichment step and by using ceftiofur in the screening plates,[18] prevalence at the flock level was relatively high and varied from 27% to 75%[13]. Unfortunately, reliable data from other countries on ESBL/AmpC-prevalence at broiler farms that could give more information about the spread of these isolates are still lacking. The aim of the present study was to establish the prevalence of ESBL- and AmpC beta-lactamase-producing *E. coli* at broiler farms in the Netherlands. Furthermore the presence of ESBL/AmpC beta-lactamase producing *E. coli* among broiler farmers was established and ESBL/AmpC-genes and plasmids present in broiler and farmer isolates were characterized and compared.



## 3.3 Material and methods

### *3.3.1 Farms*

Thirty-five broiler farmers from conventional farms with ≥ 30 000 broilers in previous rounds were randomly selected and invited to participate in the study. Questionnaires were used to obtain information on management, hygiene, disinfection and antibiotic use. During the period March to June 2009, if the farmer agreed to participate (n=26, see the Results section), farms were included and visited. The farms were dispersed throughout the Netherlands in relation to the density of broiler farms in the Netherlands (CBS) (Figure 3.1) and represent about 3.5% of Dutch broiler farms. Each farm consisted of one or more poultry houses.

### *3.3.2 Broiler isolates*

During the period March to April 2009, cloacal swabs were taken at six weeks of age from 41 birds per farm from the different poultry houses present at the farm. All swabs were suspended in 1 mL of buffered peptone water supplemented with 30% glycerol (peptone glycerol). Within one day, the first 25 samples per farm were cultured on selective agar plates (MacConkey agar (product no. 212123, Becton Dickinson) + 1 mg/L cefotaxime (Sigma-Aldrich, Germany)), and inoculated in a selective pre-enrichment broth (Luria-Bertani broth containing

1 mg/L cefotaxime). The remaining 16 swabs per farm were suspended in peptone glycerol and stored at -20°C. Agar plates and broth were incubated overnight at 37°C. When no growth was seen on the selective agar plate, the enrichment broth was inoculated on the selective agar plate and incubated overnight. If none or just a few of the 25 samples per farm yielded growth on the agar plate, the remaining 16 swabs of that farm were cultured as described above. One indole-positive *E. coli*-like colony per sample was tested for ESBL production using a combination disk test including cefotaxime (Oxoid), cefotaxime + clavulanic acid (Becton Dickinson), ceftazidime (Oxoid), ceftazidime + clavulanic acid (Becton Dickinson) and cefoxitin (Oxoid) to allow detection of AmpC-production as described in chapter 7[19]. Indole negative isolates were confirmed for *E. coli* by PCR [20]and if positive included for analysis as described above. Isolates suspended in peptone glycerol were stored at -80°C.

*3.3.3 Farmer isolates*

All farmers or caretakers were asked to participate in personal testing for ESBL/AmpC-producing *E. coli* carriage. Eighteen of the 26 farmers agreed to send in a faecal swab. These samples were processed as described for the broiler cloacal swabs. Initially one isolate per farmer was analysed. Later, four additional *E. coli* colonies per farmer were isolated from samples stored in peptone glycerol at -20°C by adding 200 µL of thawed faeces suspension to 1 mL Luria-Bertani broth containing 1 mg/L cefotaxime. After overnight incubation each sample was inoculated on MacConkey agar containing 1 mg/L cefotaxime. If present, four colonies were randomly selected and further analysed as described for the broiler isolates.

*3.3.5 Characterisation of ESBL-genes, other resistance genes, plasmids and* E. coli *genotypes*

If farmers tested positive for ESBL/AmpC-producing *E. coli*, a minimum of one isolate per phenotype from each poultry house on their farm was further characterized for ESBL/AmpC genes by microarray analysis (ATR0503, Alere, The Netherlands), PCR and sequencing[19, chapter 7]. Characterisation with the microarray also allows for the detection of other resistance genes[21]. Plasmid characterization and *E. coli* genotyping was performed only on farmer and broiler isolates carrying similar ESBL/AmpC genes as determined by transformation, PCR-based replicon typing (PBRT) and multi locus sequence typing (MLST)[22-25]. After transformation co-transfer of resistance genes other than ESBL/AmpC genes was analysed by microarray (ATR0503, Alere, the Netherlands). IncI1 and incN plasmids were further characterized by plasmid MLST (pMLST)[26,27]. IncK plasmids were further characterized by restriction fragment length polymorphism (RFLP) using *Eco*RI and *Hind*III, as no pMLST method was available for these plasmids. RFLP patterns were analysed using Bionumerics version 6.6 (Applied Maths).

*3.3.6 Questionnaires*

The data from the questionnaires were analysed by means of descriptive statistics using SPSS Statistics version 19.0.

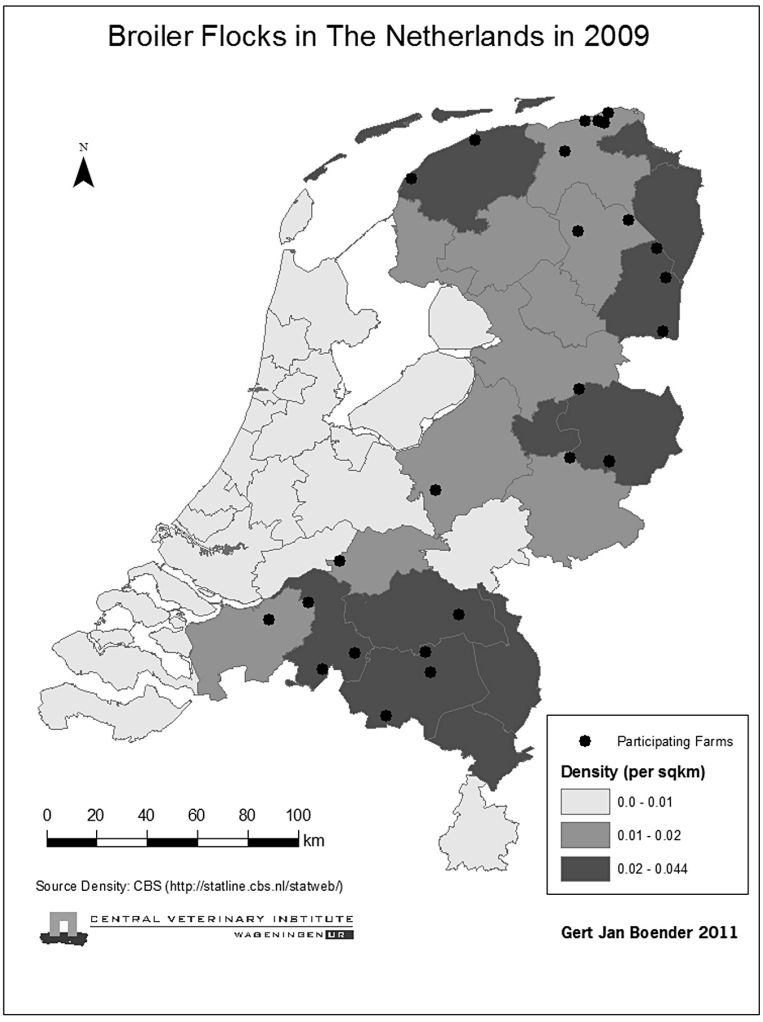


***Figure 3.1*** *Location of participating farms in the Netherlands*

### 3.4 Results

ESBL- and/or AmpC-producing *E. coli* were found on all farms, even without selective pre-enrichment. Without selective pre-enrichment the average within-farm prevalence of broilers positive for ESBL/AmpC-producing *E. coli* was 75% ±10% (data not shown). With selective pre-enrichment, the average within-farm prevalence increased to 85%±10%. On 22 farms, the within-farm prevalence was 80% or higher (Figure 3.2). The within-farm prevalence on the four additional farms was below 80% (2% on C, 32% on farm Q, 24% on farm T and 59% on farm Y). On one farm (farm C) this low prevalence was probably due to overgrowth of ESBL producers on the selective plates by swarming non-*E. coli* bacteria.

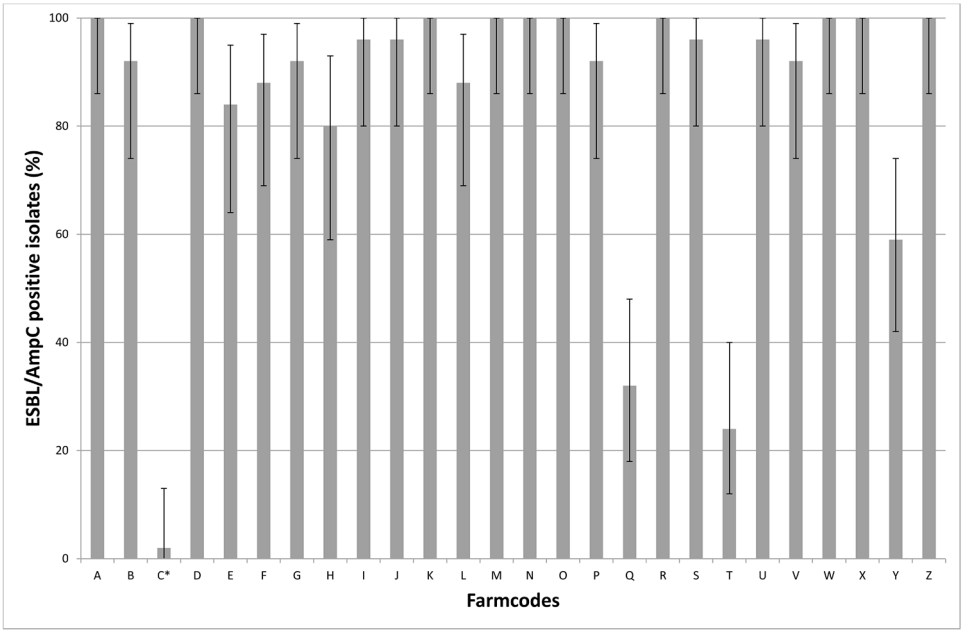


***Figure 3.2*** *Percentage of ESBL/AmpC-positive isolates from each farm using selective enrichment. At 85% of the farms the prevalence was ≥80%. * At farm C technical failures regarding isolation cannot be ruled out as a result of overgrowth of ESBL producers on the selective plates by swarming non-*E. coli *bacterial species.*

The farms varied in size (28 000 – 138 720 animals), number of houses (1 – 7 houses), and number of animals per $m^2$ (15-24 animals per $m^2$). Animals were derived from 10 different hatcheries and feeds were derived from 10 different feed companies. All farms used the all-in, all-out concept, but at 20 of the 26 farms, 10-25% of the animals were already selected to be slaughtered during the fifth week. The average downtime (period between two production cycles) was 7 days (range 4-14 days).

Hygiene measures differed between farms: 21 farms had a special room where farmers could change clothes before entering the poultry houses, and at 16 of these 21 farms there was separation between the area where farm clothes and boots were worn. Either the farmer did not use special farm-related clothes (n=1) or farm-related clothing was changed every day (n=3), every week (n=5), twice per production round (n=2) or every production round (n=15). Fourteen farmers did not change clothes when they went from one poultry house to another. At 16 farms the tools used in the poultry houses were shared between poultry houses and not cleaned or disinfected when moved between poultry houses. On all but one farms, a towel, water and soap was available before entering the poultry house, but in six farms this was not available at every poultry house. Twelve of the 26 farms had a shower at the entrance of the poultry house, but none of the farmers used the shower.

On one farm no antibiotics were used during the production round (farm K). On all other farms at least one treatment was applied. Most treatments were given as therapy for intestinal tract disorders. The antibiotics used were: ampicillin, amoxicillin, penicillin, trimethoprim/sulfachlorpyridazine, trimethoprim/sulfamethoxazole, enrofloxacin, flumequine, lincomycin/spectinomycin, neomycin, oxytetracycline, doxycycline and/or tylosin. Daily dosages per animal year (dd/ay) ranged from 0 dd/ay to 135 dd/ay, with an average of 32 dd/ay. No relation was

observed between the above-mentioned farm characteristics and the prevalence of ESBL/AmpC-positive animals, but this was due to the low variation in ESBL/AmpC prevalence among farms.

Six of the 18 farmers (33%) who participated in the study tested positive for ESBL- or plasmid-mediated AmpC beta-lactamase producing *E. coli* in their faeces. These farmers corresponded with farm codes D, E, F, H, R and V. No relation was found between farm characteristics and whether or not the farmer carried ESBL/AmpC-producing *E. coli*. Additional isolation of ESBL/AmpC-producing *E. coli* resulted in five isolates each for farmers E, F, R and V. All four additional isolates per farmer obtained from farmer E, R and V had similar ESBL or AmpC types as found previously. One isolate from farmer F contained $bla_{\text{CTX-M-1}}$, three contained $bla_{\text{CMY-2}}$ and in one isolate no ESBL-gene was found (data not shown). To determine genetic relatedness, one isolate per farmer of each ESBL or AmpC gene found, was further characterized (Table 3.1). In all cases, ESBL/AmpC-genes detected were also found to be present in their broilers. In samples from five of the six farmers, the combination of ESBL/AmpC genes found and the plasmid family on which the genes were located corresponded to genes and plasmids found in the broiler samples (farm E: $bla_{\text{CTX-M-1}}$ on incI1; farm F: $bla_{\text{CMY-2}}$ on incI1; farm H: $bla_{\text{CMY-2}}$ on incK (incK plasmids are positive by PBRT for B/O and K replicons[22]); farm V: $bla_{\text{SHV-12}}$ on incN; farm R: $bla_{\text{CTX-M-1}}$ on incI1). IncI1 and incN plasmids from the farmers and their broilers were subtyped by pMLST. On farm F and V the sequence types (STs) of the plasmids found in the farmer isolates corresponded to the ST of the plasmid found in the broiler isolates (pMLST type 12 for IncI1 and type 1 for incN respectively, Table 3.1). Analysis of the RFLP patterns of incK plasmids in isolates carrying $bla_{\text{CMY-2}}$ from broilers and the farmer on farm H showed 88.6% and 93.7% similarity (Pearson correlation) after digestion with *Eco*RI and *Hind*III respectively (data not shown), which showed that although they belong to the same plasmid family, they are of different subtypes.

MLST revealed that the STs of E. coli isolates obtained from the farmer and the broilers on the same farm were identical in the case of farms D and F (*E. coli* ST93), but differed for the other farms. However within farms D and F, *E. coli* ST93 did not contain similar ESBL-genes. In addition *E. coli* ST93 was found in a broiler isolate from farm E. Some other *E. coli* genotypes (ST359, ST770, ST442 and ST2309) occurred in farmers and/or broilers at various farms (Table 3.1).

### 3.5 Discussion

ESBL/AmpC-producing *E. coli* isolates were found in cloacal swabs from broilers at all 26 Dutch broiler farms tested. This is similar to the results of studies performed in Belgium and Spain where cloacal swabs tested positive for ESBL/AmpC-producing E. coli isolates at 5/5 and 10/10 farms, respectively [13,14]. We found that, within any flock, the prevalence of broilers carrying ESBL/AmpC-producing *E. coli* was very high. Researchers of the studies performed in Belgium and Spain, in which less selective methods were used, found a lower prevalence in positive flocks (27%-75% based on 89-100 samples per farm in Belgium and 10%-100% based on 10 samples per farm in Spain). Data collected at slaughterhouses in Denmark and Sweden using selective pre-enrichment with cephalosporins at concentrations selective for ESBL/AmpC-producers [18], suggest that the prevalence among individual broilers in these Northern countries (27% and 34% respectively in Denmark and Sweden) is lower than in the Netherlands[8,9]. Likewise broiler meat produced in Denmark is much less contaminated (3.3%-8.6%) with ESBL/AmpC-producing isolates than imported broiler meat (36% -50%) and in comparison with data reported from the Netherlands[7,8,28,29]. This clearly indicates differences in prevalence of ESBL/AmpC-producing isolates per country.

***Table 3.1*** *Characteristics of beta-lactamase genes and plasmids present in* E. coli *isolates from broilers and farmers*

| Farm | Isolate no. | Source | House no. | *E. coli* ST | beta-lactamase genes (PCR & sequencing)[a] |
|---|---|---|---|---|---|
| D | 3 | Broiler | 1 | 38 | $bla_{CMY-2}$ |
| | 6 | Broiler | 1 | 93 | $bla_{CTX-M-2}$, $bla_{TEM-1}$ |
| | 7 | Broiler | 2 | 770 | $bla_{CTX-M-1}$ |
| | 11 | Broiler | 2 | 789 | $bla_{CMY-2}$ |
| | 13 | Broiler | 3 | ND | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 14 | Broiler | 3 | ND | $bla_{CTX-M-2}$, $bla_{TEM-1}$ |
| | 20 | Broiler | 4 | 2309 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 22 | Broiler | 4 | ND | $bla_{CTX-M-2}$, $bla_{TEM-1}$[c] |
| | 586 | Farmer | NA | 93 | $bla_{CMY-2}$, $bla_{CTX-M-1}$, $bla_{TEM-1}$ |
| E | 93 | Broiler | 1 | 380 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 97 | Broiler | 2 | ND | $bla_{CTX-M-1}$ |
| | 103 | Broiler | 3 | ND | $bla_{TEM-52c}$ |
| | 111 | Broiler | 4 | 665 | $bla_{CTX-M-1}$ |
| | 114a | Farmer | NA | 93 | $bla_{CTX-M-1}$ |
| | 114b | Farmer | NA | ND | $bla_{CTX-M-1}$ |
| | 114c | Farmer | NA | ND | $bla_{CTX-M-1}$ |
| | 114d | Farmer | NA | ND | $bla_{CTX-M-1}$ |
| | 114d | Farmer | NA | ND | $bla_{CTX-M-1}$ |
| F | 26 | Broiler | 2 | ND | $bla_{CTX-M-1}$ |
| | 30 | Broiler | 3 | ND | $bla_{CTX-M-1}$ |
| | 33 | Broiler | 4 | 70 | $bla_{CMY-2}$ |
| | 36 | Broiler | 5 | ND | $bla_{CTX-M-1}$ |
| | 40 | Broiler | 6 | ND | $bla_{CTX-M-1}$ |
| | 44 | Broiler | 7 | 93 | $bla_{CTX-M-1}$ |
| | 49a | Farmer | NA | ND | none |
| | 49b | Farmer | NA | 93 | $bla_{CMY-2}$ |
| | 49c | Farmer | NA | 442 | $bla_{CTX-M-1}$ |
| | 49d | Farmer | NA | ND | $bla_{CMY-2}$ |
| | 49e | Farmer | NA | ND | $bla_{CMY-2}$ |
| H | 74 | Broiler | 1 | ND | $bla_{CMY-2}2$ |
| | 85 | Broiler | 3 | 2309 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 86 | Broiler | 3 | 420 | $bla_{SHV-12}$, $bla_{TEM-1}$ |
| | 92 | Farmer | NA | 359 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| R | 479 | Broiler | 1 | 115 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 480 | Broiler | 1 | 359 | $bla_{TEM-52}$, $bla_{TEM-1}$ |
| | 487 | Broiler | 2 | 115 | $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 488 | Broiler | 2 | ND | $bla_{TEM-52}$, $bla_{TEM-1}$ |
| | 491 | Broiler | 2 | 1358 | $bla_{CTX-M-1}$, $bla_{CMY-2}$, $bla_{TEM-1}$ |
| | 495a | Farmer | NA | 442 | $bla_{CTX-M-1}$ |
| | 495b | Farmer | NA | ND | $bla_{CTX-M-1}$ |

| Replicon types of plasmids in parent isolate (plasmid ST) | Other resistance genes (miniaturized microarray) |
|---|---|
| N, B/O, K[b], Frep, ColE | *aadA2* |
| ND | *aadA1, aadA2, dfrA1, sul1, sul2, tet*(A) |
| B/O | *aadA2, tet*(A) |
| ColE, NT | *aadA1, floR, intI2, sul2, tet*(A), *strB* |
| ND | none |
| ND | *aadA1, aadA2, dfrA1, intI1, sul1, tet*(A), *tet*(B) |
| B/O, K[b] | *aadA2, tet*(B) |
| ND | *aadA1, dfrA1, sul1, strB* |
| I1(ST7), B/O, ColE, FIB, NT | *aadA1, dfrA1, floR, intI1, sul1, sul2, tet*(A), *strB* |
| ND | *aadA1, dfrA1, sul2, tet*(A) |
| ND | *aadA1, dfrA1, sul1* |
| ND | *sul1* |
| I1(ST7), FIB, FIC | *aadA1, dfrA1, intI1, sul1, sul2, tet*(A) |
| I1(ST91), FIA | *sul2, ereB* |
| ND | *sul2* |
| ND | *sul2* |
| ND | *sul2* |
| ND | none |
| ND | *aadA4, dfrA17, intI1, tet*(A),*ereB* |
| ND | *aadA4, dfrA17, intI1, tet*(A),*ereB* |
| I1(ST12), Frep, ColE | none |
| ND | *aadA4, dfrA17, intI1, tet*(A) |
| ND | *aadA4, dfrA17, intI1, tet*(A), *ereB* |
| NTR | *aadA4, dfrA17, intI1, sul2, tet*(A), *ereB* |
| ND | *aadA1, aadA2, catA1, dfrA1, dfrA15, intI1, sul1, tet*(D), *ereB* |
| I1 (ST12), FIA, ColE | none |
| I1(ST7), ColE | *aadA1, dfrA1, dfrA5, intI1, sul1, sul2* |
| ND | none |
| ND | none |
| ND | *aadA1, dfrA1, intI1, ereB* |
| B/O, K[b] | *aadA1, dfrA1* |
| ND | *aadA1, aadA2, cmlA1, dfrA1, dfr12, intI1, sul1, sul2, sul3, tet*(A), *strA, strB* |
| I1, P, B/O/K[b] | *aadA1, catA1, dfrA1, dfrA17, sul1* |
| ND | *aadA4, dfrA17,dfrA19, intI1, sul1* |
| ND | *catA1, dfrA17, sul1, ereB* |
| ND | *aadA4, dfrA17,dfrA19, intI1* |
| ND | *catA1, dfrA17, dfrA19, ereB* |
| I1(ST3), B/O, ColE, Frep, NT | *tet*(A), *sul2* |
| I1(ST7), FIB | *aadA1, dfrA1, dfrA5, intI1, sul1, sul2, tet*(A) |
| ND | *aadA1, dfrA1, dfrA5, intI1, sul1, sul2* |

*Continued*

***Table 3.1*** *Continued*

| Farm | Isolate no. | Source | House no. | E. coli ST | beta-lactamase genes (PCR and sequencing)[2] |
|---|---|---|---|---|---|
| | 495c | Farmer | NA | ND | $bla_{CTX\text{-}M\text{-}1}$ |
| | 495d | Farmer | NA | ND | $bla_{CTX\text{-}M\text{-}1}$ |
| | 495e | Farmer | NA | ND | $bla_{CTX\text{-}M\text{-}1}$ |
| V | 445 | Broiler | 1 | ND | $bla_{SHV\text{-}12}$, $bla_{TEM\text{-}1}$[d] |
| | 448 | Broiler | 1 | 602 | $bla_{CMY\text{-}2}$ |
| | 450 | Broiler | 3 | 997 | $bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}1}$ |
| | 454 | Broiler | 4 | ND | $bla_{SHV\text{-}12}$ |
| | 459 | Broiler | 5 | ND | $bla_{SHV\text{-}12}$ |
| | 464 | Broiler | 6 | 770 | $\underline{bla_{SHV\text{-}12}}$ |
| | 469a | Farmer | NA | 69 | $\underline{bla_{SHV\text{-}12}}$ |
| | 469b | Farmer | NA | ND | $bla_{SHV\text{-}12}$ |
| | 469c | Farmer | NA | ND | $bla_{SHV\text{-}12}$ |
| | 469d | Farmer | NA | ND | $bla_{SHV\text{-}12}$ |
| | 469e | Farmer | NA | ND | $bla_{SHV\text{-}12}$ |

Compared with other European countries, the Netherlands used to use more antibiotics per kg live weight of food-producing animals[1,30]. Similarly, resistance levels in indicator bacteria derived from food-producing animals in the Netherlands are higher than in other European countries[2]. The European Food Safety Authority publishes data on antimicrobial resistance in isolates of food-producing animals in Europe. In 2009, data concerning cefotaxime resistance in *E. coli* isolates from broilers were reported for nine European countries. Only Spain reported higher levels of cefotaxime resistance than the Netherlands[2]. Methods to collect these data are harmonized within Europe and in 2009 cefotaxime resistance in *E. coli* isolates from broilers was reported in Austria, Denmark, France, Germany, Poland, Spain, Norway, Switzerland and the Netherlands[2]. The high level of antibiotics used at the broiler farms included in our study (on average, antibiotics were administered to broilers on 32 days each year) might explain (by selection or co-selection) the high prevalence of broilers carrying ESBL/AmpC beta-lactamase-producing *E. coli*. This is supported by data from Persoons *et al.*[31], who determined that use of amoxicillin and enrofloxacin was a statistically significant risk factor for high-level ceftiofur resistance in *E. coli* at broiler farms[31]. However, in our study, the prevalence of ESBL/AmpC-

| Replicon types of plasmids in parent isolate (plasmid ST) | Other resistance genes (miniaturized microarray) |
|---|---|
| ND | *aadA1, dfrA1, dfrA5, intI1, sul1, sul2* |
| ND | *aadA1, dfrA1, dfrA5, intI1, sul1* |
| ND | *aadA1, dfrA1, dfrA5, intI1, sul1, sul2* |
| ND | *aadA1, dfrA1, sul2* |
| ND | *aadA1, aadA2, intI1, sul1, sul2, tet*(A), *floR, strA* |
| ND | *aadA1, catA1, dfrA1, intI1, sul1* |
| ND | *aadA2, dfrA14, sul2, strA, strB, ereB* |
| ND | *aadA2, ereB* |
| FIB, K, B/O, ColE, N(ST1) | *aadA2* |
| FIB, Frep, N(ST1) | *aadA2* |
| ND | *aadA2* |
| ND | *aadA2* |
| ND | *aadA2* |
| ND | none |

NA= not applicable; ND, not determined: *E. coli* MLST was done only for a selection of representative farmer and broiler isolates; NT = non-typeable by PBRT[22,23]; NTR = Not transferable.
Underligning (continuous or broken lines) depicts ESBL/AmpC and other resistance genes that are co-transferred from the parent isolate on a plasmid with a certain replicon type (depicted with similar underlignment) to the transformant.
[a]With the primers used in this study no distinction was made between $bla_{CMY-2}$ and $bla_{CMY-22}$; however, for readability of the table $bla_{CMY-2}$ was inserted in the table.
[b]Positive results for replicon types B/O and K means that the plasmid belongs to incK[22].
[c]Silent mutations at nucleotide positions -28, +24, +402, compared with $bla_{TEM-1}$ reference sequence AB263754.
[d]Silent mutations at nucleotide positions +24, +234 and +402, compared with $bla_{TEM-1}$ reference sequence AB263754.

positive broilers at one farm (farm K) that used no antibiotics during the whole production period was still very high. This indicates that other factors than selection by antibiotics at the farm might affect the prevalence of these isolates. These factors may include infection control measures in the poultry production chain and antibiotic use at hatcheries, as previously described[31,32] .

As long ago as 1976, Levy *et al*. showed that resistant bacteria and plasmids bearing resistance genes could be transferred from chicken to chicken and from chicken to humans[33]. Our data confirm the hypothesis that Dutch farmers are at higher risk than the general Dutch population of carrying ESBL/AmpC-producing isolates in their gastrointestinal tract, because the prevalence in broiler farmers was 33% compared with 5% in patients admitted to hospital as described previously[7]. Colonization could have resulted from contact with broilers (Levy *et al*., suggested that colonization occurs through inhalation into the nasal passages and thence via the sputum to the gastrointestinal tract)[33]. Our results do not confirm uptake of similar strains (we did not find similarity between the gene-plasmid combinations from similar *E. coli* genotypes obtained from broiler and farmer samples at the same farm), but they do confirm that in isolates obtained from 2 out of 18 farmers ESBL genes and plasmids exhibited genetic similarity to the

isolates obtained from the broilers.

There are indications that resistant bacteria can be transferred from animals to humans via the food chain[34,35]. Therefore a high prevalence of food-producing animals carrying ESBL/AmpC-producing isolates is not desirable. Recently we compared ESBL genes, plasmids and *E. coli* genotypes from isolates of broilers and humans. One out of five *E. coli* isolates derived from human infections (mainly urinary tract infections) carry ESBL-genes and plasmids that are genetically related to isolates derived from broilers[5]. Other studies have shown that plasmid transfer does take place in models simulating the human intestine[36]. Reducing the number of multiresistant bacteria in animals, but also in the environment, seems to be a logical approach to combat the spread of antibiotic resistance.

In summary, a high proportion of broilers at broiler farms are colonized by ESBL/AmpC-producing *E. coli*. The results of this study should increase awareness of the potential risk that these bacteria pose to human health (by transmission to humans via direct contact or via the food chain). The effects of measures to reduce antibiotic use in broiler production should be investigated. Further investigation is needed to assess the role of recirculation of strains on farms and to evaluate the effect of improved infection control at farms and hatcheries. This approach will hopefully lead to a reduction in the burden of multidrug-resistant isolates in broilers, the food chain and the farm environment.

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# Chapter 4

# Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: dynamics differ between the top and the bottom

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## 4.1 Abstract

Broilers and broiler meat products are highly contaminated with Extended-Spectrum Beta-Lactamase (ESBL) or plasmid-mediated AmpC beta-lactamase producing *Escherichia coli* and are considered to be a source for human infections. As these strains are thought to be vertically transmitted, the epidemiology of ESBL/AmpC-producing *E. coli* in the Dutch broiler production pyramid was examined. Cloacal swabs of Grandparents (one- /two-days, 18 and 31 weeks old), one-day old Parents and broiler chickens of increasing age were selectively cultured to detect ESBL/AmpC-producing isolates. ESBL/AmpC-producing isolates were found at all levels in the broiler production pyramid. At broiler farms the prevalence of ESBL/AmpC-positive birds increased within the first week from 0-24% to 96-100% and stayed 100% until slaughter in contrast to a relatively stable intermediate prevalence (15-29%) found in Grandparent birds of increasing age. Interventions minimizing ESBL/AmpC contamination in broilers should focus on preventing horizontal and vertical spread, especially in relation to broiler production farms.

## 4.2 Introduction

Infections with extended spectrum beta-lactamase (ESBL) or plasmid-mediated AmpC producing isolates are found increasingly in humans[1]. During the last decade these infections have not exclusively been confined to the hospital, but community onset of infections with these organisms is also increasing[2]. Although the source of the colonization of ESBL/AmpC-producing bacteria is not completely understood, circumstantial evidence points to a food-borne source[3]. When uptake of these isolates takes place (through consumption or handling of contaminated food), these isolates are able to share their ESBL/AmpC genes with other bacteria in the gastrointestinal tract by plasmid-transfer, especially when selecting compounds, like beta-lactam antibiotics are administered[4]. ESBL/AmpC-producing bacteria in the gastrointestinal tract can act as a source for infections in other parts of the body, like the urinary tract[5]. Treatment efficacy may be impaired due to the multi-drug resistant features often found in these organisms[1].

ESBL/AmpC-producing isolates can be found in nearly all food-producing animals [6], on all kinds of meats sold at retail[7-9] and in vegetables[10]. A high prevalence of ESBL/AmpC-producing isolates is found in broilers and on broiler meat[7,11-13]. The isolates found in broilers and broiler meat carry similar ESBL-genes (mainly $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$) as found in clinical isolates in humans[8,9], moreover ESBL-genes in broiler isolates are found on similar plasmids (mainly IncI1) as in human clinical isolates[8]. This suggests that contamination of broilers and broiler meat with ESBL/AmpC-producing isolates can lead to human colonization and as a result to human infection with ESBL/AmpC-producing pathogens.

A high prevalence of broilers shedding ESBL/AmpC-producing *E. coli* is described at broiler farms in the Netherlands, Belgium and Spain[12-14]. The broiler industry has a pyramidal structure in which Pedigree chickens on the top through breeding chickens produce the broiler chickens on the bottom of the pyramid (Figure 4.1). Earlier studies have implicated a vertical transmission of *E. coli* isolates from broiler breeding chickens to their offspring[15-17]. Although much is known about the prevalence at broiler level, little is known about prevalence and characteristics of ESBL/AmpC-producing *E. coli* higher in the broiler production pyramid. In the Netherlands almost all levels of the broiler production pyramid are present (Grandparent Stock (GPS), Parent Stock (PS) and broilers). Therefore the purpose of this study was to investigate the presence and distribution of ESBL/AmpC-producing isolates in all levels of the broiler production

pyramid. Moreover in a longitudinal study at Grandparent level and at three broiler production farms the dynamics of ESBL/AmpC-producing *E. coli* among the broiler chickens was examined.

## 4.3 Materials and methods

### *4.3.3 Description of the Dutch broiler production chain*

At the top of the broiler production pyramid are the pure breeds (Pedigree) and the Great Grandparent Stock (GGPS) from which the Grandparent stock (GPS) is produced. GGPS and Pedigree farms are mostly located in the UK, Ireland, France and the United States. In the Netherlands eggs from GGPS from one breeding company are hatched at a hatchery producing GPS. However, most GPS present in the Netherlands (from other breeding companies) are imported as one-day old chickens from a hatchery located abroad. The GPS and Parent Stock (PS) are reared until the age of 18-20 weeks, after which they are moved to production farms to produce eggs that will develop into PS and broilers, respectively. The broilers are kept at commercial production farms until they go to slaughter at the age of 35-42 days. In a schematic view of the Dutch broiler production chain, the estimated average number of rearing farms, production farms and hatcheries present in the period 2009-2011 in the Netherlands is shown (Figure 4.1).



### *4.3.2 One- or two-day(s) old Grandparent chickens (GPS)*

GPS chickens were sampled from broiler breed A and B, which are by far the two dominant broiler production breeds globally. GPS chickens of broiler breed A were sampled in July 2009 at two days of age. These GPS chickens were imported from the UK and, as is routinely done for every batch of imported GPS chickens, checked for diseases by euthanizing 10 animals per farm of origin. In that way we had the opportunity to use caecal material obtained at autopsy for the detection of ESBL/AmpC-producing *E. coli*. In total 80 GPS chickens (10 from each production farm (n=8)) were investigated. Meconium samples from one-day old GPS chickens from broiler breed B were collected in September 2010 by the producer at the hatchery in the Netherlands. In total 125 meconium samples (25 from each production farm of which the GPS chickens originated (n=5)) were collected in falcon tubes and transported to the laboratory. According to the information given by primary breeding company A and B, the parents of the sampled GPS chickens had not received any antibiotic prior to sampling.

### *4.3.3 Longitudinal study in GPS of breed A*

The batch of GPS chickens from broiler breed A in which 10 animals were euthanized was also sampled at the rearing farm at 18 weeks of age (November 2009) and at the production farm at 31 weeks of age (February 2010). The animals were mixed as indicated by the arrows shown in Figure 4.2. At arrival on the rearing farm, all chickens received enrofloxacin for three days. In addition, at two and eight weeks of age, the chickens in poultry house 3 (PH3) at the rearing farm were orally treated with amoxicillin-trihydrate and phenoxymethylpenicillin respectively. From forty-one GPS chickens per poultry house, faecal samples were collected by cloacal swabs, resulting in 205 and 164 samples from GPS chickens at rearing and production farm, respectively. Initially 25 samples were processed directly in the laboratory and when not all samples were positive, the remaining 16 samples (kept in 1 mL of buffered peptone water supplemented with 30% glycerol by -20°C) were also analyzed for the presence of ESBL/AmpC-producing *E. coli*.

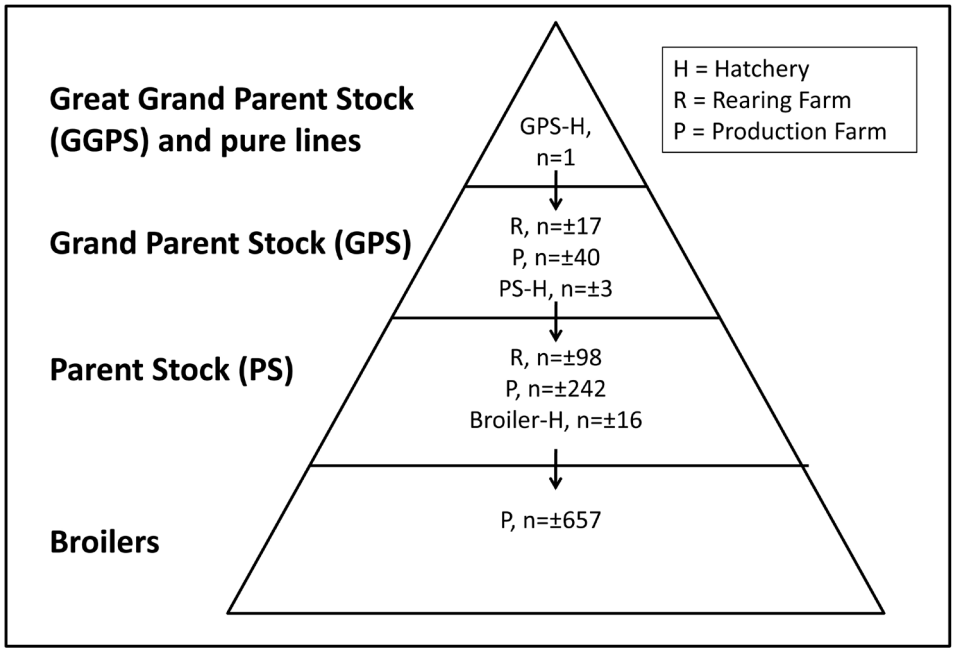


***Figure 4.1.*** *Schematic drawing of the Dutch broiler production chain. The numbers represents the average number of farms or hatcheries present in the Netherlands during the period 2009-2011. Data on the number of farms is derived from the Dutch Flock Information System (KIP-database); data on the number of hatcheries are estimated by the primary breeding companies involved in the study.*

*4.3.4 One-day old Parent chickens (PS)*

At the PS hatcheries, meconium samples of one-day old PS chickens from broiler breeds A and B were collected in the hatching units. A minimum of 25 PS chickens from each farm of origin was sampled. If the eggs from one farm of origin were divided over more than one hatching unit, 25 PS chickens from each of those hatching units were sampled. This resulted in a maximum of 75 samples per farm of origin. From broiler breed A in October 2009, the eggs were derived from nine different production farms. These eggs were divided over 21 hatching units. This resulted in 649 meconium samples (one sample was lost before analysis) that were analyzed for the presence of ESBL/AmpC-producing *E. coli*. From broiler breed B in March 2010, the eggs derived from eight farms of origin, were divided over six hatching units. This resulted in 325 meconium samples that were analyzed for the presence of ESBL/AmpC-producing *E. coli*. At both hatcheries the environment of the hatching unit (after hatching, before cleaning) was also sampled. This was done by taking five individual environmental swabs per hatching unit. Some of the parents of the PS chickens of breed B had received tylosin or enrofloxacin and some of the parents of PS chickens of breed A had received either doxycycline and tilmicosin (as two separate treatments), amoxicillin or amoxicillin combined with sulfaclozine or amoxicillin and sulfaclozine (as two separate treatments) within three months before sampling.

*4.3.5 One-day old broiler chickens*

At the broiler hatchery broiler chickens are produced. Although this hatchery hatches eggs of both broiler breeds A and B, only eggs from broiler breed A were hatched at the sampling day in February 2010. Again 25 chickens from each farm of origin were sampled. The eggs were derived from twelve different production farms divided over sixteen hatching units. This resulted in 425 meconium samples tested for the presence of ESBL/AmpC-producing *E. coli*. The environment was sampled as described for the PS hatcheries. No information about antibiotic treatments of the parents of the broilers was available.

*4.3.6 Longitudinal study at broiler farms*

Three broiler farms were included (Code A, B and C) in a longitudinal study on ESBL/AmpC prevalence at these farms. From October 2010 through January 2011 in total four poultry houses on these three farms were visited (A-1, B-1, B-2 and C-1). The broiler farms were visited at day -1 (when the broilers had not yet arrived), day 0 (the day the animals arrived in the poultry house), week 1, 2, 3, 4 and 5. At day -1, the environment from each poultry house was sampled according to Dutch regulations for *Salmonella* control. Briefly, swabs were taken from the floor of the poultry house (n=25), drinking system (n=16), feeding system (n=12), valves at the air inlet (n=5), the floor of the feed compartment (n=1), one boot of the farmer (n=1), and one sample was taken from the bedding material. All swabs were individually processed in the lab with the exception of the swabs taken in poultry house A-1. These were pooled per sample place with a maximum of five swabs in each pool. At week one to five, 25 cloacal swabs per poultry house were taken and on every visit one sample per poultry house was taken from the feed that was present inside the poultry houses. For poultry houses B-1, B-2 and C-1, also feed samples taken outside the poultry house in week five, week five and week four, respectively, were analysed. The samples were phenotypically analysed for the presence of ESBL/AmpC-producing *E. coli* and the isolates were not genetically characterized.



*4.3.7 Microbiological analysis of samples*

All faecal samples were spread on MacConkey agar (Becton Dickinson) supplemented with 1 mg/L cefotaxime (with and without aerobic pre-enrichment with Luria-Bertani broth (Becton Dickinson) containing 1 mg/L cefotaxime) and incubated aerobically overnight at 37°C. All environmental, bedding and feed samples were cultured on MacConkey agar with 1 mg/L cefotaxime (MacConkey$^{+}$) after selective pre-enrichment. All morphologically typical *E. coli* colonies on MacConkey$^{+}$ were confirmed as *E. coli* by indole test (tryptophan hydrolysis) and if negative by *E. coli* PCR [18]. One confirmed *E. coli*-type colony per sample was examined for ESBL or AmpC production by combination disc diffusion test containing cefotaxime and ceftazidime with and without clavulanic acid and cefoxitin as described [19, chapter 7]. The presence of ESBL/AmpC genes was determined for one isolate per phenotype per group of samples. These isolates were screened for ESBL/AmpC genes by miniaturized microarray (Alere, ATR0503). The presence of ESBL/AmpC genes was confirmed by PCR and sequencing as described[19, chapter 7]. To improve sequencing results, in addition to the CMY primers described in chapter 7 a new forward primer CMY-Fseq$_{838}$ 5'-TGG CGT ATT GGC GAT ATG TA -3' and reverse primer CMY-Rseq$_{857}$ 5'-TAC-ATA-TCG-CCA-ATA-CGC-CA-3' were used.

*4.3.8 Susceptibility testing*

Minimum Inhibitory Concentrations (MICs) in mg/L were determined for a panel of antibiotics (ampicillin (AMP), cefotaxime (FOT), ceftazidime (TAZ), gentamicin (GEN), tetracycline (TET), sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), naladixic acid (NAL), chloramphenicol (CHL), florfenicol (FFN), streptomycin (STR), kanamycin (KAN) and colistin (COL)) by broth microdilution using the Sensititre system as described earlier [19, chapter 7]. Wild-type susceptibility was distinguished from non-wild type susceptibility or resistance using epidemiological cut-off values according to EUCAST (www.eucast.org) or (if not available) using CLSI clinical breakpoints, respectively[20].

*4.3.9 Statistics*

The ninety-five percent confidence intervals (95% CI) are given as Clopper Pearson confidence intervals using Genstat (14th Edition).

**4.4 Results**

*4.4.1 One or two-day(s) old Grandparent chickens (GPS)*

The mean prevalence of ESBL/AmpC-producing *E. coli* in GPS chickens of broiler breed A was 23% (Table 4.1A). Prevalence at the production farms ranged from 0% (95% CI 0 – 31%) to 70% (95% CI 35 – 93%) (Figure 4.2A). For broiler breed B the mean prevalence in GPS chickens was 44% (Table 4.1A). Prevalence at the production farm ranged from 36% (95% CI 18 - 57%) to 64% (95% CI 43 – 82%) (data not shown). In both breeds, all isolates displayed solely the AmpC phenotype.

*4.4.2 Longitudinal study in GPS chickens of breed A*

Figure 4.2 shows, next to the prevalence of GPS chickens of breed A positive for ESBL/AmpC-producing isolates at the age of two days mentioned above, the prevalence at 18 weeks and 31 weeks. The prevalence at 18 weeks varied from 0% (95% CI 0 - 9%) in poultry house 1 (PH1) and poultry house 4 (PH4) to 100% (95% CI 86 – 100%) in poultry house 3 (PH3). The chickens in PH3 were treated twice for five days each with beta-lactam antibiotics (amoxicillin-trihydrate at two weeks of age and phenoxymethylpenicillin at eight weeks of age) which may have selected for ESBL/AmpC-producing *E. coli.* In the samples taken in this poultry house, most isolates (24/25) showed an AmpC phenotype, while in PH2a and PH2b, in which the chickens were not treated with antibiotics, only ESBL-phenotypes were detected (2% and 44% positive samples, respectively) (Figure 4.2B).

At 31 weeks of age (Figure 4.2C), the ESBL/AmpC prevalence varied from 2% (95% CI 0-13%) in PH2 (only ESBL phenotype) to 27% (95% CI 14-43%) in PH3 (a combination of ESBL (n=3) and AmpC (n=8) phenotypes). AmpC types were only detected in PH3 and PH4 that obtained birds from the poultry house at the rearing farm that was treated with beta-lactam antibiotics.

The AmpC-producing *E. coli* isolates found in the samples taken from the two-day-old GPS chickens were almost all found without pre-enrichment (data not shown). In contrast, the

ESBL/AmpC-positive samples taken at 18 weeks and 31 weeks were mostly detected by the use of a pre-enrichment step, except for the samples taken from the antibiotic treated chickens from PH3 at 18 weeks of age (data not shown).

*4.4.3 One-day old Parent chickens (PS)*

In the environmental samples taken from the 21 hatching units at the hatchery delivering PS chickens of broiler breed A no ESBL/AmpC-producing *E. coli* were found. Two of the 649 meconium samples (0.31%) were positive for ESBL producing *E. coli* (Table 4.1D). The PS chickens corresponding to these positive samples were derived from eggs from two different production farms. On one production farm the GPS chickens had been treated with amoxicillin (Table 4.2).

In all hatching units at the hatchery delivering PS chickens of broiler breed B, at least one of the environmental swabs was positive for AmpC-producing *E. coli* (Table 4.1D). Nineteen of the 325 broiler samples (5.8%) were positive for AmpC-producing *E. coli* (Table 4.1D). The PS chickens corresponding to these samples were derived from eggs from five different production farms that were hatched in five of the six hatching units. At two production farms the GPS chickens had been treated with tylosin and on one farm with enrofloxacin (Table 4.2).



*4.4.4 One-day old broilers*

In 12 of the 16 hatching units at least one of the environmental samples was positive for ESBL/AmpC-producing *E. coli* (data not shown). Eight of the 425 broiler samples (1.9%) were positive for ESBL/AmpC-producing isolates (Table 4.1E). The chickens corresponding to these samples were derived from eggs from three different production farms and were hatched in three of the 12 hatching units in which the environment was positive for ESBL/AmpC-producing *E. coli* (Table 4.2).

*4.4.5 Typing of ESBL/AmpC genes*

In isolates derived from GPS (broiler breed A and B), PS (broiler breed B) and broilers (broiler breed A) $bla_{CMY-2}$ was predominantly found (Table 4.1). $bla_{CMY-2}$ was the only ESBL/AmpC gene found in one- (or two-) day(s) old GPS chickens belonging to broiler breed A and B (Table 4.1A and 4.3). However at 18 weeks and at 31 weeks of age $bla_{TEM-52}$ or $bla_{TEM-52c}$ were also found in GPS chickens of broiler breed A (Table 4.1B, 4.1C and 4.2). These ESBL-genes were mainly found in the samples from the chickens not treated with beta-lactam antibiotics (Figure 4.2B). In the isolates taken from the chickens treated with beta-lactam antibiotics (in PH3), $bla_{CMY-2}$ was the dominant gene found. In the environment of the PS hatchery of broiler breed B, as in the PS chickens hatched there, only $bla_{CMY-2}$ was found (Table 4.2). $bla_{CTX-M}$ types of ESBL-genes were only found in PS chickens of broiler breed A ($bla_{CTX-M-1}$ and $bla_{CTX-M-2}$) and in environmental samples of hatching units at the broiler hatchery ($bla_{CTX-M-1}$). Although in the chickens sampled at this hatchery only $bla_{CMY-2}$ was found, in the environment, the highest diversity of ESBL/AmpC genes was found: in addition to $bla_{CTX-M-1,}$ $bla_{CMY-2}$, $bla_{TEM-20}$, $bla_{SHV-12}$ and mutations in the *ampC* promoter/attenuator (*ampC* type 3) were found (Table 4.2).

***Table 4.1*** *Summary of prevalence and characteristics of ESBL/AmpC genes found in isolates from broilers in the Dutch broiler production chain between June 2009 and December 2010.*

| Animals sampled | Year of sampling | Broiler breed | Environmental samples (n ESBL/ AmpC-positive/n hatching unit) | Total number of chicken samples |
|---|---|---|---|---|
| A: One or two-day(s) old GPS | 2009 | A | n.d. | 80 |
| | 2010 | B | n.d. | 125 |
| B: GPS 18 weeks | 2009 | A | n.d. | 189 |
| C: GPS 31 weeks | 2010 | A | n.d. | 164 |
| D: One-day old PS | 2009 | A | 5x5 pooled environmental swabs per hatching units (0/21) | 649 |
| | 2010 | B | Five environmental swabs per hatching unit (6/6, 6 isolates characterized) | 325 |
| E: One-day old broilers | 2010 | A | Five environmental swabs per hatching unit (12/16, 14 isolates characterized) | 425 |

| Number of chicken samples found positive | Prevalence in chickens | Number of chicken isolates molecular characterized | ESBL/AmpC genes in chicken samples [a] | ESBL/AmpC genes in environmental samples[b] |
|---|---|---|---|---|
| 18 | 23 | 6 | *bla*$_{CMY-2}$ (n=6) | n.d. |
| 55 | 44 | 5 | *bla*$_{CMY-2}$ (n=5) | n.d. |
| 44 | 29 | 4 | *bla*$_{TEM-52c}$(n=3), *bla*$_{CMY-2}$ (n=1) | n.d. |
| 25 | 15 | 5 | *bla*$_{TEM-52c}$(n=2), *bla*$_{TEM-52}$(n=1), *bla*$_{CMY-2}$ (n=2) | n.d. |
| 2 | 0.3 | 2 | *bla*$_{CTX-M-2}$(n=1), *bla*$_{CTX-M-1}$ (n=1) | none |
| 19 | 5.8 | 7 | *bla*$_{CMY-2}$ (n=5), *ampC* type 40 (n=2) | *bla*$_{CMY-2}$ (n=4) |
| 8 | 1.9 | 3 | *bla*$_{CMY-2}$ (n=3), | *bla*$_{CMY-2}$ (n=8), *bla*$_{TEM-20\#}$ (n=2), *bla*$_{CTX-M-1}$ (n=2), *bla*$_{SHV-12}$, *ampC* type 3 (n=1) |

GPS= Grandparent Stock, PS= Parent Stock
[a]With the primers used to sequence *bla*$_{CTX-M-2}$ no distinction can be made between *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-97}$.
[b]With the primers used to sequence *bla*$_{SHV-12}$ no distinction can be made between *bla*$_{SHV-12}$ and *bla*$_{SHV-129}$.
[#]*bla*$_{TEM-20}$ with silent mutations +144G->A, +480C->T and +723A->G compared to reference sequence *bla*$_{TEM-20}$ Y17581 (nucleotide position according to amino acid count at www.lahey/studies.org.
n.d.: not determined.

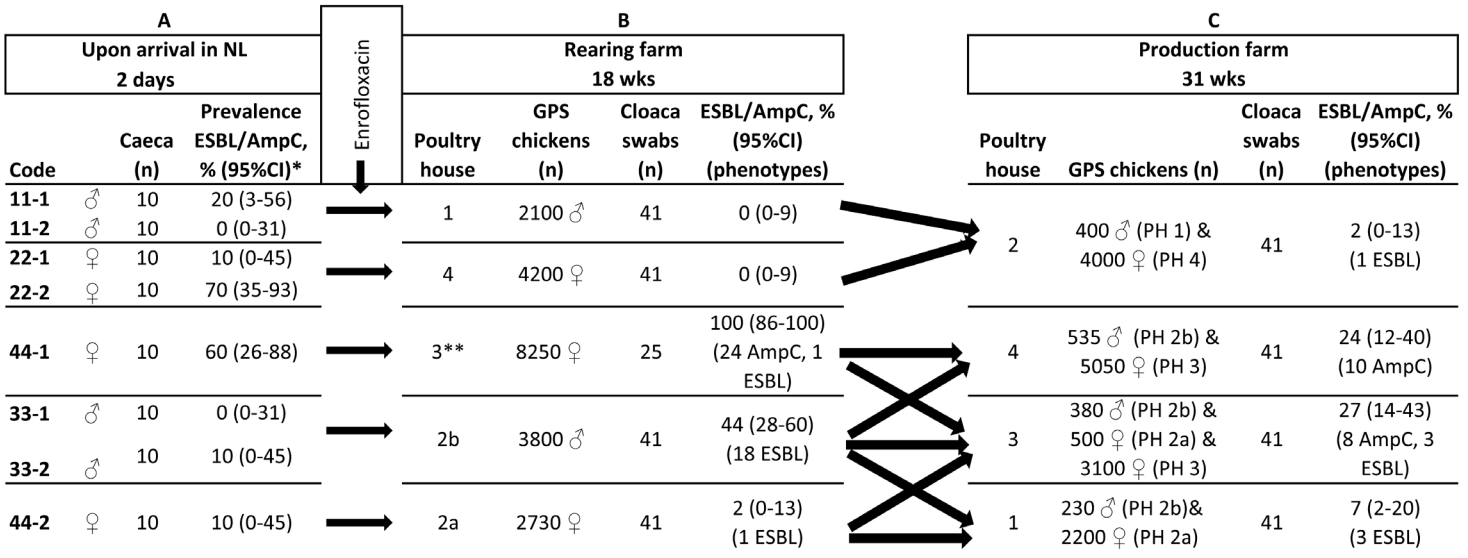


***Figure 4.2****. Schematic view of the prevalence of ESBL/AmpC-producing* E. coli *in GPS chickens of broiler breed A at 2 days, 18 weeks and 31 weeks of age. A. At arrival in the Netherlands 10 GPS chickens from each GGPS production farm of origin were euthanized by the producer for other screening tests. Caeca of those animals were analysed for the presence of ESBL/AmpC-producing* E. coli*. B. All other chickens went to the rearing farm until 20 weeks of age. At arrival on the farm they received enrofloxacin for three days. At 20 weeks of age they were transported to the production farm where they started producing eggs. At 18 weeks of age the chickens were sampled at the rearing farm. C. At the production farm the chickens were sampled at 31 weeks of age. The direction of the arrows shows how the chickens were mixed at the farms. *All isolates derived from caeca collected from two-day old GPS chickens had the AmpC phenotype. **These Grandparent Stock (GPS) chickens were also treated with amoxicillin-trihydrate and phenoxymethylpenicillin at respectively 2 and 8 weeks of age. PH = Poultry house.*

*4.4.6 Resistance patterns ESBL/AmpC-producing isolates*

ESBL/AmpC-producing *E. coli* derived from GPS chickens from broiler breed A at two-days of age were wild-type susceptible to all non-beta-lactam antibiotics tested, however isolates from these chickens analyzed at 18 weeks of age were non wild-type susceptible to the (fluoro-) quinolones and one isolate was in addition non wild-type susceptible to kanamycin (Table 4.2). Some *E. coli* isolates collected from these animals at 31 weeks of age belonging to PH1 and PH2 were susceptible to all non-beta-lactam antibiotics and a few isolates from chickens housed in PH3 and PH4 displayed non-wild-type susceptibility to the (fluoro) quinolones (Table 4.2). Information from primary breeding company A revealed use of enrofloxacin for three days to prevent mortality from *E. coli* infection at arrival on the GPS rearing farm. This treatment will select for ESBL/AmpC producers that are non-susceptible to fluoroquinolones, which were indeed detected in samples taken at 18 and 31 weeks of age (Table 4.2).

The ESBL/AmpC-producing isolates collected from one-day old GPS chickens from broiler breed B that were analyzed (n=5) showed non wild-type susceptibilities to the non-beta lactam antibiotics: nalidixic acid and ciprofloxacin (n=1), tetracycline, streptomycin and kanamycin (n=2), tetracycline, sulfamethoxazole, nalidixic acid, ciprofloxacin, streptomycin and kanamycin (n=1) and one isolate was susceptible to all non-beta-lactam antibiotics tested.

However, the analyzed isolates from broiler breed A as well as from broiler breed B at PS level displayed co-non wild-type susceptibilities to non-beta-lactam antibiotics. The ones from breed A displayed co-non wild-type susceptibility to gentamicin, tetracycline and sulfamethoxazole or to gentamicin, sulfamethoxazole, chloramphenicol and kanamycin (Table 4.2). The ones from breed B were mainly co-non wild-type susceptible to nalidixic acid and ciprofloxacin or susceptible to all non-beta-lactam antibiotics.

At broiler level, the three out of eight AmpC-producing isolates analyzed displayed either co-non wild-type susceptibility to the non-beta lactam antibiotics ciprofloxacin and naladixic acid (n=1), gentamicin, tetracycline, trimethoprim, ciprofloxacin and naladixic acid (n=1), or displayed susceptibility to all non-beta lactam antibiotics tested (n=1).

*4.4.7 Longitudinal study at broiler farms*

Environmental samples taken from the floor of the poultry house, before the start of the production period, contained ESBL/AmpC-producing *E. coli* in two poultry houses (one out of five pools of five swabs and three out of 25 individual swabs were positive in respectively poultry house A-1 and B-1, (Table 4.3A). All other environmental samples taken at the broiler farms were negative (data not shown).

Feed samples taken in the four poultry houses at the start of the production period, were all negative for ESBL/AmpC-producing *E. coli* (Table 4.3B). Feed samples taken after one week were incidentally positive for ESBL/AmpC-producing *E. coli* (Table 4.3B). This was probably the result of feed contaminated by dust and manure produced by the chickens. Feed samples, from the same batch when sampled outside the poultry house, or directly from the feeding pipe inside the poultry house were negative (Table 4.3B).

The three studied farms obtained their broilers from three different hatcheries. Prevalence of broilers positive for ESBL/AmpC-producing *E. coli* upon arrival at poultry house A-1, B-1, B-2 and C-1 was 0% (95% CI, 0-14%), 16% (95% CI 5-36%), 20% (95% CI 7-41%) and 4% (95% CI 0.1-20%), respectively. After one week prevalence was 100% (95% CI 86-100%), 100% (95% CI 86-100%), 96% (95% CI 80-100%) and 96% (95% CI 80-100%), respectively and remained 100% (95% CI 86-100%) in all poultry houses from week three onwards (Table 4.3A).

***Table 4.2*** *Results of the characterization of ESBL/AmpC genes, other resistance genes and Minimal Inhibitory Concentrations (mg/L) in isolates derived from Grandparents, Parents and broilers.*

| Broiler breed | Lab-code | Source | Code of production farm of origin | Hatching unit/poultry house number | Date of sampling | Material |
|---|---|---|---|---|---|---|
| A | 9 | GPS A 2 days | 11-1♂ | - | 2009 Jul 8 | Caeca |
| | 23 | GPS A 2 days | 22-1♀ | - | 2009 Jul 8 | Caeca |
| | 13 | GPS A 2 days | 22-2♀ | - | 2009 Jul 8 | Caeca |
| | 16 | GPS A 2 days | 33-2♂ | - | 2009 Jul 8 | Caeca |
| | 40 | GPS A 2 days | 44-2♀ | - | 2009 Jul 8 | Caeca |
| | 19 | GPS A 2 days | 44-1♀ | - | 2009 Jul 8 | Caeca |
| | 103 | GPS A 18 weeks | 33-1♂+33-2♂ | 2b | 2009 Nov 9 | Cloacal swab |
| | 138 | GPS A 18 weeks | 44-1♀ | 3 | 2009 Nov 9 | Cloacal swab |
| | 144 | GPS A 18 weeks | 44-1♀ | 3 | 2009 Nov 9 | Cloacal swab |
| | 173 | GPS A 18 weeks | 44-2♀ | 2a | 2009 Nov 9 | Cloacal swab |
| | 309 | GPS A 31 weeks | 33-1♂+33-2♂+44-2♀ | 1 | 2010 Feb 8 | Cloacal swab |
| | 313 | GPS A 31 weeks | 11-1♂+11-2♂+22-1♀+22-2♀ | 2 | 2010 Feb 8 | Cloacal swab |
| | 324 | GPS A 31 weeks | 33-1♂+33-2♂+44-1♀+44-2♀ | 3 | 2010 Feb 8 | Cloacal swab |
| | 325 | GPS A 31 weeks | 33-1♂+33-2♂+44-1♀+44-2♀ | 3 | 2010 Feb 8 | Cloacal swab |
| | 340 | GPS A 31 weeks | 33-1♂+33-2♂+44-1♀ | 4 | 2010 Feb 8 | Cloacal swab |
| | 62[d] | PS A | AA♂ | 8 | 2009 Oct 6 | Meconium |
| | 70 | PS A | CC♀ | 13 | 2009 Oct 6 | Meconium |
| | 255 | broilers A | n.a. | 6 | 2010 Feb 1 | environment hatching unit |
| | 262 | broilers A | n.a. | 7 | 2010 Feb 1 | environment hatching unit |
| | 264 | broilers A | n.a. | 8 | 2010 Feb 1 | environment hatching unit |
| | 237 | broilers A | XXX | 11 | 2010 Feb 1 | Meconium |
| | 265 | broilers A | n.a. | 11 | 2010 Feb 1 | environment hatching unit |
| | 266 | broilers A | n.a. | 11 | 2010 Feb 1 | environment hatching unit |
| | 269 | broilers A | n.a. | 12 | 2010 Feb 1 | environment hatching unit |
| | 272 | broilers A | n.a. | 13 | 2010 Feb 1 | environment hatching unit |
| | 273 | broilers A | n.a. | 14 | 2010 Feb 1 | environment hatching unit |
| | 278 | broilers A | n.a. | 41 | 2010 Feb 1 | environment hatching unit |
| | 281 | broilers A | n.a. | 46 | 2010 Feb 1 | environment hatching unit |
| | 243 | broilers A | WWW | 47 | 2010 Feb 1 | Meconium |
| | 283 | broilers A | n.a. | 47 | 2010 Feb 1 | environment hatching unit |
| | 286 | broilers A | n.a. | 47 | 2010 Feb 1 | environment hatching unit |
| | 235 | broilers A | ZZZ | 48 | 2010 Feb 1 | Meconium |

| | MIC(mg/L)[c] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Results PCR and sequencing[a,b]** | **AMP** | **FOT** | **TAZ** | **GEN** | **TET** | **SMX** | **TMP** | **CIP** | **NAL** | **CHL** | **FFN** | **STR** | **KAN** | **COL** |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 0.5 | 4 | <=8 | <=0.5 | 0.03 | 8 | 16 | 16 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | <=1 | <=8 | <=0.5 | 0.015 | <=4 | 4 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 2 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | <=1 | <=8 | <=0.5 | 0.015 | <=4 | 4 | 4 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | >16 | 1 | 4 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 0.5 | 4 | <=8 | 1 | 0.03 | 8 | 16 | 16 | 4 | <=4 | <=2 |
| *bla*$_{TEM-52c}$ | >32 | >4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.12 | >64 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | >16 | 1 | 2 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 16 | 16 | <=4 | <=2 |
| *bla*$_{TEM-52c}$ | >32 | >4 | 4 | 1 | <=1 | <=8 | <=0.5 | 0.25 | 64 | 4 | <=2 | 8 | 16 | <=2 |
| *bla*$_{TEM-52c}$ | >32 | >4 | 16 | 2 | 4 | <=8 | <=0.5 | 0.5 | >64 | 16 | 16 | 8 | <=4 | <=2 |
| *bla*$_{TEM-52c}$ | >32 | >4 | 8 | 1 | 4 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{TEM-52c}$ | >32 | >4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{TEM-52}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.25 | >64 | 8 | 8 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | <=1 | <=8 | <=0.5 | 0.25 | >64 | 4 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CTX-M-2}$ | >32 | >4 | 2 | >32 | 2 | >1024 | <=0.5 | 0.015 | <=4 | 64 | 8 | 8 | >128 | <=2 |
| *bla*$_{CTX-M-1}$ | >32 | >4 | 1 | 4 | 64 | >1024 | <=0.5 | 0.015 | <=4 | 4 | <=2 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | <=1 | <=8 | <=0.5 | 0.25 | >64 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{TEM-20*}$ | >32 | >4 | 8 | >32 | >64 | <=8 | >32 | >8 | >64 | 8 | 8 | 8 | 8 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 4 | <=8 | <=0.5 | 0.25 | >64 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.12 | 64 | 8 | 4 | 8 | <=4 | <=2 |
| *ampC* type 3 | >32 | 2 | 4 | 1 | 64 | <=8 | <=0.5 | 0.03 | <=4 | 4 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.12 | 64 | 8 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.25 | >64 | 8 | 8 | 4 | <=4 | <=2 |
| *bla*$_{TEM-20}$* | >32 | 4 | 1 | 1 | 2 | <=8 | <=0.5 | 0.5 | >64 | 8 | 16 | 8 | <=4 | <=2 |
| *bla*$_{SHV-12}$ | >32 | 4 | 16 | 1 | 4 | >1024 | >32 | 0.5 | 16 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CTX-M-1}$ | >32 | >4 | 1 | 1 | >64 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | >32 | >64 | <=8 | >32 | 8 | >64 | 8 | 8 | 8 | 8 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | >32 | >64 | <=8 | >32 | >8 | >64 | 8 | 8 | 8 | 8 | <=2 |
| *bla*$_{CTX-M-1}$ | >32 | >4 | 2 | 1 | >64 | >1024 | >32 | 0.03 | <=4 | 8 | 8 | 128 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 4 | 4 | 8 | <=4 | <=2 |

*Continued*

***Table 4.2*** *Continued*

| Broiler breed | Lab-code | Source | Code of production farm of origin | Hatching unit/poultry house number | Date of sampling | Material |
|---|---|---|---|---|---|---|
| | 287 | broilers A | n.a. | 48 | 2010 Feb 1 | environment hatching unit |
| | 293 | broilers A | n.a. | 53 | 2010 Feb 1 | environment hatching unit |
| B | 494 | GPS B | 1 | - | 2010 Nov 21 | Meconium |
| | 498 | GPS B | 2 | - | 2010 Nov 21 | Meconium |
| | 506 | GPS B | 3 | - | 2010 Nov 21 | Meconium |
| | 529 | GPS B | 4 | - | 2010 Nov 21 | Meconium |
| | 531 | GPS B | 5 | - | 2010 Nov 21 | Meconium |
| | 348 | PS B | n.a. | 27 | 2010 Mar 17 | environment hatching unit |
| | 350 | PS B | n.a. | 28 | 2010 Mar 17 | environment hatching unit |
| | 394[e] | PS B | ZZ | 28 | 2010 Mar 17 | Meconium |
| | 354 | PS B | n.a. | 29 | 2010 Mar 17 | environment hatching unit |
| | 425[e] | PS B | SS | 29 | 2010 Mar 17 | Meconium |
| | 432[f] | PS B | VV | 29 | 2010 Mar 17 | Meconium |
| | 442 | PS B | UU | 29 | 2010 Mar 17 | Meconium |
| | 361 | PS B | n.a. | 30 | 2010 Mar 17 | environment hatching unit |
| | 453[f] | PS B | VV | 30 | 2010 Mar 17 | Meconium |
| | 365 | PS B | n.a. | 31 | 2010 Mar 17 | environment hatching unit |
| | 476 | PS B | XX | 31 | 2010 Mar 17 | Meconium |
| | 369 | PS B | n.a. | 32 | 2010 Mar 17 | environment hatching unit |
| | 491 | PS B | XX | 32 | 2010 Mar 17 | Meconium |

| | MIC(mg/L)[c] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Results PCR and sequencing** | **AMP** | **FOT** | **TAZ** | **GEN** | **TET** | **SMX** | **TMP** | **CIP** | **NAL** | **CHL** | **FFN** | **STR** | **KAN** | **COL** |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 2 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 4 | 4 | 16 | 8 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | >16 | 1 | 2 | <=8 | <=0.5 | 0.25 | >64 | 4 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | >64 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 8 | >128 | >128 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | >64 | <=8 | <=0.5 | 0.03 | <=4 | 8 | 8 | >128 | >128 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | >64 | >1024 | <=0.5 | 0.12 | 32 | 8 | 4 | 128 | >128 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 4 | <=8 | <=0.5 | 0.015 | <=4 | 4 | 4 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | <=1 | >1024 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 0.5 | >64 | <=8 | <=0.5 | 0.25 | >64 | 4 | 4 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 2 | <=8 | <=0.5 | 0.12 | 32 | 4 | 4 | 16 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | 4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 8 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | 4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 4 | 8 | <=4 | <=2 |
| *ampC* type 40 | >32 | 1 | 1 | 0.5 | 2 | <=8 | <=0.5 | 0.25 | >64 | 8 | 16 | 4 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | 4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.015 | <=4 | 8 | 4 | 16 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | <=1 | <=8 | <=0.5 | 0.5 | >64 | 8 | 8 | 8 | <=4 | <=2 |
| *ampC* type 40 | >32 | 2 | 4 | 1 | <=1 | <=8 | <=0.5 | 0.25 | >64 | 4 | 4 | 16 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 8 | 1 | >64 | <=8 | <=0.5 | 0.25 | >64 | 4 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | 4 | 8 | 1 | 2 | <=8 | <=0.5 | 0.5 | >64 | 4 | 4 | 8 | <=4 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | 16 | 1 | 4 | <=8 | <=0.5 | 0.5 | >64 | 8 | 8 | 8 | >128 | <=2 |
| *bla*$_{CMY-2}$ | >32 | >4 | >16 | 1 | 4 | <=8 | <=0.5 | 0.5 | >64 | 8 | 4 | 8 | <=4 | <=2 |

[a]With the CTX-M-2 primers used for sequencing, no distinction can be made between *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-97}$.
[b]With the SHV primers used for sequencing, no distinction can be made between *bla*$_{SHV-12}$ and *bla*$_{SHV-129}$.
[c]Shaded values were considered reduced susceptible according to the EUCAST epidemiological cut-off values (www.eucast.org) and (if not available) according to CLSI clinical breakpoints (20).
[d]Isolates that were derived from offspring of breeding chickens treated with amoxicillin.
[e] Isolates that were derived from offspring of breeding chickens treated with tylosin.
[f] Isolates that were derived from offspring of breeding chickens treated with enrofloxacin.
[g]*bla*$_{TEM-20}$ with silent mutations +144G->A, +480C->T and +723A->G compared to reference sequence *bla*$_{TEM-20}$ Y17581 (nucleotide position according to amino acid count at www.lahey/studiers.org).

***Table 4.3*** *Prevalence of broilers positive for ESBL/AmpC-producing* E. coli *and the presence of these isolates in broiler feed at three commercial broiler farms measured in four poultry houses at arrival at the farm till week five between September 2010 and February 2011.*

A

| **Poultry house** | **Prevalence of ESBL/AmpC-producing E. coli in n=25 broilers (%, 95%CI), number of isolates with AmpC(A) or ESBL(E) phenotype** | | | | | |
|---|---|---|---|---|---|---|
| **Week** | **0** | **1** | **2** | **3** | **4** | **5** |
| A-1[a] | 0 (0-14) | 100 (86-100) 19A , 6E | 100 (86-100) 15A, 9E | 100 (86-100) 9A, 16E | 100 (86-100) 14A, 11E | 100 (86-100), 14A, 11E |
| B-1[a] | 16 (4.5-36) 4A | 100 (86-100) 8A, 17E | 92 (74-99) 17A, 8E | 100 (86-100) 21A, 4E | 100 (86-100) 18A, 7E | 100 (86-100) 14A, 11E |
| B-2 | 20 (6.8-41) 1A+E, 2A, 2E | 96 (80-100) 19A, 5E | 100 (86-100) 24A, 1E | 100 (86-100) 24A, 1E | 100 (86-100) 24A, 1E | 100 (86-100) 1A+E, 17A, 7E |
| C-1 | 4 (0.1-20) 1A | 96 (80-100) 1A, 23E | 100 (86-100), 3A, 22E | 100 (86-100) 3A, 22E | 100 (86-100) 9A, 16E | 100 (86-100) 1A+E, 8A, 16E |

## 4.5 Discussion

In this study we demonstrate the presence of ESBL/AmpC-producing *E. coli* isolates at all levels in the broiler production pyramid. At the top of the pyramid the prevalence is lower than found in the longitudinal study at broiler production farms at the bottom of the pyramid.

The broiler production chain is very complex with only a few primary breeding companies at the top of the pyramid that produce broilers all over the world. This results in a vulnerable system. If a disease, or in this case, antibiotic resistant bacteria enter the production chain they may be transferred globally. In 2010, Sweden reported the presence of *E. coli* carrying $bla_{CMY-2}$ positive strains in imported GPS chickens at their arrival in Sweden [21]. In the Netherlands, like in many other countries, the same hybrid chickens as in Sweden are used (www.sva.se). The

| B | | | | | |
|---|---|---|---|---|---|
| **Presence of ESBL/AmpC-producing E. coli in broiler feed[b], AmpC(A) or ESBL(E) phenotype** | | | | | |
| **0** | **1** | **2** | **3** | **4** | **5** |
| - | - | + (E) | - | - | - |
| - | + (E) | + (A) | - | - | -[c] (E) |
| - | - | - | - | - | +[c] (E) |
| - | - | - | + (E) | +[c] (E) | + (E) |

[a] '-' means absence of ESBL/AmpC-producing *E. coli* in the feed sample taken in the particular poultry house in the indicated week;''+' means presence of ESBL/AmpC-producing *E. coli* in the feed sample taken at the particular poultry house in the indicated week.

[a]in these poultry houses ESBL/AmpC-positive swabs were obtained from the floor after disinfection of the poultry house and before the chickens were housed in these poultry houses.

[b]Feed samples taken from outside the poultry house (B-1 and B-2) or taken directly from the feeding pipe inside the poultry house (C-1) were negative for ESBL/AmpC-producing *E. coli.*

present study confirms these findings and illustrates how these transferable resistance genes can spread in globally organized production systems.

In both broiler breeds examined, the plasmid-mediated AmpC gene $bla_{CMY-2}$ was imported into the Netherlands in GPS animals derived from either the UK or via eggs from the US. This *ampC*-gene was originally found in the chromosome of *Citrobacter freundii* and has spread world-wide now as a plasmid-mediated gene in many different gram-negative bacterial species. It occurs not only in isolates from poultry in Europe and the US and other parts of the world[12,22,23], but also in isolates derived from other animals and from humans [6, 24]. How it has entered the top of the poultry production pyramid is still unknown.

Isolates derived from broilers at five or six weeks of age, just before slaughter, can

carry multiple types of ESBL/AmpC genes[12,14,23]. Although at the top of the production pyramid only $bla_{CMY-2}$ was found, our results also show the presence of other genes ($bla_{TEM-52}$, $bla_{TEM-52c}$, $bla_{CTX-M-1}$ and $bla_{CTX-M-2}$) at other levels of the production pyramid, probably due to environmental contamination.

ESBL/AmpC-producing isolates often are multidrug-resistant and co-resistance can be transferred along with ESBL/AmpC genes when located on similar plasmids[25]. The $bla_{CMY-2}$ isolates from one-day old GPS birds from broiler breed A were completely susceptible to all non-beta-lactam antibiotics tested. In contrast, in the $bla_{CMY-2}$-positive isolates of GPS chickens from broiler breed B co-non wild-type susceptibilities to tetracycline, streptomycin, kanamycin, sulfamethoxazole and naladixic acid was found. Antibiotic treatment data from GGPS and pedigree stock from breed A and B showed no treatment of the particular flocks. This could indicate that the multi-resistance found in isolates from broiler breed B resulted from circulation of these isolates at the breeding farm or hatchery derived from earlier rounds.

At PS-level we again observed a difference in phenotype of the ESBL/AmpC-producing *E. coli* derived from one-day old chickens of breed A (multi co-non-wild-type susceptible) compared to the ones from breed B (solely co-non-wild type susceptible to fluoroquinolones), which might be explained by the differences in antibiotic treatments of the parents between breed A and B. Day-old chickens can inherit bacterial isolates from their parents, or from the environment [15,26]. In the hatching units of the PS-hatchery of breed A formaldehyde gas is used in low concentrations for infection control during hatching. As a result, no ESBL/AmpC-producing isolates were found in the environmental samples of the hatching units at this hatchery. This indicates that the vertical transmission route through contaminated eggs[15,17] is the most likely route of transmission in these chickens. This is important information for intervention measures and should be further investigated.

The observation in the longitudinal study at the broiler farms, that after the first week all broilers shed ESBL/AmpC-producing *E. coli* is striking. We recently described a high prevalence of six-week-old broilers (sampled a few days before slaughter) carrying ESBL/AmpC-producing *E. coli*[11]. A similar high percentage broiler meat with ESBL/AmpC producers is found in Dutch supermarkets[7-9]. This is reason for concern as the genes and plasmids found in isolates derived from broilers are also found in human clinical isolates[8, chapter 6] and indications that transmission to humans through the food chain takes place has been documented[3].

During the first hours post hatch *E. coli* isolates (either antibiotic resistant or susceptible) are taken up by the chickens from the environment, although vertical transmission of isolates has also been described[15,17]. Directly after hatch it takes between a few hours to a few days until *E. coli* has proliferated and has colonized the intestine of the chickens[27,28]. Therefore analyzing cloacal swabs a few hours post hatch might give variable results. A rapid increase of cloacal samples positive for ESBL/AmpC-producing *E. coli* in the first week at the broiler production farms might reflect the differences in colonization time between individual broilers. This may be the result of uptake of these isolates at the hatchery, or from feed and environmental sources at the broiler production farm[29]. No ESBL/AmpC-producing *E. coli* isolates were found in the feed of the broilers during the first week, but sampling of the environment of the poultry house before the birds were placed there, resulted in the confirmation of ESBL/AmpC presence in two out of four poultry houses even after intensive cleaning and disinfection of the poultry house. This indicates that recirculation of resistant strains from earlier production rounds can play a role in contaminating the consecutive flock.

The rapid increase at broiler level was in contrast to the presence of ESBL/AmpC-

producing isolates at GPS level. In the GPS chickens, except when treated with beta-lactam antibiotics, at 18 and 31 weeks of age no rapid increase in the number of chickens positive for ESBL/AmpC-producing *E. coli* was observed and the results even indicated a decrease in the number of ESBL/AmpC-producing isolates per sample. Compared to broilers, GPS chickens are housed at a very high level of biosecurity and are much less frequently treated with antibiotics. Feed composition differs also between GPS and broiler chickens. One of the main differences in the feed is the addition of anticoccidial compounds (nicarbazin, narasin, salinomycin or monensin) to the feed of broilers, while breeding chickens are vaccinated against coccidiosis[30]. Diarra and co-workers, demonstrated the presence of more ceftiofur resistant isolates in broilers fed with salinomycin compared to broilers fed either with non-supplemented feed or feed supplemented with bambermycin, penicillin, bacitracin or a combination of salinomycin plus bacitracin[31]. However data to explain this phenomenon were not given and this should be further investigated.

In a Belgian study risk factors for ceftiofur resistance at broiler farms were determined[32]. Next to antibiotic use at the farm, management factors as well as the hatchery from where the chickens originated were risk factors for the presence of a high level of ceftiofur resistance in five week old chickens at broiler production farms[32]. This last risk factor is explained by (off-label) ceftiofur use at some broiler hatcheries in Belgium, which could have selected for these isolates. In the Netherlands, like in Belgium, third generation cephalosporins are not licensed for treatment of broilers, however also in the Netherlands up to the spring of 2010 ceftiofur has been used off-label in hatcheries[33]. The relation between the use of ceftiofur at hatcheries and the occurrence of extended spectrum cephalosporin (ESC) resistant *Salmonella* and *E. coli* in broilers has previously been described in Canada[34]. Although at the time of our longitudinal study at broiler farms, ceftiofur use at hatcheries had been stopped for almost six months[33], all broilers still became rapidly positive for ESBL/AmpC-producing *E. coli*. Whether this could be due to recirculation of these strains in the environment of the hatchery or broiler farm has to be determined. It indicates that to reduce the prevalence of ESBL/AmpC-producing isolates at broiler farms other interventions than stopping antibiotic use at the hatchery are necessary as well.

To conclude, it has been demonstrated that ESBL/AmpC-producing isolates are found at every level of the broiler production pyramid. At broiler production farms these isolates spread very fast, leading to high prevalence. The high prevalence at broiler production farms are a reason for serious concern as they may enter the food chain. Vertical transmission, as well as recirculation of these isolates at farms and hatcheries may play a role. Therefore future research should not only evaluate interventions implemented at broiler farms, but should also take into account interventions implemented at hatcheries and breeding farms.

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# Chapter 5

# Rapid detection of TEM, SHV and CTX-M extended spectrum beta-lactamases in Enterobacteriaceae using ligation-mediated amplification with microarray analysis

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## 5.1 Abstract

Fast and adequate detection of extended-spectrum beta-lactamases (ESBLs) is crucial for infection control measures and the choice of antimicrobial therapy. The aim of this study was to develop and evaluate a novel ESBL assay using ligation-mediated amplification combined with microarray analysis to detect the most prevalent ESBLs in *Enterobacteriaceae*: TEM, SHV and CTX-M.

Analysis of the Lahey database revealed that the vast majority of TEM and SHV ESBLs differ from non-ESBL variants in 3 amino acid positions. TEM ESBLs have at least one of the following amino acid substitutions: R164S/H/C, G238D/N/S, and E104K. In SHV ESBLs, one or more of the following substitutions is observed: D179A/N/G, G238S/A, and E240K. Oligonucleotide probes were designed to detect these substitutions, covering 95% of ESBL TEM and 77% of ESBL SHV variants. In addition, probes were designed to distinguish between CTX-M groups 1, 2, 9 and 8/25. For evaluation of the assay, 212 *Enterobacteriaceae i*solates with various beta-lactamases were included (n=106 ESBL positive).

The sensitivity of the microarray was 101/106 (95%; 95%CI 89-98%), and the specificity 100% (95%CI 97-100%) using molecular characterization of ESBLs by PCR and sequencing as reference. Assay performance time was 8 h for 36 isolates.

This novel commercially available DNA microarray system may offer an attractive option for rapid and accurate detection of CTX-M, TEM and SHV ESBL genes in *Enterobacteriaceae* in the clinical laboratory.

## 5.2 Introduction

Rapid and adequate extended-spectrum beta-lactamase (ESBL) detection is crucial for infection control measures and the choice of antimicrobial therapy. Because phenotypic detection of ESBLs is time consuming and the results may be difficult to interpret, a faster and accurate detection method is desirable. A microarray-based genotypic test may meet these demands. However, published data on microarrays for ESBL detection are sparse.

The aim of this study was to develop and evaluate a genotypic assay, designed to identify the most prevalent ESBLs in the clinical setting (TEM, SHV and CTX-M)[1-3]. TEM and SHV ESBLs differ from their ancestral non-ESBL TEM and SHV penicillinases by one or more amino acid substitutions[4-6]. Genotypic detection of TEM and SHV ESBL mutations therefore requires identification of single nucleotide polymorphisms (SNPs). CTX-M ESBLs can be divided into five clusters, i.e. group 1, 2, 8, 9, and 25 based on their sequence similarity[7].

In this study, ligation-mediated amplification combined with detection of amplified products on a microarray was used to detect the various CTX-M groups and the ESBL-associated SNPs in TEM and SHV variants (Figure 5.1).

## 5.3 Materials and methods

### *5.3.1 Probe design*

To identify essential SNPs associated with the ESBL phenotype, sequences of all listed TEM variants (n=175) and SHV variants (n=128) in the Lahey database (http://www.lahey.org/Studies/) as of 6 September 2009 were related to phenotypes described in the literature

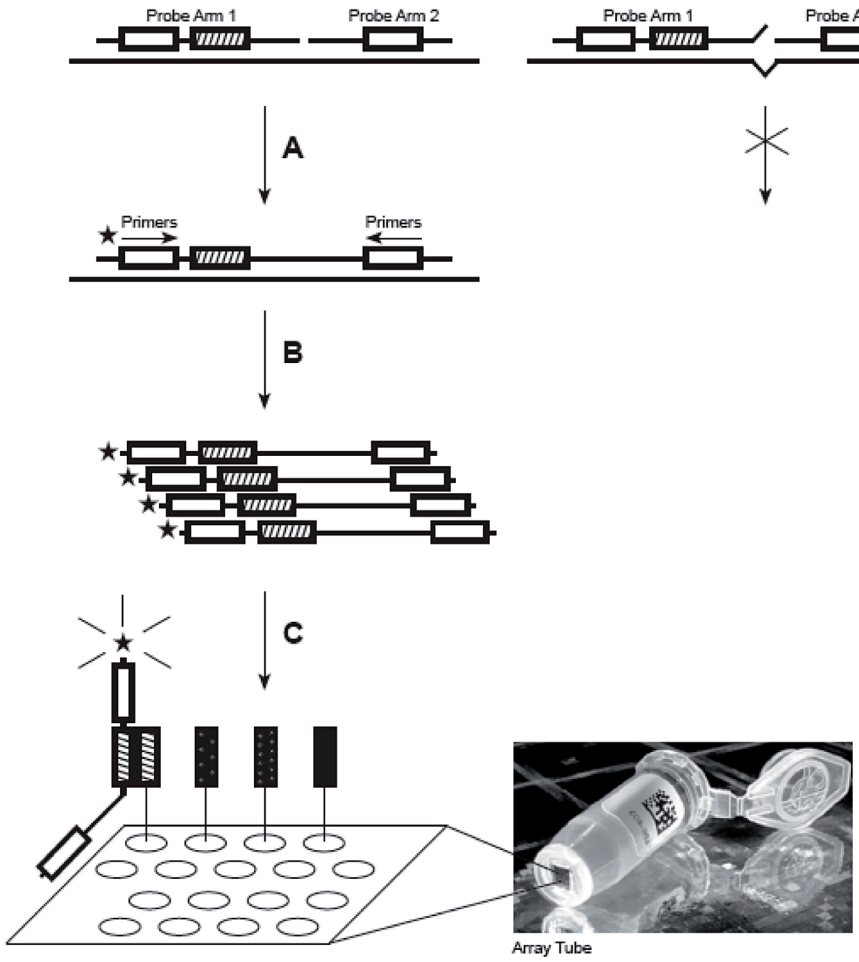



***Figure 5.1:*** *Principle of the microarray system (adapted from Wattiau* et al.[8]*). (A) Probe arms hybridise to the target sequence resulting in a double-stranded molecule with a single-stranded nick. This nick will subsequently be ligated by the DNA ligase in case of a perfect double-stranded match, connecting the two probe arms (upper left). Probe arms will not be connected in case of a mismatch (upper right). White boxes indicate generic primer binding sites that are identical in each target-specific probe. The dashed box indicates the ZIP sequence, specific for each probe, and complementary to its cZIP counterpart on the microarray. (B) Only successfully ligated probe arms will be amplified by PCR using a single primer pair, generating biotinylated products, because one of the primers has a 5-biotin moiety. A single pair of primers will co-amplify all target-specific probes, because they share the generic primer binding sites. (C) Detection of each target-specific amplification product at a specific position on the microarray is achieved through unique ZIP codes (boxes with various patterns). PCR products are hybridised to the microarray and visualized using colorimetric detection with streptavidin-horseradish peroxidase and tetramethyl benzidine.*

***Table 5.1:*** *Phenotypes of TEM variants and corresponding amino acid substitutions that may be detected by the microarray system.*

| Phenotype according to Lahey database | TEM variants | ESBL associated amino acid substitutions at positions detected by microarray | | | Expected microarray result |
|---|---|---|---|---|---|
| | | E 104 K | R 164 S/C/H | G 238 S | |
| Non ESBL | TEM-1, 2, 13, 30-40, 44, 45, 51, 54, 55, 57-59, 65, 67, 73, 74, 76-84, 90, 95, 97-99, 103, 110, 122, 127, 128, 135, 141, 145, 159, 160, 163 | - | - | - | Negative |
| ESBL | TEM-17, 18, 56, 106 | + | - | - | Positive |
| ESBL | TEM-9, 24, 26, 46, 60, 63, 64, 121, 129, 130, 131, 133, 149 | + | + S | - | Positive |
| ESBL | TEM-87 | + | + C | - | Positive |
| ESBL | TEM-6, 16, 43, 109 | + | + H | - | Positive |
| ESBL | TEM-167 | + | - | + | Positive |
| ESBL | TEM-3, 4, 15, 21, 22, 50, 52, 66, 88, 89, 92, 94, 113, 138, 139 | + | - | + | Positive |
| ESBL | TEM-8 | + | + S | + | Positive |
| ESBL | TEM-107, 134 | + | + H | + | Positive |
| ESBL | TEM-11, 27, 28, 29, 61, 75, 115, 118, 132, 147, 151, 152 | - | + H | - | Positive |
| ESBL | TEM-91, 143, 144 | - | + C | - | Positive |
| ESBL | TEM-5, 7, 10, 12, 53, 85, 86, 102, 114, 125, 136, 137, 155, 158 | - | + S | - | Positive |
| ESBL | TEM-19, 20, 25, 42, 47, 48, 49, 68, 71, 72, 93, 101, 112, 120 | - | - | + | Positive |
| **ESBL** | **TEM-126, 157[b], 164, 169[b]** | **-** | **-** | **-** | **Negative** |
| Unknown | TEM-111, 124, 142 | + | - | - | Positive |
| Unknown | TEM-123 | + | - | + | Positive |
| Unknown | TEM-165 | - | + S | - | Positive |
| Unknown | TEM-116 | - | - | - | Positive |
| Unknown | TEM-161 | + | + | - | Positive |
| Unknown | TEM-70, 96, 104, 105, 108, 117, 146, 148, 150,162,166, 168, 170-174 | - | - | - | Negative |
| Unknown[a] | TEM-14, 23, 41, 62, 69, 100, 119, 140, 153, 154, 156, 175 | Not applicable | | | |

[a] TEM number reserved or withdrawn from Lahey database; [b]ESBL phenotype according to Lahey database, but published evidence insufficient to determine phenotype (References: TEM-157[17]; TEM-169: no published data found in Pubmed or Embase).

*Table 5.2: Phenotypes of SHV variants and corresponding amino acid substitutions that may be detected by the microarray system.*

| Phenotype according to Lahey database | SHV variants | ESBL associated amino acid substitutions at positions detected by microarray | | | Expected microarray result |
|---|---|---|---|---|---|
| | | D 179 A/N/G | G 238 S/A | E 240 K | |
| Non ESBL | SHV-1, 11, 14, 19, 25, 26, 32, 33, 43, 44, 48, 49, 56, 60-62, 71-83, 85, 89 | - | - | - | Negative |
| **Non ESBL** | **SHV-10[c]** | **-** | **+ S** | **+K** | **Positive** |
| **Non ESBL** | **SHV-20[d], 21[d]** | | **+ S** | | **Positive** |
| **Non ESBL** | **SHV-22[d]** | | **+S** | **+K** | **Positive** |
| ESBL | SHV-6 | + A | - | - | Positive |
| ESBL | SHV-8 | + N | - | - | Positive |
| ESBL | SHV-24 | + G | - | - | Positive |
| ESBL | SHV-2, 2a, 3, 30, 34, 39, 86 | - | + S | - | Positive |
| ESBL | SHV-13, 102 | - | + A | - | Positive |
| ESBL | SHV-4, 5, 7, 9, 12, 15, 45, 46, 55, 64, 66, 105 | - | + S | + K | Positive |
| ESBL | SHV-18 | - | + A | + K | Positive |
| ESBL | SHV-31, 115 | - | - | + K | Positive |
| **ESBL** | **SHV-16, 27[e], 38[f], 40[e], 41[e], 42[e], 57, 70,** | **-** | **-** | **-** | **Negative** |
| Unknown | SHV-29 | - | + A | - | Positive |
| Unknown | SHV-106 | - | + S | - | Positive |
| Unknown | SHV-123, 124 | - | + S | + K | Positive |
| Unknown | SHV-97, 126 | - | - | + K | Positive |
| Unknown[a] | SHV-28, 35-37, 50-53, 59, 63, 65, 67, 69, 92-96, 101, 103, 104, 107, 108, 110, 111, 113, 114, 116, 117, 121, 125, 127 | - | - | - | Negative |
| Unknown[b] | SHV-17, 23, 47, 54, 58, 68, 84, 87, 88, 90, 91, 98-100, 109, 112, 118-120, 122 | Not applicable | | | |

[a]Detected as non-ESBL; [b]SHV number reserved or withdrawn; [c]SHV-10 has a unique substitution (S130G) reversing the extension of the beta-lactamase spectrum conferred by G238S and E240K[18]; [d]Non-ESBL phenotype according to the Lahey database, but ESBL phenotype according to literature including the reference from the Lahey database[25]; [e]ESBL phenotype according to Lahey database, but published evidence contradictory (SHV-27[10,19,20,26-28]; SHV-40[21-23]; SHV-4[10, 23]; SHV-42[23]); [f]Chromosomally encoded Class A carbapenemase[24].

(www.pubmed.gov). In line with previous reports, it was concluded that 95% (84 of 88) of the TEM variants with an established ESBL phenotype have one or more amino acid substitution at Ambler's position 104, 164, or 238, and 77% (27 of 35) of ESBL SHV variants have substitutions at position 179, 238 or 240 (Tables 5.1 and 5.2)[4, 6].

Based on these findings, 10 oligonucleotide probes (Figure 5.2) were designed for identification of TEM variants (3 probes for non-ESBL variants, 5 for ESBL variants, and 2 for discrimination of TEM-116). The probes detect the following ESBL- associated amino acid substitutions: E104K, R164S/C/H and G238S. Four rare ESBL TEM variants (TEM-126, -157, -164, -169) will not be detected because they lack these substitutions. The microarray system reports TEM-116 as ESBL-positive although the Lahey database categorizes TEM-116 as an unknown phenotype and publications on the phenotype of TEM-116 are controversial.

For identification of SHV variants, 10 oligonucleotide probes (Figure 5.2) were designed (3 probes for non-ESBL variants, one for exclusion of SHV-10, and 6 for ESBL variants) to detect the following ESBL associated substitutions: D179A/N/G, G238S/A, and E240K. The eight ESBL SHV variants that are missed by the microarray are SHV-16,-27,-38,-40,-41,-42,-57,-70. However, data on the ESBL phenotype of SHV-27, -40, -41, and -42 are controversial.

Four probes were designed for discrimination between CTX-M groups CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M 8/25. The detection of the CTX-M-8 and -25 groups was combined because of the low prevalence of these gene clusters.

*5.3.2 Consideration on design of the probes*

*TEM variants*: The Lahey database contained 175 TEM variants. According to this database, no data were available for 12 variants (reserved, withdrawn or not listed), 24 variants had an unknown phenotype, 51 variants had an non-ESBL beta-lactamase phenotype, and 88 had an ESBL phenotype of which 95% (84/88) had at least one of the following amino acid substitutions: R164 S/H/C (n= 50 variants), G238 D/N/S (n= 30 extra variants), E104K (n=4 extra variants) (Table 5.2). These substitutions were not observed in non-ESBL TEM variants. Based on these findings, 10 oligonucleotide probes (Figure 5.2) were designed for identification of TEM variants (3 probes for non-ESBL variants, 5 for ESBL variants, and 2 for identification of TEM-116). Two probes for detection of TEM-116 (presence of V84I substitution and absence of N100S substitution) were incorporated in the assay, because TEM-116 has been reported as an ESBL in several publications and >50 isolates with this TEM variant have been reported[9-12].

The microarray system reports TEM-116 as ESBL-positive and was able to detect and identify TEM-116 in one isolate (data not shown). However, the Lahey database categorizes TEM-116 as an unknown phenotype and publications on the phenotype of TEM-116 are controversial. Contamination of several *Taq* polymerases by TEM-116 may have played a role in experiments attempting to establish the ESBL phenotype of this TEM variant[13].

Probes for detection of substitutions G238D and G238N, described exclusively in TEM-111 and TEM-142, (unknown phenotypes according to the Lahey database; no published data available) were not incorporated in the assay, because these two TEM variants also harbour the E104K substitution, allowing detection by the microarray system.

Four ESBL TEM variants (TEM-126, -157, -164 and -169) do not contain any of the ESBL-associated substitutions mentioned above, and are therefore not detected by the array (Table 5.1). In the literature, TEM-126[14, 15]and TEM 164[16], have each been reported in one clinical isolate. TEM-157 and TEM-169 also have an ESBL phenotype according to Lahey database, but

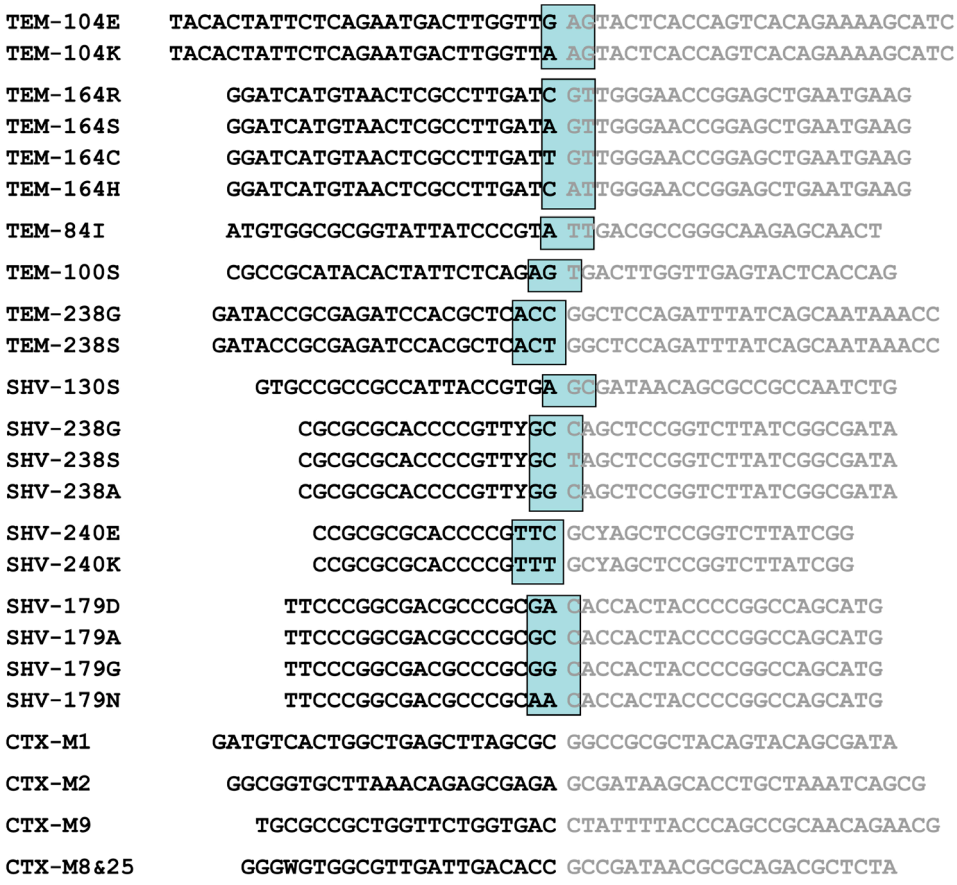



***Figure 5.2*** *Nucleotide sequences of the 24 probes for ligation-mediated amplification and considerations on design of the probes. The part of the probe complementary to TEM, SHV and CTX-M genes is shown. The part of the probe containing the primer binding sites, and ZIP codes for hybridization to micro-array are not shown. To the left, probe names are shown with gene name (TEM, SHV, CTX-M1, 2, 9 and 8/25) and relevant amino acid position of the mutations followed by a single letter indicating the amino acid codon detected by the probe. The upstream and downstream target-specific segments of each probe are shown. Boxes in the TEM and SHV probes represent codons detected by the probes. Note that probes for TEM-238, SHV-238 and SHV-240 are in antisense orientation.*

published evidence is insufficient to determine the phenotype of TEM-157[17], and no published data were found on TEM-169 in Pubmed or Embase. Finally, 5 TEM variants (TEM-111, -123, -124, -142, and -165) with unknown phenotypes according to the Lahey database contain at least one ESBL-associated substitution and will therefore be identified as ESBL positive by the assay. In the literature, SNPs at TEM positions 237 and 240 have also been associated with the ESBL phenotype. However, since ESBLs with substitutions at position 237 and 240 also have substitutions at position 104, 164 or 238 (http://www.lahey.org/Studies/), probes for detection

of these SNPs were not included in the assay.

*SHV Variants:* The Lahey database contained 128 SHV variants. According to this database, no data were available for 20 variants, 38 variants had an unknown phenotype, 35 variants had a non-ESBL phenotype, and 35 had an ESBL phenotype of which 77% (27/35) had at least one of the following mutations on amino acid position: D179 A/N/G (n=3 variants), G238 S/A (n=22 extra variants) and E240K (n=2 extra variants) (Table 5.2). SHV-10 has a non-ESBL phenotype, although it harbours the G238S and E240K substitutions due to a unique compensatory mutation at amino-acid position 130 (S to G) precluding the extension of the beta-lactamase spectrum[18].

For identification of SHV variants, 10 oligonucleotide probes were designed (3 probes for non ESBL variants, one for exclusion of SHV-10, and 6 probes for ESBL variants) (Figure 5.2). Since ESBL variants SHV-16, 57, and 70 do not possess one of the ESBL-associated substitutions mentioned above, the microarray system does not identify these SHV variants as ESBL. Five other SHV-genes (SHV-27, -38, -40, -41, -42) with an ESBL phenotype according to the Lahey database also lack these substitutions and are consequently not identified as ESBL. However, the published evidence on the phenotype is contradictory for four of these variants: SHV-27[10, 19, 20], SHV-40[21-23], SHV-41[10, 23], SHV-42[23], and SHV-38 is a chromosomally encoded Class A carbapenemase[24]. On the other hand, SHV-20,-21, and -22 are categorized all as non-ESBL in the Lahey database, while they are reported as ESBL in the literature including the reference from the Lahey database[25]. In line with the published data, these three SHV variants are detected as ESBL-positive by the microarray system, since they all harbour at least one ESBL-associated mutation (Table 5.2).

*CTX-M variants:* An alignment was made of the CTX-M sequences mentioned in the Lahey database using ClustalX Software and 4 probes were designed based on conserved regions, where near sequence identity is observed within each group and there is strong discrimination between the groups (CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M 8/25 group). The detection of the CTX-M-8 and -25 groups was combined because of the low prevalence of these gene clusters.

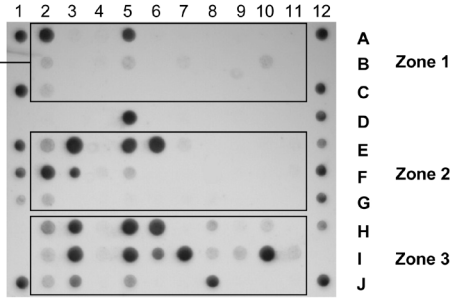
### *5.3.2 Ligation-mediated amplification and microarray analysis*

The ESBL Array (Check-Points B.V., Wageningen, the Netherlands) was supplied as a kit. Microarray images were generated using a microarray reader (ArrayTube Reader, ClonDiag Chip Technologies, Jena, Germany) connected to a computer running dedicated software for analysis of the images. The software indicates whether a TEM-, SHV- or CTX-M ESBL or a combination is detected and specifies the CTX-M group. By visual inspection of the microarray pictures the accuracy of individual probes in identifying specific SNPs was determined. Three isolates can be analysed in parallel on one microarray (Figure 5.3). The performance time of the microarray was 8 h per 36 isolates (3h DNA isolation; and 5 h ligation, amplification and detection).

### *5.3.3 Test isolates*

A total of 212 genotypically and phenotypically well-characterized *Enterobacteriaceae* were tested (Tables 5.3 and 5.4). The collection contained 106 ESBL-positive and 106 ESBL-negative isolates (1 isolate per patient) of human (132) and veterinary (80) origin. An overview of the ESBL genes present is displayed in Table 5.1. With this collection, 20 of the 24 probes for ESBL detection could be evaluated, while 4 probes detecting TEM R164C and SHV D179A/N/G could not be tested.

The ESBL-negative isolates included *Enterobacteriaceae* with plasmid-mediated and chromosomal AmpC production, OXA beta-lactamases, non-ESBL TEM or SHV variants, ampicillin-susceptible *Escherichia coli* isolates, and 7 *Klebsiella oxytoca* K1 hyperproducers.




**Lay-out of zone 1**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HybC | TEM 104E (non ESBL) | TEM E104K (ESBL) | DNA C | TEM 164R (non ESBL) | TEM R164S (ESBL) | TEM R164C (ESBL) | TEM R164H (ESBL) | TEM V84I (non ESBL) | TEM N100S (non ESBL) |
| pos C | TEM 238 G (non ESBL) | TEM G238S (ESBL) | DNA C | SHV 130S (non ESBL) | SHV 238G (non ESBL) | SHV G238S (ESBL) | SHV G238A (ESBL) | SHV 240E (non ESBL) | SHV E240K (ESBL) |
| pos C | SHV 179D (non-ESBL) | SHV D179A (ESBL) | Neg C | SHV D179G (ESBL) | SHV D179N (ESBL) | CTX-M1 | CTX-M2 | CTX-M9 | CTX-M8+25 |

***Figure 5.3*** *Example of microarray picture (upper panel). The microarray contains three zones, each with 30 identical probe targets, allowing parallel analysis of three isolates. The analysis of three isolates on one microarray is possible because for each isolate a ligation-mediated PCR reaction is performed in a separate tube using probe pairs with unique ZIP codes for each isolate. Subsequently, the PCR reaction products from the three individual isolates are pooled for hybridization and detection in one Array Tube. The layout of zone 1 is shown (lower panel). Each zone contains 24 probe targets for ESBL detection and six control spots. The control spots were designed to assess the following steps:*

HybC*: Biotinylated probe complementary to spotted oligonucleotide at indicated array position.*

Pos C*: Internal reaction control consisting of DNA probe and complementary target DNA. Upon successful completion of the A-step a small amount of product will be amplified in the subsequent PCR that will be detected at the indicated positions on the microarray.*

Neg C: *Internal reaction control consisting of DNA probe but lacking any complementary target DNA. The intensity of the signal at the indicated array positions specify the background noise*

DNA C*: Two DNA probes targeted at highly conserved sequences in* Enterobacteriaceae *and non-fermenters. At least one of these probes is expected to give a spot at the indicated positions in case DNA of* Enterobacteriaceae *or non-fermenters is present in the reaction.*

*The positions in column 1 and 12 (upper panel) contain biotinylated oligonucleotides spotted on the microarray that are used as control spots for the staining process and reference spots for image analysis software. To allow visual inspection of the DNA microarray pictures spots interpreted as positive by the software are marked with a green circle (not shown). The isolates analysed in the picture were ESBL negative (zone 1), non-ESBL TEM positive (zone 2) and CTX-M-1 group positive, combined with a non-ESBL TEM and a non-ESBL SHV (zone 3).*

*The software reported the isolate as "ESBL" if a) at least one of the ESBL-associated substitutions is detected in TEM (E104K, R164S, R164C, R164H, G238S) or SHV (G238S, G238A, E240K, D179A, D179G, D179N) b) a CTX-M gene is detected c) a TEM-116 was identified based on presence of the V84I substitution in TEM without the N100S substitution (Table 5.1, Figure 5.3). The software indicated whether a (combination of) TEM-, SHV- or CTX-M ESBL had been detected, and specified the CTX-M group (CTX-M group 1, 2, 9, or combined 8/25). If SHV or TEM genes were detected without any of the ESBL-associated substitutions, the test result was "SHV no ESBL", or "TEM no ESBL", respectively. The assay was not able to provide a Lahey number for TEM and SHV genes (e.g. TEM-6 or SHV-2), because multiple TEM and SHV ESBL variants share the same SNPs on the positions detected by the assay (Tables 5.1 and 5.2). Furthermore, if e.g. two TEM ESBL SNPs are detected, it is impossible to distinguish between the presence of two TEM genes with one SNP per gene versus the presence of one TEM gene with two SNPs. The software reported "No ESBL" if no TEM, SHV and CTX-M genes were detected. The software advised to repeat the assay when a) the result was "ESBL suspected" i.e. in case one of the ESBL-associated spots on the array appeared positive but the pattern of spots was otherwise not consistent with a known ESBL, b) when artefacts e.g. air bubbles hampered microarray reading and interpretation, or c) when the amount of DNA input was insufficient.*

***Table 5.3:*** *Characteristics of the 106 ESBL Positive Test Isolates (bacterial species, beta-lactamases, ESBL genotype, ESBL phenotype, and array result).*

| Isolate number | Species | Beta-lactamase | Beta-lactamase type | ESBL geno-type | ESBL pheno-type | ESBL array | Origin |
|---|---|---|---|---|---|---|---|
| 1 - 3 | *E. coli* | CTX-M-1 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | A,B |
| 4 | *E. coli* | CTX-M-10 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | A |
| 5 - 9 | *E. coli* | CTX-M-15 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | A,B |
| 10 | *E. coli* | CTX-M-3 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | B |
| 11, 12 | *K. pneumoniae* | CTX-M-15 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | C |
| 13 | *Salmonella enterica* | CTX-M-1 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | B |
| 14 | *Salmonella enterica* | CTX-M-15 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | B |
| 15 | *Salmonella enterica* | CTX-M-28 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | B |
| 16 | *Salmonella enterica* | CTX-M-3 | ESBL CTX-M-Group 1 | Pos | Pos | Pos | B |
| 17 | *E. coli* | CTX-M-15, TEM-1 | ESBL CTX-M-Group 1, Non ESBL TEM | Pos | Pos | Pos | A |
| 18 - 20 | *E. coli* | CTX-M-2 | ESBL CTX-M-Group 2 | Pos | Pos | Pos | A,B |
| 21 | *P. mirabilis* | CTX-M-2 | ESBL CTX-M-Group 2 | Pos | Pos | Pos | A |
| 22 | *Salmonella enterica* | CTX-M-5 | ESBL CTX-M-Group 2 | Pos | Pos | Pos | B |
| 23 | *Salmonella enterica* | CTX-M-2, SHV-2 | ESBL CTX-M-Group 2, ESBL SHV | Pos | Pos | Pos | B |
| 24 | *P. mirabilis* | CTX-M-39 | ESBL CTX-M-Group 25 | Pos | Pos | **Neg** | C |
| 25 | *Salmonella enterica* | CTX-M-3 | ESBL CTX-M-Group 3 | Pos | Pos | Pos | B |
| 26 | *E. coli* | CTX-M-8 | ESBL CTX-M-Group 8 | Pos | Pos | Pos | A |
| 27 | *Salmonella enterica* | CTX-M-8 | ESBL CTX-M-Group 8 | Pos | Pos | Pos | B |
| 28 | *E. cloacae* | CTX-M-9 | ESBL CTX-M-Group 9 | Pos | **Non det** | Pos | A |
| 29, 30 | *E. cloacae* | CTX-M-9 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | C |
| 31 - 33 | *E. coli* | CTX-M-14 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | A,B,C |
| 34, 35 | *E. coli* | CTX-M-16 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | A,C |
| 36 | *E. coli* | CTX-M-27 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | A |
| 37 - 40 | *E. coli* | CTX-M-9 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | A,B |
| 41 | *Salmonella enterica* | CTX-M-14 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | B |
| 42 | *Salmonella enterica* | CTX-M-9 | ESBL CTX-M-Group 9 | Pos | Pos | Pos | B |
| 43 | *C. freundii* | SHV-2a | ESBL SHV | Pos | Pos | Pos | B |
| 44 - 48 | *E. cloacae* | SHV-12 | ESBL SHV | Pos | Pos | Pos | A |
| 49 | *E. cloacae* | SHV-2 | ESBL SHV | Pos | Pos | Pos | A |
| 50 | *E. coli* | SHV 5a, SHV-12 | ESBL SHV | Pos | Pos | Pos | B |
| 51 -53 | *E. coli* | SHV-12 | ESBL SHV | Pos | Pos | Pos | A,B |
| 54 | *E. coli* | SHV-3 | ESBL SHV | Pos | Pos | Pos | B |
| 55, 56 | *E. coli* | SHV-4 | ESBL SHV | Pos | Pos | Pos | A,C |
| 57 - 59 | *E. coli* | SHV-5 | ESBL SHV | Pos | Pos | Pos | A,B |
| 60 | *E. coli* | SHV-57 | ESBL SHV | Pos | Pos | **Neg** | B |
| 61 -63 | *K. pneumoniae* | SHV-12 | ESBL SHV | Pos | Pos | Pos | C |
| 64 | *K. pneumoniae* | SHV-18 | ESBL SHV | Pos | Pos | Pos | C |
| 65, 66 | *K. pneumoniae* | SHV-2 | ESBL SHV | Pos | Pos | Pos | C |
| 67, 68 | *K. pneumoniae* | SHV-5 | ESBL SHV | Pos | Pos | Pos | C |
| 69, 70 | *Salmonella enterica* | SHV-2 | ESBL SHV | Pos | Pos | Pos | B |
| 71 | *Salmonella enterica* | SHV-4 | ESBL SHV | Pos | Pos | Pos | B |

*Continued*

***Table 5.3*** *Continued*

| Isolate number | Species | Beta-lactamase | Beta-lactamase type | ESBL geno-type | ESBL pheno-type | ESBL array | Origin |
|---|---|---|---|---|---|---|---|
| 72 | *K. pneumoniae* | CTX-M-15, SHV-5 | ESBL SHV, CTX-M-Group 1 | Pos | Pos | Pos | A |
| 73, 74 | *K. pneumoniae* | SHV-12, CTX-M-1 | ESBL SHV, CTX-M-Group 1 | Pos | Pos | Pos | A |
| 75 | *K. oxytoca* | SHV-5, TEM-1 | ESBL SHV, Non ESBL TEM | Pos | Pos | Pos | A |
| 76 | *C. freundii* | TEM-52 | ESBL TEM | Pos | Pos | Pos | B |
| 77 | *E. cloacae* | TEM-19 | ESBL TEM | Pos | Pos | Pos | A |
| 78 | *E. coli* | TEM-10 | ESBL TEM | Pos | Pos | Pos | C |
| 79 | *E. coli* | TEM-12 | ESBL TEM | Pos | Pos | Pos | C |
| 80, 81 | *E. coli* | TEM-3 | ESBL TEM | Pos | Pos | Pos | B,C |
| 82, 83 | *E. coli* | TEM-4 | ESBL TEM | Pos | Pos | Pos | B,C |
| 84 | *E. coli* | TEM-5 | ESBL TEM | Pos | Pos | **Neg** | B |
| 85 | *E. coli* | TEM-52 | ESBL TEM | Pos | Pos | Pos | A |
| 86 | *E. coli* | TEM-52 | ESBL TEM | Pos | Pos | Pos | B |
| 87 | *E. coli* | TEM-6 | ESBL TEM | Pos | Pos | Pos | B |
| 88 | *E. coli* | TEM-7 | ESBL TEM | Pos | Pos | **Neg** | B |
| 89 | *E. coli* | TEM-7 | ESBL TEM | Pos | Pos | Pos | C |
| 90 | *E. coli* | TEM-7 | ESBL TEM | Pos | Pos | Pos | C |
| 91 | *E. coli* | TEM-8 | ESBL TEM | Pos | Pos | Pos | B |
| 92, 93 | *E. coli* | TEM-9 | ESBL TEM | Pos | Pos | Pos | B,C |
| 94 | *K. pneumoniae* | TEM-18 | ESBL TEM | Pos | Pos | Pos | C |
| 95 | *K. pneumoniae* | TEM-19 | ESBL TEM | Pos | Pos | Pos | C |
| 96 | *K. pneumoniae* | TEM-3 | ESBL TEM | Pos | Pos | Pos | C |
| 97 | *P. mirabilis* | TEM-43 | ESBL TEM | Pos | Pos | Pos | C |
| 98 | *P. mirabilis* | TEM-52 | ESBL TEM | Pos | Pos | Pos | C |
| 99 | *P. mirabilis* | TEM-72 | ESBL TEM | Pos | Pos | **Neg** | C |
| 100, 101 | *Salmonella enterica* | TEM-20 | ESBL TEM | Pos | Pos | Pos | B |
| 102, 103 | *Salmonella enterica* | TEM-52 | ESBL TEM | Pos | Pos | Pos | B |
| 104 | *Salmonella enterica* | TEM-63 | ESBL TEM | Pos | Pos | Pos | B |
| 105 | *E. coli* | CMY-10, CTX-M-9 | Plasmid mediated AmpC, ESBL CTX-M-Group 9 | Pos | Pos | Pos | B |
| 106 | *E. coli* | ACC-1, SHV-2a | Plasmid mediated AmpC, ESBL SHV | Pos | **Neg** | Pos | B |

Pos = Positive; neg = negative; Non det = non determinable; Origin A = Academic Medical Centre and VU University Medical Centre, Amsterdam, the Netherlands; Origin B = Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands; Origin C = University Centre Utrecht, the Netherlands.

***Table 5.4:*** *ESBL negative isolates (n=106)*

| Isolate number | Species | Beta-lactamase | Beta-lactamase type | ESBL geno-type | ESBL pheno-type | ESBL array | Origin |
|---|---|---|---|---|---|---|---|
| 107 | *C. freundii* | Chromosomal AmpC | Chromosomal AmpC | Neg | Neg | Neg | A |
| 108 | *E. cloacae* | Chromosomal AmpC | Chromosomal AmpC | Neg | Neg | Neg | C |
| 109 - 113 | *E. coli* | Chromosomal AmpC | Chromosomal AmpC | Neg | Neg | Neg | B |
| 114 - 120 | *K. oxytoca* | K1 hyperproducer | K1 | Neg | **Pos** | Neg | C |
| 121 | *E. coli* | No TEM, SHV or CTX-M genes | No TEM, SHV or CTX-M genes | Neg | **Pos** | Neg | C |
| 122 - 170 | *E. coli* | No TEM, SHV or CTX-M genes | No TEM, SHV or CTX-M genes | Neg | neg | Neg | C |
| 171, 172 | *P. mirabilis* | No TEM, SHV or CTX-M genes | No TEM, SHV or CTX-M genes | Neg | Neg | Neg | A |
| 173, 174 | *E. coli* | SHV-1 | Non ESBL SHV | Neg | Neg | Neg | B |
| 175 | *K. oxytoca* | SHV-11 | Non ESBL SHV | Neg | Neg | Neg | C |
| 176 | *E. cloacae* | TEM-1 | Non ESBL TEM | Neg | Neg | Neg | A |
| 177 - 184 | *E. coli* | Tem-1 | Non ESBL TEM | Neg | Neg | Neg | A,B |
| 185 -187 | *E. coli* | TEM-2 | Non ESBL TEM | Neg | Neg | Neg | B,C |
| 188, 189 | *Salmonella enterica* | TEM-1b | Non ESBL TEM | Neg | Neg | Neg | B |
| 190 | *E. coli* | OXA-2 | OXA | Neg | Neg | Neg | B |
| 191 | *E. coli* | OXA-23 | OXA | Neg | Neg | Neg | B |
| 192 | *E. coli* | OXA-30 | OXA | Neg | Neg | Neg | B |
| 193 | *E. coli* | OXA-5 | OXA | Neg | Neg | Neg | B |
| 194 | *E. coli* | OXA-7 | OXA | Neg | Neg | Neg | B |
| 195 | *E. coli* | OXA-9 | OXA | Neg | Neg | Neg | B |
| 196, 197 | *E. coli* | ACC-1 | plasmid mediated AmpC | Neg | Neg | Neg | B,C |
| 198, 199 | *E. coli* | ACT-1 | plasmid mediated AmpC | Neg | Neg | Neg | B,C |
| 200 | *E. coli* | CMY-1 | plasmid mediated AmpC | Neg | Neg | Neg | B |
| 201, 202 | *E. coli* | CMY-2 | plasmid mediated AmpC | Neg | Neg | Neg | B,C |
| 203, 204 | *E. coli* | DHA-1 | plasmid mediated AmpC | Neg | Neg | Neg | B,C |
| 205 | *E. coli* | MIR-1 | plasmid mediated AmpC | Neg | Neg | Neg | C |
| 206 | *E. coli* | MOX-1 | plasmid mediated AmpC | Neg | Neg | Neg | C |
| 207, 208 | *Salmonella enterica* | ACC-1 | plasmid mediated AmpC | Neg | Neg | Neg | B |
| 209 | *Salmonella enterica* | CMY-18 | plasmid mediated AmpC | Neg | Neg | Neg | B |
| 210 - 212 | *Salmonella enterica* | CMY-2 | plasmid mediated AmpC | Neg | Neg | Neg | B |

Origin A = Academic Medical Centre and VU University Medical Centre, Amsterdam, the Netherlands; Origin B = Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands; Origin C = University Centre Utrecht, the Netherlands.

*5.3.4 Phenotypic characterization*

To compare the results of the microarray system with the ESBL phenotype, the Phoenix automated system with the NMIC-ID75 card (Becton Dickinson Diagnostic Systems, Baltimore, MD, USA) was used to determine MICs. Confirmatory tests for ESBL production were performed if the ESBL MIC of ceftriaxone or ceftazidime was >1 mg/L. As confirmation, three ESBL Etests (AB Biodisk, Solna, Sweden) with ceftazidime, cefotaxime, and cefepime, each +/- clavulanic acid, were used.

*5.3.5 DNA isolation and sequencing*

DNA isolation was performed using Nucleospin Tissue Columns (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The presence of TEM, SHV, and CTX-M was determined using PCR and sequencing of the same batch of DNA as used in the microarray (references in Table 5.5).

*5.3.6 Statistics*

Test characteristics (sensitivity and specificity) are presented with 95% confidence intervals (CIs).



***Table 5.5*** *Primers for PCR and sequencing of TEM, SHV and CTX-M genes.*

| Gene | Primer | Sequence | Reference |
|---|---|---|---|
| TEM | TEM-F | 5'-GCGGAACCCCTATTTG-3' | [29] |
| TEM | TEM-R | 5'-ACCAATGCTTAATCAGTGAG-3' | [29] |
| SHV | SHV-F1 | 5'-CTTTACTCGCCTTTATCG-3' | [11] |
| SHV | SHV-R1 | 5'-TCCCGCAGATAAATCACCA-3' | [11] |
| CTX-M | CTX-M-F | 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' | [30] |
| CTX-M | CTX-M-R | 5'-TGGGTRAARTARGTSACCAGAAYSAGCGG-3' | [30] |
| CTX-M1 group | CTX-M-10-1F | 5'-ATGGTTAAAAAATCACTGCG-3' | [31] |
| CTX-M1 group | CTX-M-10-4R | 5'-AAACCGTTGGTGACGAT-3' | [31] |
| CTX-M2 group | CTX-M-2F | 5'-ATGATGACTCAGAGCATTCG-3' | [32] |
| CTX-M2 group | CTX-M-2R | 5'-TTATTGCATCAGAAACCGTG-3' | [32] |
| CTX-M8 group | CTX-Mgp8-F | 5'-TGATGAGACATCGCGTTAAG-3' | [33] |
| CTX-M8 group | CTX-Mgp8-R | 5'-TAACCGTCGGTGACGATTTT-3' | [33] |
| CTX-M9 group | CTX-M-9-1F | 5'-TGGTGACAAAGAGAGTGCAACG-3' | [31] |
| CTX-M9 group | CTX-M-9-MF | 5'-GGAGGCGTGACGGCT TTT-3' | [31] |

## 5.4 Results

***Table 5.6*** *Detection of ESBL associated substitutions by microarray in 106 ESBL positive isolates (110 genes since 4 isolates harboured 2 ESBL genes).*

| TEM ESBL genes in collection | ESBL-associated substitutions detectable by array | Number of isolates | ESBL-positive result in array |
|---|---|---|---|
| TEM-18 | E104K | 1 | 1 |
| TEM-9, -63 | E104K, R164S | 3 | 3[a] |
| TEM-6, -43 | E104K, R164H | 2 | 2 |
| TEM-3, -4, -52 | E104K, G238S | 11 | 11 |
| TEM-8 | E104K, R164S, G238S | 1 | 1 |
| TEM-5, -7, -10, -12 | R164S | 6 | 4[b] |
| TEM-19, -20, -72 | G238S | 5 | 4[b] |
| **Sensitivity of individual probes** | | | |

| SHV ESBL genes in collection | ESBL-associated substitutions detectable by array | Number of isolates | ESBL-positive result in array |
|---|---|---|---|
| SHV-2, -2a, -3 | G238S | 11 | 11 |
| SHV-4, -5, -12 | G238S, E240K | 22 | 22[a] |
| SHV-18 | G238A, E240K | 1 | 1 |
| SHV-57 | not detectable by array | 1 | 0 |
| **Sensitivity of individual probes** | | | |

| CTX-M genes in collection | CTX-M-group | Number of isolates | ESBL-positive result in array |
|---|---|---|---|
| CTX-M-1, -3, -10, -15, -28 | CTX-M-1 | 21 | 21/21 |
| CTX-M-2, -5 | CTX-M-2 | 6 | 6/6 |
| CTX-M-9, -14, -16, -27 | CTX-M-9 | 16 | 16/16 |
| CTX-M-8, -39 | CTX-M-8/25 | 3 | 2/3[b] |
| **Sensitivity of individual probes** | | | |

| Number of ESBL substitutions detected/number of expected ESBL substitutions | | | |
|---|---|---|---|
| **E104K** | **R164S** | **R164H** | **G238S** |
| 1/1 | --- | --- | --- |
| 3/3 | 1/3[a] | --- | --- |
| 2/2 | --- | 2/2 | --- |
| 11/11 | --- | --- | 11/11 |
| 1/1 | 1/1 | --- | 1/1 |
| --- | 4/6[a] | --- | --- |
| --- | --- | --- | 4/5[b] |
| **18/18 (100%)** | **6/10 (60%)** | **2/2 (100%)** | **16/17 (94%)** |

| Number of ESBL substitutions detected/number of expected ESBL substitutions | | | |
|---|---|---|---|
| | **G238S** | **G238A** | **E240K** |
| | 11/11 | --- | --- |
| | 21/22[a] | --- | 22/22 |
| | --- | 1/1 | 1/1 |
| | --- | --- | --- |
| | **32/33 (97%)** | **1/1 (100%)** | **23/23 (100%)** |

| Number of ESBL substitutions detected/number of expected ESBL substitutions | | | |
|---|---|---|---|
| **CTX-M-1** | **CTX-M-2** | **CTX-M-9** | **CTX-M-8/25** |
| 21/21 | --- | --- | --- |
| --- | 6/6 | --- | --- |
| --- | --- | 16/16 | --- |
| --- | --- | --- | 2/3[b] |
| **21/21 (100%)** | **6/6 (100%)** | **16/16 (100%)** | **2/3 (67%)** |

[a]The failure to detect the R164S substitution in two TEM-9 harbouring isolates did not result in an ESBL-negative test result due to detection of the E104K substitution in these isolates. Similarly, the failure to detect the G238S substitution in a SHV-12-producing isolate did not result in an ESBL-negative test result due to identification of the E240K substitution.

[b]The R164S mutation was not demonstrated in one TEM-5-producing and one TEM-7-producing isolates. The G238S substitution was not detected in a TEM-72-positive isolate resulting in a false ESBL-negative array result.

*5.4.1 Test characteristics of the microarray*

Using sequencing of the ESBL gene and the corresponding phenotype in the Lahey database as the reference test, the sensitivity of the microarray was 101/106 (95%; 95%CI 89-98%) and the specificity was 100% (95%CI 97-100%).

A false-negative result was obtained in five ESBL-positive isolates. One SHV-57-positive isolate was not detected, because SHV-57 lacks the ESBL-associated SNPs detected in the array system. In one TEM-5 and one TEM-7 isolate, the R164S mutation was not detected, and in one TEM-72 isolate the G238S mutation was not detected. Finally, one CTX-M-39 gene was not detected in one isolate (Table 5.6). In the isolates with a false-negative result, the array test was repeated twice, including a new DNA extraction. Since PCR and sequencing were also repeated (with a positive result) on the same DNA batches, plasmid instability was not a likely explanation for these negative results.

*5.4.2 Accuracy of individual probes to identify specific SNPs*

In total, 8 of the 110 ESBL genes in the isolate collection were not, or incompletely, detected. For five isolates this resulted in a false-negative result, while for three isolates (two TEM-9-positive isolates and one SHV-12-positive isolate) the microarray result was not influenced because another ESBL substitution was detected (Table 5.6).

The specificity of the probes was high. For only two isolates spots were detected not in accordance with the sequencing results. An additional TEM E104K spot was detected in a TEM-19 isolate and a TEM G238S spot in a TEM-18 isolate.

*5.4.3 Test characteristics of the phenotypic assay*

The sensitivity of phenotypic ESBL testing was 104/106 (98%; 95% CI 93-99%) and the specificity 98/106 (92%; 95%CI 86-96%).

In one CTX-M-9 producing *Enterobacter cloacae* isolate, all 3 ESBL Etests were non-determinable, and in one *E. coli* isolate, containing a SHV-2 ESBL and an ACC-1 plasmid-mediated AmpC, the ESBL Etests were negative. Using the microarray system, the CTX-M-9 and SHV-2 were correctly detected in these isolates.

As expected, the seven K1-hyperproducing *Klebsiella oxytoca* isolates were ESBL positive in the phenotypic tests, but negative in the microarray system. One *E. coli* isolate displayed positive ESBL Etests, although no TEM, SHV or CTX-M genes were detected by either PCR or the microarray system.

**5.5 Discussion**

This study describes the first commercially available microarray system enabling the detection of ESBLs belonging to the three most prevalent ESBL families: TEM, SHV and CTX-M. Analysing 212 well-characterized *Enterobacteriaceae* isolates, this test had a sensitivity of 95% and a specificity of 100%.

A microarray has advantages in comparison with phenotypic testing for ESBL production. First, the results could be obtained within the same working day, whereas phenotypic confirmatory tests require an overnight incubation. Secondly, the microarray is accurate for

species producing beta-lactamases that may interfere with phenotypic tests for ESBL production, such as K1 hyperproducing *K. oxytoca* and AmpC-producing isolates. Thirdly, the microarray identifies ESBL families and provides information on the SNPs in the TEM and SHV ESBL gene(s), which may be useful for infection control purposes.

The assay also has some disadvantages. An intrinsic limitation of this type of genotypic assay is the limited number of ESBLs (or ESBL families) that may be detected. The array was designed to include the most prevalent ESBLs, resulting in coverage of 95% (84/88) of the TEM ESBLs, and 77% (27/35) of the SHV ESBLs described in the Lahey database. Recently another microarray (not commercially available) was described enabling detection of 99% of the TEM ESBLs and 94% of the SHV ESBLs in the Lahey database[34]. The flexibility of the microarray system presented here, however, allows easy extension of the assay with additional probes.

Another issue is the potential sensitivity for cross-contamination from previous amplification products. To prevent this, the use of two separate rooms proved to be imperative (one room for DNA isolation and ligation, and one for amplification, hybridization and detection).

Another limitation of this study was the fact that 4 of the 24 probes in the microarray system were not evaluated, because isolates with the target mutations were not available. However, these probes have been tested successfully by the manufacturer on artificial DNA sequences (data not shown).

In conclusion, this microarray accurately detects and identifies the three most prevalent ESBL gene families in *Enterobacteriaceae.* The simple and straightforward protocol makes this system a promising tool for detection of ESBLs in a clinical microbiology laboratory.



### 5.6 Acknowledgements

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# Chapter 6

# Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains

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## 6.1 Abstract

Intestinal carriage of extended spectrum beta-lactamase (ESBL)-producing bacteria in food-producing animals and contamination of retail meat may contribute to increased incidences of infections with ESBL-producing bacteria in humans. Therefore, distribution of ESBL genes, plasmids and strain genotypes in *Escherichia coli* obtained from poultry and retail chicken meat in the Netherlands was determined and defined as 'poultry-associated'(PA). Subsequently, the proportion of *E. coli* isolates with PA ESBL genes, plasmids and strains was quantified in a representative sample of clinical isolates. The *E. coli* were derived from 98 retail chicken meat samples, a prevalence survey among poultry, and 516 human clinical samples from 31 laboratories collected during a 3-month period in 2009. Isolates were analysed using an ESBL-specific microarray, sequencing of ESBL genes, PCR-based replicon typing of plasmids, plasmid multi-locus sequence typing (pMLST) and strain genotyping (MLST).

Six ESBL genes were defined as PA ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{SHV-2}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-20}}$, $bla_{\text{TEM-52}}$): 35% of the human isolates contained PA ESBL genes and 19% contained PA ESBL genes located on IncI1 plasmids that were genetically indistinguishable from those obtained from poultry (meat). Of these ESBL genes, 86% were $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ genes, which were also the predominant genes in poultry (78%) and retail chicken meat (75%). Of the retail meat samples, 94% contained ESBL-producing isolates of which 39% belonged to *E. coli* genotypes also present in human samples. These findings are suggestive for transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain.

## 6.2 Introduction

There is a worldwide increase in infections caused by Gram-negative bacteria producing extended spectrum beta-lactamases (ESBLs), even in a low-resistance country such as the Netherlands[1]. This is remarkable because the Netherlands have low levels of antibiotic usage and have been successful in controlling nosocomial spread of other multi-resistant bacteria[2-4].

In contrast to human antibiotic use, antibiotic use in the poultry industry is higher in the Netherlands than in any other European country[5] .The prevalence of ESBL-producing *Escherichia coli* in the gastrointestinal tract of healthy food-producing animals, especially poultry, increased from 3% in 2003 to 15% in 2008 and in 2009 ESBL-producing bacteria were detected in 26 of 26 broiler farms studied[6, 7,Chapter 3]. Furthermore, contamination of retail chicken meat with ESBL-producing Gram-negative bacteria has been documented in several countries[8-10].

For these reasons the poultry industry has been considered a potential reservoir of ESBL-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated meat. We therefore determined the distribution of ESBL genes, plasmids and strain genotypes in *E. coli* obtained from poultry and retail chicken meat in the Netherlands and defined these as "poultry associated" (PA). Subsequently, we quantified the proportion of *E. coli* isolates with PA related ESBL genes, plasmids and strains in a large and representative sample of clinical *E. coli* isolates from Dutch patients.

## 6.3 Methods

### *6.3.1 Isolates*

#### *6.3.1.1 Retail chicken meat*

Between April and June 2010, 98 fresh raw chicken breasts were purchased in 12 stores in Utrecht, the Netherlands. Seventy-eight of the samples were purchased at nine stores belonging to six supermarket chains (Dutch market share of 90%) and 20 from three different butcheries. Information about the region where the chickens were raised was available for 30 supermarket samples (27% Netherlands, 73% Benelux).

From each sample 25 g meat was homogenized with 225 ml peptone water in a stomacher. After overnight pre-enrichment, the homogenate was cultured on selective ESBL agar plates (ESBL Brillance, Oxoid, Basingstoke, Hants, UK). ESBL production was confirmed using Etests (BioMérieux, Marcy l'Etoile, France). Species identification was performed by MALDI-TOF (Bruker Daltonics, Bremen, Germany).

#### *6.3.1.2 Poultry*

The poultry isolates were derived from the Dutch surveillance program on antibiotic resistance in bacteria isolated in food-producing animals in 2006 [11]. The sampling strategy in this program aims to obtain annual collections of *E. coli* and *Salmonella enterica*, representative of the Dutch food-producing animal bacterial populations. Twelve percent (22 *E. coli* and 22 *S. enterica*) of all isolates were cefotaxime resistant. ESBL genes were identified in 35 of these: 17 (49%) $bla_{\text{CTX-M-1}}$, ten (29%) $bla_{\text{TEM-52}}$, four (11%) $bla_{\text{TEM-20}}$, three (9%) $bla_{\text{CTX-M-2}}$, and one (3%) $bla_{\text{SHV-2}}$ [12, Chapter 2]. The 27 $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ positive isolates were included.

#### *6.3.1.3 Human*

From 1 February 2009 until 1May 2009, 31 Dutch laboratories submitted all *E. coli* with a positive ESBL screen test ((MIC >1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek-2 expert system) [13]. For each isolate the following data were collected: age, gender, material, and institution (hospital, general practitioner (GP), long term care facility (LTCF)). From each laboratory the first 25 consecutive isolates (if available), one isolate per patient, were included. The participating laboratories are geographically dispersed over the Netherlands and represent a mixture of secondary and tertiary care hospitals, LTCFs and GPs. The laboratories serve a total of 58 hospitals, covering approximately 45% of all hospital beds in the Netherlands.

### *6.3.2 Molecular analyses*

The presence of ESBL genes was determined by microarray analysis [14] and gene sequencing. All *human* isolates were investigated by microarray and sequencing was performed on a random selection of 50%. Among the *retail* isolates all morphologically different ESBL positive *E. coli* from three meat samples of each available packaging type (whole breast or sliced) per store were analyzed by sequencing.

Plasmid analysis was performed on a random selection of human and poultry isolates carrying either a $bla_{CTX\text{-}M\text{-}1}$ or a $bla_{TEM\text{-}52}$ gene. All plasmids were characterized using PCR based Replicon Typing (PBRT)[12, 15]. The association between ESBL gene and plasmids was determined either by Southern blot hybridization or transformation[12]. IncI1 plasmids were typed by plasmid Multi-Locus Sequence Typing (PMLST)[16].

Isolates were genotyped by MLST (http://www.mlst.net). Among the *human* isolates 27 were genotyped: isolates with documented presence of $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$ genes on an IncI1 plasmid (n=15) and a random selection (n=12) of all other isolates carrying a $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$. Among the *poultry* isolates, all 22 isolates with either $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$ located on IncI1 plasmids were selected for genotyping. From the *retail* isolates with a $bla_{CTX\text{-}M\text{-}1,}$ $bla_{SHV\text{-}12}$ or $bla_{TEM\text{-}52}$ gene, 23 isolates were randomly selected for genotyping.

## 6.4 Results

### *6.4.1 Distribution of ESBL genes*

#### *6.4.1.1 Retail chicken meat samples*

Of the 98 chicken retail meat samples, 92 (94%) samples contained at least one *E. coli* isolate with an ESBL phenotype, yielding 163 isolates (average number per sample 2; range 1-4). From 48 samples 81 isolates cultured were further analysed. The array confirmed the presence of an ESBL gene in all isolates: 40 CTX-M-1-group, 21 TEM-3-group, 13 SHV-4-group, three SHV-2-group, three CTX-M-2-group, and one TEM-19-group. By sequencing one ESBL gene was identified in each of these six different ESBL groups: $bla_{CTX\text{-}M\text{-}1}$, $bla_{TEM\text{-}52}$, $bla_{SHV\text{-}12}$, $bla_{SHV\text{-}2}$, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{TEM\text{-}20}$, respectively. These genes were considered as PA. The $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ accounted for 75% of the genes (Table 6.1). $Bla_{SHV\text{-}12}$ gene was not detected in poultry in 2006, but has been detected in poultry isolates obtained in 2009 (D. Mevius, personal communication).

#### *6.4.1.2 Human samples*

In the study period, 1017 *E. coli* were ESBL screen positive from which 516 were included (Figure 6.1). The median number per laboratory was 17 (range 7-25) and per hospital 10 (range 0-21). The proportion of isolates derived from non-university hospitals was 54%, from General Practitioners 30%, from university medical centers 6%, from LTCFs 5% (5% unknown).

Based on the microarray results, 409 (79%) isolates contained an ESBL-gene, and in 344 (84%) of these the ESBL genes were potentially PA (Figure 6.1: rows A and B). Sequence results of 208 randomly selected isolates identified five ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{TEM\text{-}52}$, $bla_{SHV\text{-}2}$ or $bla_{SHV\text{-}12}$) of the six PA genes (Figure 6.1: row C). The $bla_{TEM\text{-}20}$ gene was not detected in any of the human isolates.

The proportion of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes among all ESBL genes detected in clinical isolates was similar in five different age groups (0-4, 20-39, 40-59, 60-79,>80 years) and in four different geographic regions. The proportion of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes was similar among isolates submitted by General Practitioners (33%; 23/70; 95% CI: 22-44), non-academic hospitals (26%; 27/104; 95% CI: 18-34), LTCFs (26%; 4/14; 95% CI: 5-52) and academic hospitals (37%; 3/8; 95% CI: 4-71). The 27 isolates that were MLST genotyped were obtained from 17 different laboratories. Of these, 23 (85%) were urine isolates, 19 (70%) came from GPs and none came from the same facility.

***Table 6.1*** *Distributions of extended spectrum beta-lactamse (ESBL) genes in* Escherichia coli *and* Salmonella spp*. isolates from poultry, poultry retail meat samples and from human origin based on array results combined with sequence results.*

| | **Poultry** | **Poultry meat samples[a]** | **Human[a]** |
|---|---|---|---|
| Poultry-associated ESBL genes | n=35 | n=81 | n=409 |
| $bla_{\text{CTX-M-1}}$ (%) | 49 | 49 | 24 |
| $bla_{\text{TEM-52}}$ (%) | 29 | 26 | 6 |
| $bla_{\text{SHV-12}}$ (%) | 0 | 16 | 4 |
| $bla_{\text{SHV-2}}$ (%) | 11 | 4 | 0,4 |
| $bla_{\text{CTX-M-2}}$ (%) | 9 | 4 | 0,2 |
| $bla_{\text{TEM-20}}$ (%) | 3 | 1 | 0 |
| Total (%) | 100 | 100 | 35 |

The number of isolates analysed by array among meat and human isolates was 81 and 409, respectively. The number of isolates analysed by sequencing among poultry, meat and human isolates was 35 (100%), 81 (100%) and 208 (51%), respectively. [a]Percentages are extrapolations based on array results and sequence results. For calculation of the percentages see also Figure 6.1. For example percentage of $bla_{CTX\text{-}M\text{-}1}$ in human isolates = 0.84 x 0.85 x 0.34 = 24%.

*6.4.2 Plasmid analysis and isolate typing*

*6.4.2.1 Human isolates*

PBRT was performed on 15 of 51 human isolates with a $bla_{\text{CTX-M-1}}$ gene and on six of 14 human isolates with a $bla_{\text{TEM-52}}$ gene (Table 6.3; Figure 6.1: rows C and D). Nine of the 15 $bla_{\text{CTX-M-1}}$ genes and all six of the $bla_{\text{TEM-52}}$ (i.e. 15/21; 71%) were located on an IncI1 plasmid.

The pMLST demonstrated that seven of the nine $bla_{\text{CTX-M-1}}$/IncI1 plasmids (78%) belonged to pMLST Clonal Complex CC7 and PMLST sequence type ST7 (CC7/ST7), one to CC3/ST3 and one to CC31/ST35 (Table 6.3; Figure 6.1: row E).

Isolate genotyping demonstrated that six of the seven CC7/ST7 isolates belonged to the PA MLST types: ST10 (n=1), ST58 (n=3), ST117 (n=2) (Table 6.3; Figure 6.1: row F).

The pMLST analysis of the six $bla_{\text{TEM-52}}$ /IncI1 plasmids demonstrated that five were ST36 (CC5) and one was ST10 (CC5), which differ in a single locus (one mutation in the *sogS*-gene).

Genotyping revealed that two isolates belonged to PA ST10 (Table 6.3; Figure 6.1: rows E and F).Typing by MLST of 13 randomly selected isolates demonstrated among ten $bla_{\text{CTX-M-1}}$ positive isolates three PA genotypes (ST117, ST57, ST354) and among three $bla_{\text{TEM-52}}$ positive isolates one PA genotype (ST23).

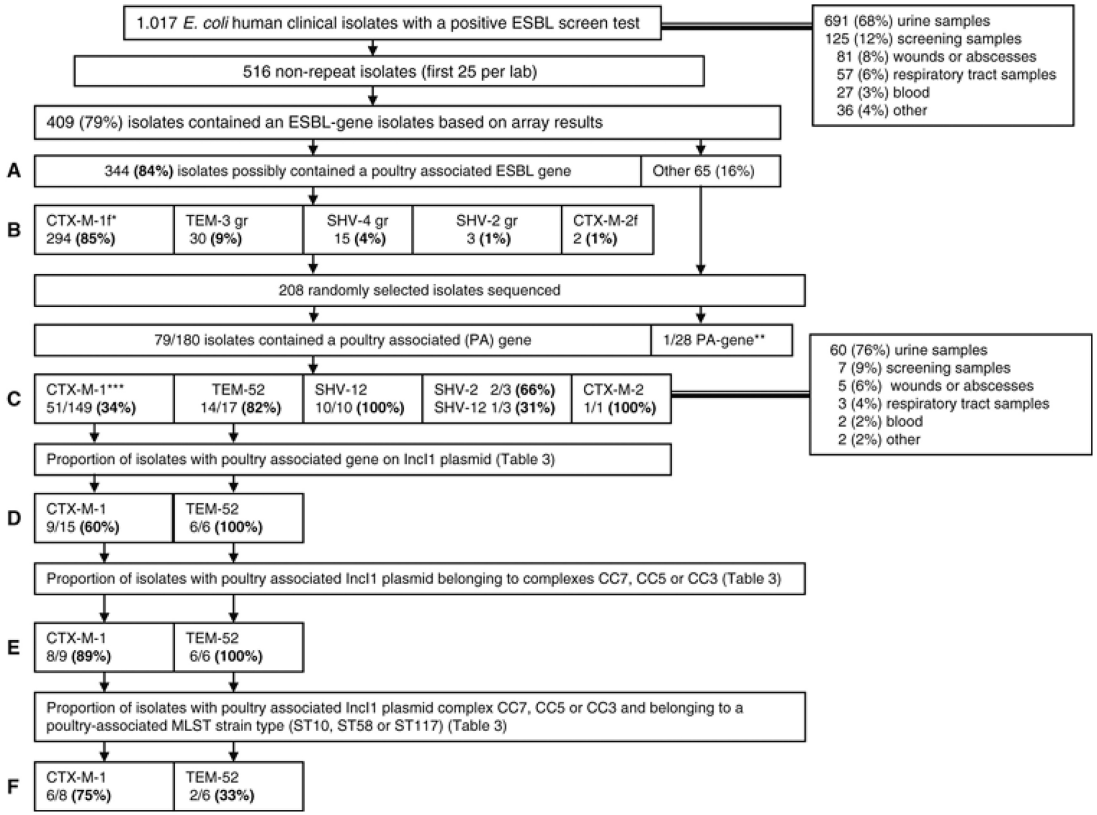


***Figure 6.1*** *Schematic view of methods and numbers of isolates used to determine the proportion of human Escherichia coli isolates with poultry-associated extended spectrum beta-lactamase (ESBL) genes, plasmids and isolate genotypes. Percentages in bold were used to determine proportions (Table 6.2). * Nine isolates harboured next to the CTX-M-1 group another possibly poultry-associated gene: SHV-4-group gene (n=5), a TEM-3-group gene (n=3) and SHV-2-group gene (n=1); ** One isolate carried according to the array an ESBL gene of the SHV-31 group but sequencing results indicated the presence of a* $bla_{SHV-12}$*; ***Three isolates harbored two poultry-associated genes:* $bla_{CTX-M-1}$ *plus* $bla_{SHV-12}$ *(n=2) and* $bla_{CTX-M-1}$ *plus* $bla_{TEM-52}$ *(n=1).*

*6.4.2.2 Poultry isolates*

The PBRT was performed on all 27 $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ containing *E. coli* and *Salmonella*. Sixteen (of 17) $bla_{CTX\text{-}M\text{-}1}$ and six (of 10) $bla_{TEM\text{-}52}$ genes were located on an IncI1 plasmid (22/27; 81%) (Table 6.3).

Plasmid MLST of the 16 $bla_{CTX\text{-}M\text{-}1}$/IncI1 plasmids demonstrated that 12 (75%) (eight *E. coli,* four *Salmonella*) belonged to CC7/ST7 and one to CC7/ST30 (ST30 is a single-locus variant of ST7). One plasmid belonged to CC3/ST3 and two were non-tyepable.

Genotyping by MLST of the eight CC7/ST7 *E. coli* revealed ST10, ST48, ST58, ST117, and four STs not found among clinical or meat samples.

The pMLST of the six $bla_{TEM\text{-}52}$ /IncI1 plasmids demonstrated that all six (two *E. coli*, four *Salmonella*) belonged to CC5/ST10. One of the *E. coli* belonged to genotype ST10.

*6.4.2.3 Retail meat*

Isolate genotyping was performed on 23 retail *E. coli* (nine $bla_{CTX\text{-}M\text{-}1}$ (five stores), seven $bla_{TEM\text{-}52}$ (four stores) seven $bla_{SHV\text{-}12}$ (five stores)). Nine (39%) belonged to MLST types also found in human isolates: ST10 (n=4), ST23 (n=1), ST57 (n=1), ST117 (n=2), and ST354 (n=1). One isolate belonged to ST48, which was like ST10 and ST117 also identified among the poultry isolates.

*6.4.3 Genetic correlation between human, chicken meat and poultry isolates*

These data revealed four sets of *E. coli* isolates of human and animal origin with indistinguishable ESBL genes, plasmids and isolate genotypes: (i) *E. coli* ST10 with $bla_{CTX\text{-}M\text{-}1}$ and IncI1/ST7 as human blood culture isolate and a poultry isolate, (ii) *E. coli* ST58 with $bla_{CTX\text{-}M\text{-}1}$ and IncI1/ST7 as three human urine isolates from three different laboratories and a poultry isolate, (iii) *E. coli* ST117 with $bla_{CTX\text{-}M\text{-}1}$ and IncI1/ST7 as two human isolates from different laboratories and a poultry isolate, and (iv) *E. coli* ST10 with $bla_{TEM\text{-}52}$ and IncI/ST10/36 was detected in two human urine samples from two laboratories and a poultry isolate. These four MLST genotype/ESBL gene combinations were also found in retail meat isolates (Table 6.3).

*6.4.4 Quantification of the proportion of PA genes, plasmids and strains in human isolates.*

Based on these data we quantified the proportion of human ESBL-producing *E. coli* with PA genes, plasmids and isolates (Figure 6.1 and Table 6.2). On the level of ESBL genes 35% (95% CI : 30-39%) of the human ESBL isolates contained PA ESBL genes and $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ accounted for the majority (30/35; 86%) (Tables 6.1, 6.2).

Plasmid analysis was limited to $bla_{TEM\text{-}52}$-positive and $bla_{CTX\text{-}M\text{-}1}$-positive isolates. On the level of these two ESBL genes and plasmid family (i.e. IncI1) the proportion of human isolates genetically related to poultry isolates was 20% (95% CI: 17-25%). On the level of these ESBL genes, the presence of IncI1 plasmid and similar plasmid sequence types (CC3, CC5 or CC7) this proportion was 19% (95% CI: 15-23%). Finally, at the level of these ESBL genes, plasmid typing and MLST of the isolate, this proportion was 11% (95% CI: 8-14%) (Table 6.2).

Of the five ESBL-producing *E. coli* bloodstream isolates that were sequenced two contained a PA ESBL gene: $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$.The $bla_{CTX\text{-}M\text{-}1}$ was located on the same plasmid (IncI1), from the same plasmid sequence type (CC7), and belonged to the same MLST cluster

(ST10) as was detected in a poultry isolate (Table 6.3). No plasmid analysis was performed on the $bla_{TEM-52}$-positive blood culture isolate, but all other isolates with $bla_{TEM-52}$ that were investigated had the same plasmids as found in poultry isolates (Figure 6.1: rows D and E).

***Table 6.2*** *The proportion of human extended spectrum beta-lactamase (ESBL)-positive Escherichia coli isolates that is genetically related to ESBL-positive poultry isolates on the level of gene, plasmid and strain genotype[a].*

| **Level of genetic typing** | **% of human isolates with poultry associated genetic element[a]** |
|---|---|
| ESBL genes ($bla_{CTX-M-1}$, $bla_{TEM-52}$, $bla_{SHV-12}$, $bla_{SHV-2}$ and $bla_{CTX-M-2}$) | 35% (see Table 6.1) |
| $bla_{CTX-M-1}$ and $bla_{TEM-52}$ genes | 30% (23.7% blaCTX-M-1; 6.2%blaTEM-52) |
| $bla_{CTX-M-1}$ and $bla_{TEM-52}$ genes on IncI1 plasmid | 20% (14.2% blaCTX-M-1; 6.2% blaTEM-52) |
| $bla_{CTX-M-1}$ and $bla_{TEM-52}$ genes on IncI1 plasmid belonging to complex CC7 or CC3 and CC5 respectiveley | 19% (12.6% blaCTX--1; 6.2% blaTEM-52) |
| $bla_{CTX-M-1}$ and $bla_{TEM-52}$ genes on IncI1 plasmid belonging to complex CC7 or CC3 and CC5 respectively in a poultry-associated MLST strain (ST10, ST58 or ST117) | 11% (9.5% blaCTX-M-1; 2.0% blaTEM-52) |

MLST, multi-locus sequence typing; [a]Percentages are extrapolations based on array results, sequence results and results of plasmid characterization and strain typing. For calculation of the percentages see Figure 6.1. For example percentage of $bla_{TEM-52}$ genes on IncI1 plasmid belonging to complex CC5 in to poultry identical MLST strains = 0.84 (row A) x 0.09 (row B) x 0.82 (row C) x 1 (row D) x 1 (row E) x 0.33 (row F) = 2.0%.

## 6.5 Discussion

In a representative sample of human ESBL-positive *E. coli* isolates in the Netherlands, 35% contained ESBL genes and 19% contained ESBL genes located on plasmids that were genetically indistinguishable from those obtained in poultry isolates. The majority of these ESBL genes (86%) were $bla_{CTX-M-1}$ and $bla_{TEM-52}$ genes, also the predominant genes in poultry (77%) and retail chicken meat (75%). Furthermore, 94% of a representative sample of chicken meat was contaminated with ESBL-producing *E. coli,* of which 39% belonged to genotypes also found in human samples. These findings are suggestive for transmission of ESBL-producing *E. coli* from poultry to humans, most likely through the food chain. Although our findings do not unequivocally prove that the poultry reservoir is the source of infections in humans, there are four lines of circumstantial evidence that do support such a sequence of events.

First, the potential of animal-derived *Enterobacteriaceae* to cause infections in humans has been established in community outbreaks of *Salmonella* and enteropathogenic *E coli* [17], and associations between *E. coli* colonization and infection in humans and exposure to retail chicken and other food sources have been reported [18-20].

Second, the prevalence of $bla_{CTX\text{-}M\text{-}1}$ genes (24%) and $bla_{TEM\text{-}52}$ (6%) among human *E. coli* is higher in the Netherlands than in most other countries[21-26].

Third, the increase of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes among human *E. coli* corroborates with an increase of these ESBL genes in poultry isolates in the Netherlands. The prevalence of cefotaxime-resistant *E. coli* in Dutch poultry started to increase in 2003[6] and in a human surveillance study among 21 laboratories in the Netherlands in 2006, proportions of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ producing *E. coli* were 9% and 3%, respectively (Sandra Bernards; personal communication).

Fourth, in one study people working with poultry seemed to have a higher risk for intestinal carriage of ESBL producing bacteria [7, Chapter 3].

Our study was restricted to Dutch patients, poultry and poultry meat products. Yet, ESBL carriage by poultry and contamination of retail meat with ESBL-producing bacteria has also been demonstrated in other European countries[8, 9, 21, 25, 27-29]

Our study has limitations. First, the spectrum of PA ESBL genes was based on a single study in poultry in 2006 and the analysis of 98 retail chicken meat samples in 2010, and this spectrum was compared with human isolates obtained between February and May 2009. Naturally, it is impossible to directly link carriage among poultry in 2006 to contaminated meat samples in 2010 to infected humans in 2009. Yet, although the ESBL epidemiology is rapidly evolving, it seems unlikely that the spectrum of genes present in these three compartments has changed dramatically over the period of 4 years. In fact, the five genes identified in poultry in 2006 were all identified on meat in 2010, in both compartments $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes accounted for 78% and 75% of ESBL genes and in both compartments strains with the same genotype were detected.

Second, the plasmid analysis was limited to a small selection of isolates with $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes, only. The latter was a consequence of the extreme labour-intensity of these analyses.

Strengths of our study include the detailed molecular analyses and the inclusion of human isolates from a nationwide surveillance program covering all aspects of the healthcare system and with an unbiased selection of isolates allowing, for the first time, the possibility to quantify the association between genetic relationships and incidence of infections in humans.

For example, during the study period 27 patients had an *E. coli* bacteraemia with a positive ESBL screen test. If, based on our results, 79% of these isolates contained an ESBL gene, this would imply 21 patients with ESBL-bacteremia. The ESBL genes from five of these isolates were sequenced and at least one and possibly two were PA. When extrapolated, at least one of 21 (5%), but possibly eight (38%) patients would have suffered an episode of PA *E. coli* bacteremia. As the participating laboratories cover nearly half of the Dutch hospital beds this would mean between two and 16 patients in the Netherlands between February and May 2009.



### 6.6 Acknowledgements

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***Table 6.3*** *Results of strain (multi-locus sequence typing) and plasmid typing (Inc-group and plasmid multi-locus sequence typing) of $bla_{CTX\text{-}M\text{-}1}$-producing and $bla_{TEM\text{-}52}$-producing Escherichia coli isolates from human patients and E. coli and Salmonella enterica isolates from poultry sources.*

| ESBL-gene | Strain Code | Origin | Species | Material |
|---|---|---|---|---|
| $bla_{CTX\text{-}M\text{-}1}$ | 148 | Human | *E. coli* | Blood |
| | 38.27 | Poultry | *E. coli* | Caecum |
| | 53a, 54a | Retail | *E. coli* | Chicken meat |
| | 1365 | Human | *E. coli* | Urine |
| | 1350 | Human | *E. coli* | Urine |
| | 1240 | Human | *E. coli* | Urine |
| | 38.16 | Poultry | *E. coli* | Caecum |
| | 1240 | Human | *E. coli* | Urine |
| | 897 | Human | *E. coli* | Respiratory tract |
| | 1047 | Human | *E. coli* | Rectal swab |
| | 38.52 | Poultry | *E. coli* | Caecum |
| | 623 | Human | *E. coli* | Urine |
| | 39.26 | Poultry | *E. coli* | Caecum |
| | 38.53 | Poultry | *E. coli* | Caecum |
| | 38.49 | Poultry | *E. coli* | Caecum |
| | 39.02 | Poultry | *E. coli* | Caecum |
| | 39.05 | Poultry | *E. coli* | Caecum |
| | 1247 | Human | *E. coli* | Urine |
| | 162.03 | Poultry | *S.* Java | Unknown |
| | 175.77 | Poultry | *S.* Infantis | Unknown |
| | 187.45 | Poultry | *S.* Infantis | Caecum |
| | 187.46 | Poultry | *S.* Infantis | Caecum |
| | 39.51 | Poultry | *E. coli* | Caecum |
| | 691 | Human | *E. coli* | Urine |
| | 1503 | Human | *E. coli* | Urine |
| | 39.47 | Poultry | *E. coli* | Meat |
| | 186.74 | Poultry | *S.* Java[b] | Caecum |
| | 450 | Human | *E. coli* | Urine |
| | 186.27 | Poultry | *S.* Agona | Caecum |
| | 990 | Human | *E. coli* | Urine |
| | 1198 | Human | *E. coli* | Urine |
| | 312 | Human | *E. coli* | Urine |
| | 60 | Human | *E. coli* | Urine |
| | 1455 | Human | *E. coli* | Urine |
| | 627 | Human | *E. coli* | Urine |
| | 13,591,416,152,179 | Human | *E. coli* | Urine |
| | 666,152,387 | Human | *E. coli* | Urine |
| | 52a,54a,72a,71 | Retail | *E. coli* | Chicken meat |
| | 60,61,63a,69,39b | Retail | *E. coli* | Chicken meat |

| Plasmid typing | | IncI1 typing | | *E. coli* strain typing |
|---|---|---|---|---|
| **ESBL localization** | **Plasmid size (kb)** | **Clonal Complex** | **Sequence type** | **Sequence type** |
| IncI1 | 100 | **CC7** | ST7 | 10 |
| IncI1 | 88 | **CC7** | ST7 | 10 |
| n.d. | n.d. | n.d. | n.d. | 10(n=2) |
| IncI1 | 100 | **CC7** | ST7 | 58 |
| IncI1 | 100 | **CC7** | ST7 | 58 |
| IncI1 | 95 | **CC7** | ST7 | 58 |
| IncI1 | 100 | **CC7** | ST7 | 58 |
| n.d. | n.d. | n.d. | n.d. | 58 |
| IncI1 | 100 | **CC7** | ST7 | 117 |
| IncI1 | 100 | **CC7** | ST7 | 117 |
| IncI1 | 100 | **CC7** | ST7 | 117 |
| n.d. | n.d. | n.d. | n.d. | 117 |
| IncI1 | 100 | **CC7** | ST7 | 48 |
| IncI1 | 100 | **CC7** | ST7 | 155 |
| IncI1 | 97 | **CC7** | ST7 | 641 |
| IncI1 | 110 | **CC7** | ST7 | 665 |
| IncI1 | 97 | **CC7** | ST7 | 752 |
| IncI1 | 100 | **CC7** | ST7 | 767 |
| IncI1 | 97 | **CC7** | ST7 | n.d. |
| IncI1 | 100 | **CC7** | ST7 | n.d. |
| IncI1 | 100 | **CC7** | ST7 | n.d. |
| IncI1 | 100 | **CC7** | ST7 | n.d. |
| IncI1 | 95 | **CC7** | ST30 | 155 |
| IncI1 | 90 | CC31 | ST35 | 131 |
| n.d. | n.d. | n.d. | n.d. | 131 |
| IncI1 | 97 | n.d. | n.t.[a] | 117 |
| IncI1 | 97 | n.d. | n.t.[a] | n.d. |
| IncI1 | 95 | **CC3** | ST3 | 167 |
| IncI1 | 110 | **CC3** | ST3 | n.d. |
| IncB/O | 95 | n.d. | n.d. | n.d. |
| IncB/O | 95 | n.d. | n.d. | n.d. |
| IncB/O | 100 | n.d. | n.d. | n.d. |
| IncN | 30 | n.d. | n.d. | n.d. |
| IncN | 35 | n.d. | n.d. | n.d. |
| n.t. | 30 | n.d. | n.d. | n.d. |
| n.d. | n.d. | n.d. | n.d. | 69(n=2),57,162 |
| n.d. | n.d. | n.d. | n.d. | 354.453.545 |
| n.d. | n.d. | n.d. | n.d. | 23(n=2), 624,1564 |
| n.d. | n.d. | n.d. | n.d. | 1594 (n=2),1901, n.t. (n=2) |

*Continued*

***Table 6.3*** *Continued*

| ESBL-gene | Strain Code | Origin | Species | Material |
|---|---|---|---|---|
| $bla_{TEM-52}$ | 38.34 | Poultry | *E. coli* | Caecum |
| | 320 | Human | *E. coli* | Urine |
| | 681 | Human | *E. coli* | Urine |
| | 85b | Retail | *E. coli* | Chicken meat |
| | 68 | Human | *E. coli* | Urine |
| | 39.76 | Poultry | *E. coli* | Caecum |
| | 166.01 | Poultry | *S.* Java[b] | Meat |
| | 166.22 | Poultry | *S.* Java[b] | Meat |
| | 162.19 | Poultry | *S.* Infantis | Unknown |
| | 173.44 | Poultry | *S.* Infantis | Caecum |
| | 85 | Human | *E. coli* | Urine |
| | 91 | Human | *E. coli* | Urine |
| | 1362 | Human | *E. coli* | Urine |
| | 229, 194 | Human | *E. coli* | Urine |
| | 45a,47a,83a,90,95a | Retail | *E. coli* | Chicken meat |

| Plasmid typing | | IncI1 typing | | *E. coli* strain typing |
|---|---|---|---|---|
| **ESBL localization** | **Plasmid size (kb)** | **Clonal Complex** | **Sequence type** | **Sequence type** |
| IncI1 | 97 | **CC5** | ST10 | 10 |
| IncI1 | 95 | **CC5** | ST36 | 10 |
| IncI1 | 95 | **CC5** | ST36 | 10 |
| n.d. | n.d. | n.d. | n.d. | 10 |
| IncI1 | 95 | **CC5** | ST10 | 156 |
| IncI1 | 90 | **CC5** | ST10 | 752 |
| IncI1 | 82 | **CC5** | ST10 | n.d. |
| IncI1 | 82 | **CC5** | ST10 | n.d. |
| IncI1 | 82 | **CC5** | ST10 | n.d. |
| IncI1 | 90 | **CC5** | ST10 | n.d. |
| IncI1 | 90 | **CC5** | ST36 | 131 |
| IncI1 | 90 | **CC5** | ST36 | n.t.[c] |
| IncI1 | 90 | **CC5** | ST36 | 453 |
| n.d. | n.d. | n.d. | n.d. | 23.744 |
| n.d. | n.d. | n.d. | n.d. | 23,48,117,1403,n.t. |

ESBL, extended spectrum beta-lactamase; n.d. not determined; n.t., non-typable; [a]Four sequences in conformance with pMLST ST7, but for locus ardA no sequence results were obtained; [b]*Salmonella enterica* serovar paratyphi B variant Java; [c]Six sequences in conformance with MLST ST767, but for locus *icd* no sequence results were obtained. This table shows the genetic correlation between *E. coli* from patients and retail meat and *E. coli* and *Salmonella* from poultry carrying $bla_{\text{CTX-M-1}}$ or $bla_{\text{TEM-52}}$. The *E. coli* isolates were compared by Multi Locus Sequence Typing (http://www.mlst.net). All IncI1 plasmids were compared by pMLST and three genetically related clusters were found, indicated by bold face text: CC7, CC3 and CC5. There were four sets of *E. coli* isolates, of human and animal origin, with undistinguishable ESBL genes, plasmids and isolated genotypes, indicated in the table by different shading patterns (light gray, MLST ST10 (n=2); middle gray, MLST 58; dark gray, MLST 117).

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# Chapter 7

# Occurrence and characteristics of extended spectrum beta-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses

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## 7.1 Abstract

The objective of this study was to investigate the occurrence and characteristics of extended spectrum beta-lactamase (ESBL) and AmpC-producing *Enterobacteriaceae* isolates in clinical samples of companion animals and horses and compare the results with ESBL/AmpC-producing isolates described in humans.

Between October 2007 and August 2009, 2700 *Enterobacteriaceae* derived from clinical infections in companion animals and horses were collected. Isolates displaying inhibition zones of ≤ 25 mm for ceftiofur and/or cefquinome by disk diffusion were included. ESBL/AmpC production was confirmed by combination disk tests. The presence of resistance genes was identified by microarray, PCR and sequencing, *Escherichia coli* genotypes by multi locus sequence typing and antimicrobial susceptibility by broth microdilution.

Sixty-five isolates from dogs (n=38), cats (n=14), horses (n=12) and a turtle were included. Six *Enterobacteriaceae* species were observed, mostly derived from urinary tract infections (n=32). All, except 10 isolates tested resistant to cefotaxime and ceftazidime by broth microdilution using clinical breakpoints. ESBL/AmpC genes observed were $bla_{\text{CTXM-1, -2, -9, -14,-15,}}$ $bla_{\text{TEM-52}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{CMY-39}}$. $bla_{\text{CTX-M-1}}$ was predominant (n=17). $bla_{\text{CTX-M-9}}$ occurred in combination with *qnrA1* in 3 of the 11 *Enterobacter cloacae* isolates. Twenty-eight different *E. coli* sequence types (STs) were found. *E. coli* carrying $bla_{\text{CTX-M-1}}$, belonged to 13 STs of which 3 were previously described in Dutch poultry and patients.

This is the first study among a large collection of Dutch companion animals and horses characterizing ESBL/AmpC-producing isolates. A similarity in resistance genes and *E. coli* STs among these isolates and isolates from Dutch poultry and humans may suggest exchange of resistance between different reservoirs.

## 7.2 Introduction

Members of the family *Enterobacteriaceae* commonly express plasmid-encoded broad-spectrum beta-lactamases (TEM-1, TEM-2, and SHV-1) that confer resistance to amino-penicillins and first generation cephalosporins, but not to third- and fourth-generation cephalosporins. The introduction of third-generation cephalosporins in the 1980s was a milestone in antimicrobial chemotherapy and improved the treatment options in human and veterinary medicine[1]. Unfortunately, resistance to extended spectrum cephalosporins (ESCs) emerged a few years later. Resistance to ESCs in *Enterobacteriaceae* is most often related to the production of extended-spectrum beta-lactamases (ESBL) or AmpC beta-lactamases. ESBLs confer resistance to amino-penicillins, cephalosporins and monobactams and are inhibited by clavulanic acid. AmpC beta-lactamases have a broader spectrum of resistance, including the cephamycins, and are not inhibited by beta-lactamase inhibitors. The production of ESBLs and AmpC beta-lactamases is often plasmid mediated. Moreover, these plasmids frequently carry genes encoding resistance to other drug classes, such as fluoroquinolones, aminoglycosides, sulfa-derivatives and trimethoprim[2,3]. Therefore treatment options for infections caused by ESBL- and/or AmpC-producing organisms are limited.

Initially these organisms were associated with hospitals and institutional care in humans, but they are now increasingly found in the community and in food-producing animals, particularly poultry, suggesting an exchange of organisms or genes between the different reservoirs, or a different antibiotic use behaviour[4]. In the Netherlands, ESCs are authorized for

use in food-producing animals (ceftiofur, cefquinome) and companion animals (cefovecin).

Resistance to ESC has been studied in detail in Gram-negative bacteria isolated from humans and food-producing animals[5, 6, Chapter 2&6]. However, data on ESBL and AmpC beta-lactamase-producing *Enterobacteriaceae* in companion animals and horses are limited[7]. The objective of this study was to investigate the occurrence of ESBL/AmpC-producing organisms in clinical samples, to further characterize these isolates and compare the results with ESBL and AmpC-producing isolates described in humans.

## 7.3 Material and methods

### *7.3.1 Bacterial isolates*

The Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University investigates samples from all over the Netherlands. Per year approximately 10 000 samples are submitted for bacteriological analysis and about 48% of these samples originate from dogs, 18% from cats, 12% from horses, 10% from food-producing animals and 12% from other animals including reptiles, birds and mammals other than those mentioned. From October 2007 to August 2009, 10 755 isolates derived from approximately 18 000 samples, of which 2700 were *Enterobacteriaceae*, were tested for susceptibility to a wide range of antimicrobial agents by disk diffusion using a semi-confluent inoculum of the bacteria on Iso-Sensitest agar (bioTRADING, Mijdrecht, the Netherlands) with Neo-Sensitabs (Rosco, Diagnostica, Taastrup, Denmark). Species were identified by conventional biochemical methods. *Salmonella* serovar identification was performed using microtiter and slide agglutination methods (O- and H- group antigens) according to the latest version of the Kauffman-White scheme. All *Enterobacteriaceae* isolates that displayed an inhibition zone ≤ 25 mm for ceftiofur (dog/cat/turtle isolates) and/or cefquinome (horse isolates) were included in the study and stored at -80°C until further analysis.

### *7.3.2 Phenotypic ESBL/AmpC testing.*

These isolates were tested phenotypically for ESBL production by combination disk tests using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson) according to CLSI guidelines[8]. In addition, a cefoxitin disk (30 µg, Becton Dickinson) was added to this test, to detect AmpC phenotypes. All isolates classified as intermediate or resistant using CLSI criteria (≤ 17 mm) to cefoxitin were suspected to be AmpC producers.

### *7.3.3* E. coli *genotyping*

In order to compare the genotypes of the *E. coli* isolates found in this study with ESBL-producing *E. coli* in former human studies, all *E. coli* strains were genotyped by multi locus sequence typing (MLST) as described previously[9]. Sequences were uploaded on the MLST website (http://mlst.ucc.ie/.

### *7.3.4 Susceptibility test*

All isolates were tested for antimicrobial susceptibility by determining MICs using broth microdilution, according to the international standard ISO 20776-1:2006. MICs were determined

for ampicillin (concentration range of 0.5 - 32 mg/L), cefotaxime (0.06 - 4 mg/L), ceftazidime (0.25 - 16 mg/L), ciprofloxacin (0.008 - 4 mg/L) , nalidixic acid (4 - 64 mg/L), colistin (2 - 4 mg/L), gentamicin (0.25 - 32 mg/L), kanamycin (4 - 128 mg/L), sulfamethoxazole (8 - 1024 mg/L), trimethoprim (0.5 - 32 mg/L), streptomycin (2 - 128 mg/L), tetracycline (1 - 64 mg/L), chloramphenicol (2 - 64 mg/L) and florfenicol (2 - 64 mg/L) using *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 as control isolates. Multidrug resistance was defined as resistance to three or more antimicrobial agents included in the following list of eight antibiotics: cefotaxime (R > 2 mg/L), ciprofloxacin (R > 1 mg/L), colistin (R > 2 mg/L), gentamicin (R > 4 mg/L), sulfamethoxazole (R > 256 mg/L), trimethoprim (R > 4 mg/L), tetracycline (R > 8 mg/L) and chloramphenicol (R > 8 mg/L). Isolates were classified as resistant based on EUCAST clinical breakpoints (www.eucast.org) and if not available (for sulfamethoxazole and tetracycline), CLSI clinical breakpoints[8]. *Serratia marcescens* is intrinsically resistant to colistin and *Proteus mirabilis* to both colistin and tetracycline[10]. These bacteria species were not included to calculate multiresistance.

*7.3.5 beta-lactamase identification*

All isolates were screened for $bla_{SHV}$, $bla_{LEN}$, $bla_{TEM}$, $bla_{OXA}$, $bla_{CTX-M}$, $bla_{DHA}$, $bla_{ACC}$, $bla_{MOX}$, $bla_{FOX}$ or $bla_{CMY}$ gene families by miniaturized microarray (Identibac, AMR-ve 05 genotyping, Alere International, Tilburg, the Netherlands)[11]. The gene families that responded positively in the array were further typed by PCR and sequencing using primers displayed in Table 7.1. PCR consisted of 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at temperatures mentioned in Table 7.1 and 60 s of extension at 72°C) after one step of 5 minutes at 94°C. Amplicons were purified and sequenced as described previously[5]. All *E. coli* isolates with AmpC phenotypes that could not be attributed to the presence of a plasmid-mediated AmpC gene were tested for mutations in the *ampC* promoter/attenuator region as described previously[12]. PCRs to detect $bla_{SHV}$, $bla_{CTX-M}$ or $bla_{TEM}$-genes were performed in isolates with an ESBL phenotype in which the array was negative for probes encoding ESBL-gene families (Table 7.1).

*7.3.6 Additional resistance gene identification*

Antibiotic resistance genes were detected using the same microarray that was used for the screening of beta-lactamase genes. This array included probes for 40 non-beta-lactam resistance gene families known to occur in Gram-negative bacteria[11]. Isolates with positive signals for *qnr*-genes, which encode for plasmid-mediated quinolone resistance genes, were further analysed by PCR and sequencing using primers for *qnrA*, *qnrB* and *qnrS* as displayed in Table 7.1 and as described previously[13].

## 7.4 Results

During the study period (October 2007 – August 2009), 2% of all *Enterobacteriaceae* (n=65) displayed inhibition zones of ≤ 25 mm for ceftiofur or cefquinome. Between January 2008 and August 2009, this was found for 3% of the *Enterobacteriaceae* derived from dogs, 4% from cats, and 8% from horses. For 2007 these data were not available. In 2007, a total of eight isolates matched the inclusion criteria. In 2008, 28 isolates were included, and in 2009, 29 isolates. All

***Table 7.1*** *Primers and PCR conditions used in this study to detect ESBL, plasmid mediated AmpC, chromosomal* ampC *and* qnr *genes.*

| Target | Primer | Annealing (°C) | Sequence (5'-3') | Product size (bp) | Reference |
|---|---|---|---|---|---|
| **CTX-M-families** | CTX-M-F | 55 | ATG TGC AGY ACC AGT AAR GTK ATG GC | 592 | 5 |
| | CTX-M-R | | TGG GTR AAR TAR GTS ACC AGA AYS AGC GG | | |
| **CTX-M-1** | CTX-1-SEQ-F | 60 | CCC ATG GTT AAA AAA TCA CTG C | >1000 | 28 |
| | CTX-1-SEQ-R | | CAG CGC TTT TGC CGT CTA AG | | |
| **CTX-M-2** | CTX-M-2F | 55 | ATG ATG ACT CAG AGC ATT CG | 886 | 5 |
| | CTX-M-2R | | TTA TTG CAT CAG AAA CCG TG | | |
| **CTX-M-9** | CTX-M-9-1F | 55 | TGG TGA CAA AGA GAG TGC AAC G | 875 | 20 |
| | CTX-M-4R | | TCA CAG GCC TTC GGC GAT | | |
| **CTX-M-14/17** | CTX-M-$9_{792}$F | 55 | CTA TTT TAC CCA GCC GCA AC | 238 | This study |
| | CTX-M-$9_{1029}$R | | GTT ATG GAG CCA CGG TTG AT | | |
| **SHV** | SHV-F | 55 | TTA TCT CCC TGT TAG CCA CC | 796 | 5 |
| | SHV-R | | GAT TTG CTG ATT TCG CTC GG | | |
| **TEM** | TEM-F | 55 | GCG GAA CCC CTA TTT G | 964 | 5 |
| | TEM-R | | ACC ATT GCT TAA TCA GTG AG | | |
| **CMY** | CMY-F | 58 | ATG ATG AAA AAA TCG TTA TGC TGC | 1138 | 5 |
| | CMY-R | | GCT TTT CAA GAA TGC GCC AGG | | |
| **Chromosomal *ampC*** | $AmpC1_{-71}$ | 55 | AAT GGG TTT TCT ACG GTC TG | 191 | 12 |
| | $AmpC2_{120}$ | | GGG CAG CAA ATG TGG AGC AA | | |
| **OXA-1** | OXA-1-F | 55 | ATG AAA AAC ACA ATA CAT ATC AAC TTC GC | 820 | 29 |
| | OXA-1-R | | GTG TGT TTA GAA TGG TGA TCG CAT T | | |
| **OXA-2** | OXA-2-F | 55 | ACG ATA GTT GTG GCA GAC GAA C | 601 | 29 |
| | OXA-2-R | | ATY CTG TTT GGC GTA TCR ATA TTC | | |
| ***qnrA*** | *qnrA*-F | 53 | ATT TCT CAC GCC AGG ATT TG | 516 | 13 |
| | *qnrA*-F | | GAT CGG CAA AGG TTA GGT CA | | |
| ***qnrB*** | *qnrB*-F | 53 | GAT CGT GAA AGC CAG AAA GG | 469 | 13 |
| | *qnrB*-R | | ACG ATG CCT GGT AGT TGT CC | | |
| ***qnrS*** | *qnrS*-F | 53 | ACG ACA TTC GTC AAC TGC AA | 417 | 13 |
| | *qnrS*-R | | TAA ATT GGC ACC CTG TAG GC | | |

***Table 7.2*** *Characteristics of* Enterobacteriaceae *isolates displaying inhibition zones of ≤ 25 mm for ceftiofur or cefquinome from clinical infections in companion animals and horses.*

| Phenotype | *bla*- and *qnr* genes | Number of isolates | Species | *E. coli* MLST |
|---|---|---|---|---|
| ESBL (n=29) | CTX-M-1 | 7 | *E. coli* | 117, 162, 141, 770, 2030, 2226, 461 |
| | CTX-M-1, TEM-1 | 9 | *E. coli* | 88, 162 (n=2), 457, 362, 34(n=3), 58 |
| | CTX-M-1, CTX-M-14, TEM-1 | 1 | *E. coli* | 1287 |
| | CTX-M-1, TEM-80 | 1 | *E. coli* | 461 |
| | CTX-M-14 | 1 | *E. coli* | 1287 |
| | CTX-M-15 | 1 | *E. coli* | 648 |
| | CTX-M-15, TEM-1 | 2 | *E. coli* | 88, 156 |
| | CTX-M-15, TEM-1, OXA-1 | 1 | *E. coli* | 131 |
| | CTX-M-2, TEM-1 | 2 | *E. coli* | 156 (n=2) |
| | TEM-52 | 3 | *E. coli (n=2), S. enterica (n=1)* | 58, 93 |
| | TEM-1, OXA-1 | 1 | *E. coli* | 117 |
| AmpC (n=22) | CMY-2 | 6 | *E. coli (n=3), P. mirabilis (n=3)* | 297, 372, 2227 |
| | CMY-2, OXA-1 | 1 | *E. coli* | 88 |
| | CMY-2, SHV-1 | 1 | *E. coli* | 12 |
| | CMY-2, TEM-1 | 2 | *E. coli* | 68 (n=2) |
| | CMY-39, *qnr*B17var[a] | 1 | *C. freundii* | n.a. |
| | Chromosomal *ampC* mutations[b] | 3 | *E. coli* | 372, 58, 88 |
| | Chromosomal *ampC* type 3[c], TEM-1 | 1 | *E. coli* | 539 |
| | TEM-1 | 1 | *E. cloacae* | n.a. |
| | TEM-1, *qnr*S1/S3 | 2 | *E. cloacae* | n.a. |
| | none | 4 | *E. cloacae* | n.a. |
| ESBL/AmpC (n=6) | CTX-M-9 | 1 | *E. cloacae* | n.a. |
| | CTX-M-9, *qnr*A1 | 3 | *E. cloacae* | n.a. |
| | CTX-M-15, CMY-2, TEM-1 | 1 | *E. coli* | 648 |
| | CTX-M-14, TEM-1, chromosomal *ampC* type 4[c] | 1 | *E. coli* | 405 |
| IRE (n=2) | TEM-30 | 1 | *E. coli* | 88 |
| | none | 1 | *S. marcescens* | n.a. |
| Susceptible (n=6) | OXA-1 | 1 | *E. coli* | 88 |
| | TEM-1 | 3 | *E. coli* | 448, 950, 1642 |
| | TEM-1, OXA-1 | 1 | *E. coli* | 1642 |
| | none | 1 | *E. coli* | 2225 |

| Year of isolation | Source | Material |
|---|---|---|
| 2008 (n=5), 2009 (n=2) | dog (n=2), cat (n=2), horse (n=3) | peritoneal fluid (n=2), urine (n=2), wound (n=1), uterus (n=2) |
| 2008 (n=3), 2009 (n=6) | dog (n=4), horse (n=5) | urine (n=4), blood (n=1), uterus (n=3), trachea (n=1) |
| 2008 | horse | wound |
| 2009 | dog | wound |
| 2008 | horse | wound |
| 2008 | dog | throat |
| 2008 (n=2) | dog (n=2) | urine (n=2) |
| 2008 | dog | urine |
| 2007, 2008 | cat, horse | urine, wound |
| 2007 (n=1), 2009 (n=2) | dog (n=2), cat (n=1) | urine (n=2), faeces (n=1) |
| 2007 | dog | peritoneal fluid |
| 2008 (n=3), 2009 (n=3) | dog (n=5), cat (n=1) | urine (n=6) |
| 2007 | dog | urine |
| 2008 | cat | Wound |
| 2008, 2009 | dog (n=2) | urine (n=1), wound (n=1) |
| 2007 | turtle | Trachea |
| 2008 (n=1), 2009 (n=2) | dog (n=2), cat (n=1) | peritoneal fluid (n=1), bile (n=1), urine (n=1) |
| 2009 | dog | urine |
| 2009 | horse | Wound |
| 2008 | cat (n=2) | Wound (n=2) |
| 2008 (n=1), 2009 (n=3) | dog (n=2), cat (n=2) | urine (n=4) |
| 2008 | cat | Wound |
| 2008 (n=1), 2009 (n=2) | dog (n=3) | blood , trachea, urine |
| 2009 | dog | Wound |
| 2007 | dog | peritoneal fluid |
| 2007 | dog | Wound |
| 2007 | dog | wound |
| 2009 | dog | urine |
| 2008 (n=1), 2009 (n=2) | dog (n=3) | urine (n=3) |
| 2009 | cat | Wound |
| 2008 | cat | urine |

IRE=Inhibitor Resistant ESBL; *E. coli* = *Escherichia coli*; *S. enterica*= *Salmonella enterica* subspecies *enterica* 4,[5],12:b:-; *P. mirabilis* = *Proteus mirabilis*; *E. cloacae* = *Enterobacter cloacae*; *C. freundii* = *Citrobacter freundii*; *S. marcescens* = *Serratia marcescens*; n.a. = not applicable; [a]Sequence is similar to *qnrB17*, but has 1 silent mutation at position 169 GC**G** -> GC**A**; [b]Chromosomal *ampC* mutations type 2, 3 and 31 according to Mulvey *et al.* [14]; [c] Chromosomal *ampC* types refer to mutations in the chromosomal *ampC* gene as described by Mulvey *et al*.[14].

65 isolates originated from clinical infections from dogs (n=38), cats (n=14), horses (n=12) and a turtle. Except for two horses, all animals belonged to different owners and were submitted by 37 veterinary clinics located in 11 provinces in the Netherlands. One isolate was cultured from a dog that lived in an animal shelter in Germany.

Information about antibiotic treatment was not available for all patients, but given the fact that most veterinary practices send in their samples only when initial antibiotic treatment fails, the isolates obtained are considered to be derived from patients that have received one or more antibiotic treatments.

The isolates were cultured from urine (n=32), wound (n=15), peritoneal fluid (n=5), uterus (n=5), trachea (n=3), blood (n=2), faeces (n=1), bile (n=1) and a throat sample (n=1). Most isolates were *E. coli* (n=48). Other isolates were identified as *Enterobacter cloacae* (n=11), *Proteus mirabilis* (n=3), *Salmonella enterica* subspecies *enterica* 4,[5],12:b:- (n=1), *Serratia marcescens* (n=1) and *Citrobacter freundii* (n=1).

Combination disk tests resulted in 29 isolates displaying an ESBL-phenotype, 22 an AmpC-phenotype, six a mixed ESBL/AmpC phenotype (displaying synergy with clavulanic acid and resistance to cefoxitin) and two displayed inconclusive results in the confirmation test (resistant to cefotaxime and/or ceftazidime, but no synergy with clavulanic acid and susceptible to cefoxitin). This was designated to be an inhibitor resistant ESBL (IRE) type. In the remaining six isolates (four from dogs and two from cats), although with inhibition zones of ≤ 25 mm for ceftiofur, the ESBL phenotypic test was negative. These six isolates had MICs between ≤ 0.06 and 0.5 mg/L for cefotaxime and between ≤ 0.25 and 0.5 mg/L for ceftazidime, and molecular analysis of these isolates did not result in the detection of an ESBL and/or AmpC gene. The isolates that showed either an ESBL, ESBL/AmpC or IRE type were all resistant to cefotaxime (MIC > 2 mg/L) and/or ceftazidime (MIC > 4 mg/L). Four isolates displaying an AmpC phenotype had cefotaxime and ceftazidime MIC values below the clinical breakpoints. In sum, of 2700 isolates screened, 55 isolates (2.0 %) were resistant to either cefotaxime or ceftazidime.

In the 29 isolates with an ESBL phenotype the following ESBL genes were found: $bla_{\text{CTX-M-1}}$ (n=17), $bla_{\text{CTX-M-15}}$ (n=4), $bla_{\text{CTX-M-14}}$ (n=1), $bla_{\text{CTX-M-2}}$ (n=2), $bla_{\text{TEM-52}}$ (n=3) and a combination of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-14}}$ (n=1). In one isolate no ESBL gene could be detected (Table 7.2). No $bla_{\text{SHV}}$ or $bla_{\text{OXA}}$ genes encoding for ESBL production were detected.

Among the 22 isolates with an AmpC phenotype, 11 carried an AmpC gene ($bla_{\text{CMY-2}}$ (n=10) and $bla_{\text{CMY-39}}$ (n=1)) and one of these isolates (a *P. mirabilis* with $bla_{\text{CMY-2}}$) was clinically susceptible to cefotaxime and ceftaxidime (Table 7.2). Four isolates with an AmpC phenotype had mutations in the promoter region of the chromosomal *ampC* gene. These four isolates had mutations in the *ampC* gene promoter belonging to sequence types (STs) 2, 3 (n=2) and 31 as described by Mulvey *et al*.[14]. Two of those isolates (one with type 2 and one with type 3) were clinically susceptible to cefotaxime and ceftazidime (Table 7.3). The remaining seven isolates displaying an AmpC phenotype all belonged all to *E. cloacae*. In these isolates no plasmid-mediated AmpC gene was detected with the methods used (Table 7.2 and 7.3). One isolate of this group had MICs of 1 mg/L and 0.5 mg/L for cefotaxime and ceftazidime, respectively.

Among the isolates showing a combined ESBL/AmpC phenotype, $bla_{\text{CTX-M-9}}$ (n=4, all *E. cloacae*), $bla_{\text{CTX-M-14}}$ in combination with type 4 mutations in the *ampC* attenuator region [14] (n=1) and a combination of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ were detected (n=1) (Tables 7.2 and 7.3).

Among the two isolates with inconclusive results in the combination disk test (IRE), one carried $bla_{\text{TEM-30}}$ and in the other isolate (*S. marcescens*) no ESBL or AmpC genes were observed (Table 7.2).

Most isolates were multidrug resistant: 74% percent of all 61 isolates included in the calculation (all isolates except *P. mirabilis* and *S. marcescens*) were resistant to three or more antimicrobial classes and 32% were resistant to more than five classes of antibiotics. Among the isolates included in this calculation, nine were clinically susceptible to cefotaxime and/or ceftazidime, six of these were resistant to three or more antibiotics and one of them was resistant to more than five antibiotics. Besides beta-lactamase genes, the following gene-families were observed, encoding resistance to trimethoprim (*dfrA1* (23%), *dfrA12* (5%), *dfrA14* (3%), *dfrA17* (25%), *dfr A19* (2%), *dfrV* (6%)), sulfonamides (*sul1*(42%), *sul2* (12%), *sul3* (2%), fluoroquinolones (*qnr,* detects *qnrA*-genes (6%), *qnrB* (2%), *qnrS* (3%)), tetracyclines (*tetA* (6%), *tetB* (25%), *tetC* (2%)), aminoglycosides (*aadA1* (29%), *aadA2* (17%), *aadA4* (14%), *aac(6')-Ib* (8%), *strA* (3%), *strB* (25%), *ant2a* (8%)), chloramphenicol (*cmlA1* (3%), *catA1* (25%), *catB3* (2%)), florfenicol (*floR* (5%)), and macrolides (*ermB* (3%), *ereA* (3%), *ereB* (20%)). In addition, integrase genes *intI1* (29%) and *intI2* (3%) were found (Table 7.3). PCR and sequencing of the seven isolates positive for a *qnr* gene in the array demonstrated the presence of three *qnrA1* genes; all three were detected in *E. cloacae* isolates from dogs also carrying $bla_{\text{CTX-M-9}}$. Two *qnrS1/S3* genes (no distinction could be made between *qnrS1* and *qnrS3* with the primers used) were observed in *E. cloacae* carrying $bla_{\text{TEM-1}}$ isolated from cats and one *qnrB17*-variant (*qnrB17* with one silent mutation at position 169 GC**G** → GC**A**) was found in *C. freundii* carrying $bla_{\text{CMY-39}}$ isolated form a turtle. In one isolate (ID 32, Table 7.3), although positive for the *qnr*-probe in the array, no *qnr* PCR product could be amplified with the primers used in this study (Table 7.1). This strain was susceptible to ciprofloxacin (MIC 0.015 mg/L) as well as to nalidixic acid (MIC ≤ 4 mg/L) (Table 7.1), confirming the absence of a *qnr* gene.

MLST of the *E. coli* isolates resulted in 28 different STs (Table 7.2).The predominant STs were ST88 (n=6), ST162 (n=3), ST58 (n=3), ST34 (n=3) and ST156 (n=3). Three new STs were identified: ST2225, ST2226, and ST2227. Except for the two isolates from the horses owned by the same person, which both belonged to ST34, no relation was found between STs and the area where the animals lived, or where the isolates were collected (data not shown).

### 7.5 Discussion

A prevalence of 2% ESC resistance in clinical isolates derived from companion animals is comparable to what is found in other countries[15, 16]. In this study we used ceftiofur or cefquinome to detect resistance to ESCs. Veterinary diagnostic laboratories often use cephalosporins that are commonly used in veterinary practice to perform susceptibility tests. However, these antibiotics have been shown to be less suitable as indicator cephalosporins for isolates with ESBL or plasmid-mediated AmpC beta-lactamases[17]. In our study, screening with these cephalosporins using non-standardized interpretive criteria resulted in 19 (29%) apparently false-positive isolates, in which no ESBL and/or plasmid mediated AmpC genes could be detected by array or PCR and sequencing. Six of these (all *E. coli*) were negative in the phenotypic confirmation test and had MICs below clinical breakpoints for cefotaxime and ceftazidime. Four other isolates (two *E. coli*, one *P. mirabilis* and one *E. cloaceae*), although displaying an AmpC phenotype, also had MICs below the clinical breakpoints for both ESCs. This resulted in a misclassification of 15% (10/65) of the isolates being resistant to ESC using ≤25 mm as cut-off value for ceftiofur or cefquinome in the disk diffusion test with Neo-Sensitabs (Rosco). To have fewer false-positive results in the future both the method and the interpretative criteria should be critically analysed and possibly reconsidered.

Overall a variety of ESBL genes was found within six bacterial species isolated from four animal species. $bla_{CTX\text{-}M\text{-}1}$ was the predominant ESC resistance gene found (26%). This gene was also the predominant ESBL gene among *Enterobacteriaceae* studied in Dutch horses in 2003-05[18]. At that time, only $bla_{CTX\text{-}M\text{-}1}$ was isolated. Our study shows that in later years $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}14}$ also occur in *Enterobacteriaceae* from Dutch horses.

$bla_{CTX\text{-}M\text{-}1}$ was recently described as the predominant ESBL gene in cefotaxime-resistant *E. coli* and *S. enterica* isolates (49%) of Dutch poultry[5] and in poultry meat isolates (49%) derived from Dutch supermarkets[6]. In Dutch patients, $bla_{CTX\text{-}M\text{-}1}$ was not predominant in clinical infections (21 – 24%)[6, 19], but it was the predominant gene (46%) found in ESC resistant *E. coli* derived from rectal swabs of human patients in Dutch hospitals[19]. Our results show that $bla_{CTX\text{-}M\text{-}1}$ was present among others in *E. coli* ST117 (n=2), ST58 (n=3) and ST162 (n=3). These *E. coli* genotypes are described in Dutch poultry (ST117 and ST58) and patients (ST117, ST58 and ST162) carrying $bla_{CTX\text{-}M\text{-}1}$ [6,Chapter 6].This suggests a clonal spread of these *E. coli* genotypes carrying $bla_{CTX\text{-}M\text{-}1}$ between different hosts.

A remarkable finding in our study was that all $bla_{CTX\text{-}M\text{-}9}$ genes (n=4) occurred in *E. cloacae* isolates, three derived from dogs and one from a cat. The canine isolates also harboured the plasmid-mediated quinolone resistance gene *qnrA1*. The combined presence of $bla_{CTX\text{-}M\text{-}9}$ and *qnrA1* has been described in outbreak strains of *Enterobacter hormaechei* (initially also classified as *E. cloacae*) in a Dutch hospital at Utrecht University Medical Centre carrying a conjugative plasmid pQC of the IncHI2 family with several complex integrons containing *aadB*, $bla_{CTX\text{-}M\text{-}9}$ and *qnrA1*[20, 21] .The three canine isolates were not screened for the *aadB* gene, but had non-wild type MICs of gentamicin, and two isolates contained a class 1 integron. To our knowledge this has not been reported previously in clinical isolates from dogs. The dogs and their owners originated from three different areas in the Netherlands and none of them originated from the area around Utrecht, which confirms the observed nationwide outbreak of *qnrA1*-positive multidrug-resistant *E. cloacae* in human patients[22]. Moreover, in 2008 an incHI2 plasmid containing *qnrA1* and $bla_{CTX\text{-}M\text{-}9}$ was found in *Salmonella* paratyphi B var. Java isolated from broilers[23 , 24]. Together with our data this suggests that a plasmid with this combination of resistance genes is not only present in different areas within the Netherlands, but has also disseminated in different hosts, including humans, food-producing animals and companion animals.

Another interesting finding in our study was the presence of $bla_{CTX\text{-}M\text{-}15}$ in different *E. coli* genotypes. Only one isolate derived from a dog carrying $bla_{CTX\text{-}M\text{-}15}$ belonged to uropathogenic *E. coli* ST131. This ST has emerged globally in hospital and community settings and has been described frequently in humans[25] and incidentally in isolates derived from dogs and horses in several European countries[25-27], emphasizing the intra-species and pan-European spread of this resistant clone. Our data, however, confirm that $bla_{CTX\text{-}M\text{-}15}$ in *E. coli* of dogs is not limited to *E. coli* ST131, and that other clones like ST88, ST156 and ST648 play a role in the dissemination of this resistance gene as well.

This is the first study providing information about the genes and genotypes involved in ESBL- or AmpC-producing isolates from Dutch companion animals and horses in a large group of isolates. The most prevalent gene found, $bla_{CTX\text{-}M\text{-}1}$ was previously found in Dutch human and poultry isolates. This, together with the presence of the combination of $bla_{CTX\text{-}M\text{-}9}$ and *qnrA1* in *E. cloacae* isolates, which was previously described in human isolates, suggests transmission between the different reservoirs or the existence of a common source. The fact that companion animals often live in close contact with their owners makes the occurrence of transmission between them even more likely. This study shows that prudent usage of antibiotics in companion

animals and horses should be emphasized and continued susceptibility surveillance to ESCs is recommended.

### 7.6 Acknowledgements

This work was partly presented in an oral presentation at the Scientific Spring Meeting of the Dutch Society for Medical Microbiology and the Dutch Society for Microbiology in Arnhem, The Netherlands, 2011 (abstract number 0194) and in a poster at the fourth symposium on Antimicrobial Resistance in Animals and the Environment (ARAE) in Tours, France, 2011(abstract number P55).

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***Table 7.3*** *Results of genotyping, microarray analysis, ESBL/AmpC characterization and MIC determination of* Enterobacteriaceae *isolates displaying inhibition zones of ≤ 25 mm for ceftiofur and/or cefquinome derived from clinical infections in companion animals and horses.*

| Phenotype | ID | Species | *E. coli* MLST | Year of Isolation | Source | Material | ESBL/ AmpC/ *qnr* genes | Other resistance gene families (microarray) |
|---|---|---|---|---|---|---|---|---|
| ESBL | 36 | *E. coli* | 117 | 2008 | dog | peritoneal fluid | CTX-M-1 | *tetA* |
| | 51 | *E. coli* | 162 | 2009 | dog | peritoneal fluid | CTX-M-1 | *aadA1, dfrA1, sul1* |
| | 22 | *E. coli* | 141 | 2008 | cat | urine | CTX-M-1 | *aadA1, dfrA1, dfrV, intI1, sul1* |
| | 9 | *E. coli* | 770 | 2008 | cat | urine | CTX-M-1 | *aadA1, dfrA1¸intI1, sul1, sul2* |
| | 57 | *E. coli* | 2030 | 2009 | horse | wound | CTX-M-1 | *aadA4, catA1, dfrA1, dfrA17, sul1, sul2, tetB, intI1, strB* |
| | 23 | *E. coli* | 2226 | 2008 | horse | uterus | CTX-M-1 | *aadA4, catA1, dfrA17, intI1, sul1, tetB* |
| | 38 | *E. coli* | 461 | 2008 | horse | uterus | CTX-M-1 | *catA1, dfrA14, dfrA17, sul2, ereB, tetB, strB* |
| | 50 | *E. coli* | 88 | 2009 | dog | urine | CTX-M-1, TEM-1 | *dfrA17, ereB* |
| | 39 | *E. coli* | 162 | 2008 | dog | urine | CTX-M-1, TEM-1 | *aadA1, dfrA1, sul1, tetB* |
| | 62 | *E. coli* | 162 | 2009 | dog | urine | CTX-M-1, TEM-1 | *catA1, dfrA17, ereB, tetB* |
| | 53 | *E. coli* | 457 | 2009 | dog | urine | CTX-M-1, TEM-1 | *dfrA17* |
| | 18 | *E. coli* | 362 | 2008 | horse | blood | CTX-M-1, TEM-1 | *aadA4, catA1, dfrA17, sul1, tetB, strB* |
| | 61 | *E. coli* | 34 | 2009 | horse | trachea | CTX-M-1, TEM-1 | *catA1, dfrA17, ereB* |
| | 63 | *E. coli* | 34 | 2009 | horse | uterus | CTX-M-1, TEM-1 | *dfrA17* |
| | 68 | *E. coli* | 34 | 2009 | horse | uterus | CTX-M-1, TEM-1 | *catA1, dfrA17, intI1, sul1* |
| | 31 | *E. coli* | 58 | 2008 | horse | uterus | CTX-M-1, TEM-1 | *dfrV, intI1, sul2, strB* |
| | 17 | *E. coli* | 1287 | 2008 | horse | wound | CTX-M-1, CTX-M-14, TEM-1 | *aadA1, aadA4, catA1, dfrA1, dfrA17, intI1, sul1, sul2, tetB, strB* |

| MIC (mg/L) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMP | CTX | CAZ | CIP | NAL | CST | GEN | KAN | SMX | TMP | STR | TET | CHL | FFN |
| >32 | >4 | 4 | 0.015 | ≤4 | ≤2 | 1 | ≤4 | ≤8 | ≤0.5 | 8 | >64 | 8 | 8 |
| >32 | >4 | 1 | >8 | >64 | ≤2 | 0.5 | ≤4 | >1024 | >32 | 16 | 2 | 8 | 8 |
| >32 | >4 | 1 | 0.015 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | >32 | 16 | 2 | 4 | 4 |
| >32 | >4 | 0.5 | ≤0.008 | ≤4 | ≤2 | 1 | ≤4 | >1024 | >32 | 16 | 2 | 4 | 4 |
| >32 | >4 | 2 | 0.015 | ≤4 | ≤2 | >32 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | ≤0.25 | ≤0.008 | ≤4 | ≤2 | >32 | >128 | >1024 | >32 | 128 | >64 | >64 | 4 |
| >32 | >4 | 4 | 0.03 | ≤4 | ≤2 | >32 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 1 | 0.12 | 64 | ≤2 | 1 | ≤4 | >1024 | >32 | 32 | 64 | 4 | 4 |
| >32 | >4 | 1 | 0.015 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | >32 | 128 | >64 | 8 | 8 |
| >32 | >4 | 2 | 8 | >64 | ≤2 | 1 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 2 | 0.015 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | >32 | 16 | >64 | 8 | 8 |
| >32 | >4 | 1 | 0.015 | ≤4 | ≤2 | 16 | >128 | >1024 | >32 | >128 | >64 | >64 | 4 |
| >32 | >4 | 0.5 | 0.015 | ≤4 | ≤2 | >32 | ≤4 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 0.5 | 0.015 | ≤4 | ≤2 | 32 | ≤4 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 1 | 0.015 | ≤4 | ≤2 | >32 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 8 | 0.03 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | >32 | >128 | 2 | 8 | 8 |
| >32 | >4 | 8 | >8 | >64 | ≤2 | >32 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |

*Continued*

***Table 7.3*** *Continued*

| Phenotype | ID | Species | *E. coli* MLST | Year of Isolation | Source | Material | ESBL/ AmpC/ *qnr* genes | Other resistance gene families (microarray) |
|---|---|---|---|---|---|---|---|---|
| | 55 | *E. coli* | 461 | 2009 | dog | wound | CTX-M-1, TEM-80 | *dfrV* |
| | 12 | *E. coli* | 1287 | 2008 | horse | wound | CTX-M-14 | *aadA1, catA1, dfrA1, tetB* |
| | 41 | *E. coli* | 648 | 2008 | dog | throat | CTX-M-15 | *aadA14, dfrA17, sul1, ermB, tetB* |
| | 11 | *E. coli* | 88 | 2008 | dog | urine | CTX-M-15, TEM-1 | none |
| | 37 | *E. coli* | 156 | 2008 | dog | urine | CTX-M-15, TEM-1 | *aadA4, catA1, dfrA17, sul1, tetB* |
| | 35 | *E. coli* | 131 | 2008 | dog | urine | CTX-M-15, TEM-1, OXA-1 | *catB3, dfr12, sul1, aac6Ib* |
| | 5 | *E. coli* | 156 | 2007 | cat | urine | CTX-M-2, TEM-1 | *aadA1, aadA2, dfrA1, cmlA1, intI1, sul1, sul2, sul3, tetA* |
| | 20 | *E. coli* | 156 | 2008 | horse | wound | CTX-M-2, TEM-1 | *aadA1, ant2a, catA1, dfrA1, floR, intI1, sul1, tetB, strB* |
| | 44 | *E. coli* | 58 | 2009 | dog | urine | TEM-52 | *ereB* |
| | 43 | *E. coli* | 93 | 2009 | dog | urine | TEM-52 | none |
| | 3 | *S. enterica* | n.a. | 2007 | cat | faeces | TEM-52 | *aadA1, aadA2, dfrA1, intI2* |
| | 4 | *E. coli* | 117 | 2007 | dog | peritoneal fluid | TEM-1, OXA-1 | *aadA1, aadA2, intI1, sul1, strB* |
| AmpC | 59 | *E. coli* | 297 | 2009 | dog | urine | CMY-2 | *ereB* |
| | 15 | *E. coli* | 372 | 2008 | dog | urine | CMY-2 | none |
| | 48 | *E. coli* | 2227 | 2009 | dog | urine | CMY-2 | *ereB* |
| | 13 | *P. mirabilis* | n.a. | 2008 | dog | urine | CMY-2 | no signal |
| | 47 | *P. mirabilis* | n.a. | 2009 | dog | urine | CMY-2 | none |
| | 24 | *P. mirabilis* | n.a. | 2008 | cat | urine | CMY-2 | *aadA1, aadA2, dfrA1, intI2* |
| | 8 | *E. coli* | 88 | 2007 | dog | urine | CMY-2, OXA-1 | *aadA1, aadA2, catA1, intI1, sul1, ereB, tetB* |
| | 32 | *E. coli* | 12 | 2008 | cat | wound | CMY-2, SHV-1 | *aadA1, intI1, sul1, qnr, ereA* |

| MIC (mg/L) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AMP** | **CTX** | **CAZ** | **CIP** | **NAL** | **CST** | **GEN** | **KAN** | **SMX** | **TMP** | **STR** | **TET** | **CHL** | **FFN** |
| >32 | >4 | 1 | 0.015 | ≤4 | ≤2 | 2 | >128 | >1024 | >32 | >128 | >64 | 8 | 8 |
| >32 | >4 | 1 | >8 | >64 | ≤2 | 32 | >128 | >1024 | >32 | >128 | >64 | >64 | 8 |
| >32 | >4 | 8 | >8 | >64 | ≤2 | 0.5 | ≤4 | >1024 | >32 | >128 | >64 | 16 | 16 |
| >32 | >4 | 16 | 0.03 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | ≤0.5 | 64 | 64 | 8 | 8 |
| >32 | >4 | 16 | >8 | >64 | ≤2 | >32 | 32 | >1024 | >32 | 64 | >64 | >64 | 32 |
| >32 | >4 | >16 | >8 | >64 | ≤2 | 32 | 64 | >1024 | >32 | 32 | 64 | 8 | 8 |
| >32 | >4 | 8 | >8 | >64 | ≤2 | ≤0.25 | ≤4 | >1024 | >32 | 32 | >64 | 32 | 16 |
| >32 | >4 | 4 | >8 | >64 | ≤2 | >32 | >128 | >1024 | >32 | 128 | >64 | >64 | >64 |
| >32 | >4 | 16 | 0.5 | >64 | ≤2 | 1 | ≤4 | >1024 | >32 | >128 | >64 | 16 | 16 |
| >32 | >4 | >16 | 0.5 | >64 | ≤2 | 0.5 | 8 | >1024 | ≤0.5 | 8 | 4 | 32 | 32 |
| >32 | >4 | >16 | 0.06 | 8 | ≤2 | ≤0.25 | ≤4 | ≤8 | >32 | 128 | 4 | 16 | 8 |
| >32 | 4 | 0.5 | 1 | >64 | nd | 1 | >128 | >1024 | ≤0.5 | >128 | 4 | 16 | 8 |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | 1 | ≤4 | ≤8 | ≤0.5 | 8 | 4 | 16 | 16 |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | 0.5 | ≤4 | ≤8 | ≤0.5 | 8 | 2 | 4 | 4 |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | 0.5 | ≤4 | ≤8 | ≤0.5 | 16 | 2 | 8 | 8 |
| >32 | >4 | 4 | 0.03 | ≤4 | >4 | 2 | ≤4 | 16 | ≤0.5 | 8 | 64 | 16 | 4 |
| >32 | >4 | >16 | 0.12 | ≤4 | >4 | 2 | ≤4 | ≤8 | ≤0.5 | 16 | >64 | 8 | 4 |
| >32 | >4 | 1 | 0.03 | ≤4 | >4 | 1 | >128 | ≤8 | >32 | 64 | 64 | 16 | 4 |
| >32 | >4 | >16 | 0.06 | ≤4 | ≤2 | 0.5 | >128 | >1024 | ≤0.5 | 16 | >64 | >64 | 16 |
| >32 | >4 | >16 | 0.015 | ≤4 | ≤2 | 1 | ≤4 | >1024 | ≤0.5 | 32 | 2 | 8 | 8 |

*Continued*

***Table 7.3*** *Continued*

| Pheno-type | ID | Species | *E. coli* MLST | Year of Isola-tion | Source | Material | ESBL/ AmpC/ *qnr* genes | Other resistance gene families (microarray) |
|---|---|---|---|---|---|---|---|---|
| | 29 | *E. coli* | 68 | 2008 | dog | urine | CMY-2, TEM-1 | *sul2, ereA, tetB, strA, strB* |
| | 45 | *E. coli* | 68 | 2009 | dog | wound | CMY-2, TEM-1 | *dfrA17* |
| | 6 | *C. freundii* | n.a. | 2007 | turtle | trachea | CMY-39, *qnrB17var*[a] | *qnrB* |
| | 46 | *E. coli* | 372 | 2009 | dog | perito-neal fluid | *ampC* type 2[b] | none |
| | 70 | *E. coli* | 58 | 2009 | cat | bile | *ampC* type 3[b] | *tetB, strB* |
| | 34 | *E. coli* | 88 | 2008 | dog | urine | *ampC* type 31[b] | *floR, sul2* |
| | 64 | *E. coli* | 539 | 2009 | dog | urine | *ampC* type 3[b], TEM-1 | *aadA1, dfrA1, ereB* |
| | 25 | *E. cloacae* | n.a. | 2008 | cat | wound | TEM-1, *qnrS1/S3* | *aadA2, dfr12, intI1, sul1, qnrS, strB* |
| | 33 | *E. cloacae* | n.a. | 2008 | cat | wound | TEM-1, *qnrS1/S3* | *dfrA14, qnrS, strB* |
| | 54 | *E. cloacae* | n.a. | 2009 | horse | wound | TEM-1 | none |
| | 27 | *E. cloacae* | n.a. | 2008 | dog | urine | none | *ereB* |
| | 66 | *E. cloacae* | n.a. | 2009 | dog | urine | none | none |
| | 42 | *E. cloacae* | n.a. | 2009 | cat | urine | none | *tetC, strB, ereB* |
| | 67 | *E. cloacae* | n.a. | 2009 | cat | urine | none | none |
| ESBL/ AmpC | 73 | *E. coli* | 648 | 2009 | dog | wound | CTX-M-15, CMY-2, TEM-1 | *aadA4, dfrA17, aac6IB, tetB* |
| | 21 | *E. cloa-cae* | n.a. | 2008 | dog | blood | CTX-M-9, *qnrA1* | *aadA2, ant2a, catA1, intI1, sul1, qnr, aac6IB* |
| | 69 | *E. cloa-cae* | n.a. | 2009 | dog | trachea | CTX-M-9, *qnrA1* | *aadA2, ant2la, intI1, sul1, qnr, aac6Ib* |
| | 72 | *E. cloa-cae,* | n.a. | 2009 | dog | urine | CTX-M-9, *qnrA1* | *aadA2, ant2la, sul1, qnr, strA, strB* |
| | 19 | *E. cloa-cae* | n.a. | 2008 | cat | wound | CTX-M-9 | *aadA2, ant2la, intI1, sul1* |
| | 7 | *E. coli* | 405 | 2007 | dog | perito-nea fluid | CTX-M-14, TEM-1, *ampC* type 4[b] | *aadA4, dfr12, dfrA17, dfrA19, cmlA1, intI1, sul1, aac6Ib, ermB, tetA, strB* |

| MIC (mg/L) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AMP** | **CTX** | **CAZ** | **CIP** | **NAL** | **CST** | **GEN** | **KAN** | **SMX** | **TMP** | **STR** | **TET** | **CHL** | **FFN** |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | 0.5 | ≤4 | >1024 | >32 | >128 | >64 | 8 | 8 |
| >32 | >4 | >16 | >8 | >64 | ≤2 | >32 | >128 | >1024 | >32 | >128 | 8 | 16 | 16 |
| >32 | >4 | >16 | 0.25 | 8 | ≤2 | ≤0.25 | 8 | ≤8 | ≤0.5 | 32 | 4 | 16 | 16 |
| >32 | 1 | 1 | 0.015 | ≤4 | ≤2 | 1 | ≤4 | ≤8 | ≤0.5 | 4 | 2 | 8 | 8 |
| >32 | 1 | 4 | 0.015 | ≤4 | ≤2 | 0.5 | ≤4 | ≤8 | ≤0.5 | >128 | >64 | 8 | 8 |
| >32 | >4 | >16 | 0.25 | >64 | ≤2 | 1 | ≤4 | >1024 | >32 | >128 | 2 | >64 | >64 |
| >32 | 2 | 8 | >8 | >64 | ≤2 | 0.5 | ≤4 | >1024 | >32 | 32 | >64 | 8 | 8 |
| >32 | >4 | 2 | >8 | >64 | ≤2 | ≤0.25 | >128 | >1024 | >32 | 128 | 16 | >64 | 32 |
| >32 | >4 | >16 | 1 | 8 | ≤2 | ≤0.25 | ≤4 | >1024 | >32 | >128 | 4 | >64 | 8 |
| >32 | 1 | 0.5 | 0.015 | ≤4 | ≤2 | >32 | 16 | >1024 | >32 | 4 | 4 | 8 | 8 |
| >32 | >4 | >16 | 0.03 | 8 | ≤2 | ≤0.25 | ≤4 | ≤8 | 4 | 4 | 4 | 8 | 16 |
| >32 | 4 | 1 | 2 | >64 | ≤2 | 0.5 | ≤4 | ≤8 | 2 | 4 | 8 | 32 | 32 |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | ≤0.25 | ≤4 | ≤8 | 1 | >128 | 64 | 8 | 8 |
| >32 | >4 | >16 | 0.03 | ≤4 | ≤2 | 1 | ≤4 | ≤8 | ≤0.5 | 4 | 4 | 8 | 8 |
| >32 | >4 | >16 | >8 | >64 | ≤2 | >32 | 128 | >1024 | >32 | >128 | >64 | 16 | 16 |
| >32 | >4 | >16 | 2 | >64 | ≤2 | 16 | 128 | >1024 | >32 | 64 | 64 | >64 | 8 |
| >32 | >4 | 2 | 0.5 | 16 | ≤2 | 8 | 128 | >1024 | >32 | 64 | 4 | 8 | 8 |
| >32 | >4 | 2 | 2 | >64 | ≤2 | 4 | 16 | >1024 | ≤0.5 | 128 | 2 | 8 | 8 |
| >32 | >4 | 2 | 0.25 | >64 | ≤2 | 2 | 16 | 1024 | ≤0.5 | 32 | 4 | 8 | 8 |
| >32 | >4 | 2 | >8 | >64 | ≤2 | >32 | 64 | >1024 | >32 | 128 | >64 | 16 | 16 |

*Continued*

***Table 7.3*** *Continued*

| Phenotype | ID | Species | *E. coli* MLST | Year of Isolation | Source | Material | ESBL/ AmpC/ *qnr* genes | Other resistance gene families (microarray) |
|---|---|---|---|---|---|---|---|---|
| IRE | 1 | *S. marcescens* | n.a. | 2007 | dog | wound | none | *aadA1, aadA2, dfrA1, intI1, sul1* |
| | 2 | *E. coli* | 88 | 2007 | dog | wound | TEM-30 | *aadA1, catA1, dfrA1, sul1* |
| No ESBL/ | 49 | *E. coli* | 88 | 2009 | dog | urine | OXA-1 | *aadA1, catA1, ereB, tetB* |
| AmpC/ | 58 | *E. coli* | 448 | 2009 | dog | urine | TEM-1 | *dfrV* |
| IRE | 26 | *E. coli* | 950 | 2008 | dog | urine | TEM-1 | *aadA1, catA1, dfrA1, intI1, sul, strB* |
| | 52 | *E. coli* | 1642 | 2009 | dog | urine | TEM-1 | none |
| | 71 | *E. coli* | 1642 | 2009 | cat | wound | TEM-1, OXA-1 | *aadA1, aadA4, floR, sul1, tetA, strB* |
| | 14 | *E. coli* | 2225 | 2008 | cat | urine | none | *ereB* |

| MIC (mg/L) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AMP** | **CTX** | **CAZ** | **CIP** | **NAL** | **CST** | **GEN** | **KAN** | **SMX** | **TMP** | **STR** | **TET** | **CHL** | **FFN** |
| >32 | >4 | 0.5 | 8 | >64 | >4 | 1 | >128 | >1024 | >32 | 128 | 8 | 16 | 16 |
| >32 | 2 | 16 | >8 | >64 | ≤2 | 0.5 | >128 | >1024 | >32 | 128 | >64 | >64 | 8 |
| >32 | 0.5 | 0.5 | >8 | >64 | ≤2 | 0.5 | ≤4 | >1024 | ≤0.5 | 32 | >64 | >64 | 8 |
| >32 | 0.12 | 0.5 | >8 | >64 | ≤2 | 0.5 | ≤4 | >1024 | >32 | >128 | >64 | 8 | 8 |
| >32 | 0.25 | 0.5 | >8 | >64 | ≤2 | 16 | >128 | >1024 | >32 | 128 | 64 | >64 | 16 |
| >32 | 0.5 | ≤0.25 | >8 | >64 | ≤2 | 0.5 | >128 | >1024 | >32 | >128 | >64 | >64 | >64 |
| >32 | 0.25 | ≤0.25 | >8 | >64 | ≤2 | 0.5 | >128 | >1024 | >32 | >128 | >64 | >64 | >64 |
| 2 | ≤0.06 | ≤0.25 | 0.25 | >64 | ≤2 | 0.5 | ≤4 | >1024 | ≤0.5 | 8 | 4 | 8 | 8 |

IRE = Inhibitor Resistant ESBL; *E. coli* = *Escherichia coli*; *S. enterica* = *Salmonella enterica* subspecies *enterica* 4,[5],12:b:-; *P. mirabilis* = *Proteus mirabilis*; *E. cloacae* = *Enterobacter cloacae*; *C. freundii* = *Citrobacter freundii*; *S. marcescens* = *Serratia marcescens;* nd = not determined; AMP=Ampicillin; CTX=Cefotaxime; CAZ=Ceftazidime; CIP=Ciprofloxacin; NAL=Nalidixic acid; CST=Colistin; GEN=Gentamicin; KAN=Kanamycin; SMX=Sulfamethoxazole; TMP=Trimethoprim; STR=Streptomycin; TET=Tetracycline; CHL=Chloramphenicol; FFN=Florfenicol; [a]Sequence is similar to *qnrB17*, but has one silent mutation at position 169 GC**G** -> GC**A**; [b]Chromosomal *ampC* mutations as described by Mulvey and co-workers[14].

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# Chapter 8

## General discussion

## 8. General discussion

Production of beta-lactamases is the predominant resistance mechanism of Gram-negative bacteria to beta-lactam antibiotics. TEM-1-like beta-lactamases are an example of these beta-lactamases. With the development of extended spectrum cephalosporins like third- and fourth-generation cephalosporins as ceftriaxone, cefotaxime, ceftazidime and cefquinome resistance to TEM-1-like beta-lactamases produced by Gram-negative bacteria seemed no longer a problem. However, within a few years, after the introduction of these compounds bacteria started to produce beta–lactamases with an extended spectrum of activity, that were able to inactivate these new antibiotic compounds also. Nowadays this resistance (mainly conferred by the production of extended spectrum beta-lactamases (ESBLs) and AmpC-beta-lactamases) is spread worldwide[1].

Gram-negative bacteria can cause serious infections in humans and beta-lactam antibiotics are regularly used for the treatment of these infections. Resistance to these antibiotics can lead to impaired treatments[2]. In general, *Enterobacteriaceae* resistant to third generation cephalosporins are significantly more often resistant to other classes of antibiotics, like fluoroquinolones, aminoglycosides and trimethoprim-sulfa combinations, compared to cephalosporin susceptible isolates[3]. This limits the treatment options further, resulting in the use of last resort antimicrobials like carbapenems. As a result, increasing resistance to carbapenems in *Enterobacteriaceae* (mainly in *Klebsiella pneumoniae*) causing infections in humans is currently described worldwide[4]. Serious concern therefore exists about the recent finding of the first carbapenemase variants (VIM-1 and OXA-23) in animal isolates in Germany and France[5-7].

At first, *Enterobacteriaceae* resistant to third generation cephalosporins were mainly confined to hospital settings, but since the late 1990s these isolates have also emerged in community-settings, mainly causing urinary tract infections caused by CTX-M-producing *E. coli* [8, 9]. Animals are considered to contain a reservoir of ESBL/AmpC producing isolates[10]. Some authors have reported the transfer of these isolates to humans via direct contact with animals[11]. It is also suggested that transmission can take place via food[12], mainly of poultry origin[8, 13, 14].

### 8.1 Main findings in this thesis

Concern about the increase in ESBL-producing isolates in human patients in the Netherlands[15] and the simultaneous increase in cefotaxime resistance in *Salmonella* and commensal *E. coli* isolates from broiler chickens[16] initiated the research projects described in this thesis. The first finding was the presence of similar combinations of ESBL/AmpC genes and plasmids found in commensal *E. coli* and in *Salmonella* isolates both derived from Dutch broilers in 2006. The cause of this increase in cefotaxime resistance in both bacterial species was mainly due to an increase in isolates carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ on IncI1 plasmids (**Chapter 2**). Other combinations, like $bla_{\text{CTX-M-2}}$ on IncHI2/P, $bla_{\text{CMY-2}}$ on IncK, $bla_{\text{TEM-20}}$ on IncI1, $bla_{\text{ACC-1}}$ on small non-typeable plasmids and $bla_{\text{SHV-2}}$ on IncK plasmids, were also found either in both or one of the bacterial species. It was confirmed that these isolates are multidrug resistant as described earlier[3, 17], but unexpectedly in *E. coli* isolates these co-resistances were never located on the same plasmid that carried the ESBL or AmpC gene and in *Salmonella* isolates seldomly. We found some exceptions, which included resistance to sulfamethoxazole, tetracycline and/or trimethoprim in a few *Salmonella* strains carrying $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$ or $bla_{\text{TEM-20}}$. **Because similar combinations of ESBL/AmpC genes and plasmids were found in both *E. coli* and *Salmonella*, we concluded**

**that plasmid transfer between isolates of different bacterial species most likely occurs in the gastrointestinal tract of broilers including transfer to *Salmonella* strains pathogenic for humans.**

From the Dutch monitoring results on antimicrobial resistance in food-producing animals (MARAN 2009) it was known that in 2007 around 20% of all commensal *E. coli* from broilers randomly isolated in caecal samples taken at slaughterhouses (in which every isolate represents one flock of broilers) displayed resistance to cefotaxime[16]. These data, however, did not provide information on the prevalence of broilers carrying these isolates at broiler farms. This information was obtained by a study performed in 2009, in which selective media were used to isolate cefotaxime resistant *E. coli* from faecal material from broilers at broiler farms (**Chapter 3**). The intention of this study was to determine the prevalence of broilers carrying ESBL/AmpC producing *E. coli* in their faeces on farms. To be able to elucidate risk factors that might play a role in contracting these resistant isolates in broilers we aimed to find farms with high or low within-farm prevalence. The main finding of this study was that **all broiler farms had broilers carrying ESBL/AmpC-producing isolates in their faeces and on more than 85% of the farms the percentage of broilers that were ESBL/AmpC positive was ≥ 80%.** There were only four farms with a low within-farm prevalence. This number of low prevalence farms was very small and the characteristics with respect of management and hygiene on these farms did not differ from the rest of the farms, therefore risk factors for the prevalence of broilers carrying ESBL/AmpC-producing isolates could not be determined. Transfer of resistant isolates from broilers to broiler farmers had been described earlier[18, 19], therefore it was not surprising to find that **33% of the broiler farmers are also carriers of ESBL/AmpC producing isolates themselves**, which is much higher than found in the Dutch community (5-10% [13, 20]). Although plasmid subtyping revealed the presence of only two isolates derived from two farmers with ESBL/AmpC genes located on similar plasmid subtypes as in the broiler isolates, the high prevalence in broiler farmers compared to the general Dutch population indicates the uptake of these isolates also in the other farmers. Uptake of isolates from former production rounds might explain the differences found. Cephalosporins are not licensed for use in broiler production. Therefore a high percentage of broilers carrying ESBL/AmpC-producing isolates at broiler farms was not expected. However it turned out that until spring 2010, ceftiofur was used at Dutch hatcheries to prevent early mortality in one-day old broiler chickens[21]. This is also described to have occurred in Belgian hatcheries, leading to a high prevalence of ceftiofur resistant isolates at Belgian broiler farms[22]. Antibiotic usage at the broiler farms, also by non-beta-lactam antibiotic usage, due to the multidrug-resistant character of these isolates, could have maintained the presence of these isolates in broilers.

Broilers are produced in a pyramidal structure with breeding lines at the top where genetic selection takes place to produce fast and efficient growing broilers at the bottom. Vertical transmission of resistant bacteria had been described earlier[23-26], but the presence of ESBL/AmpC-producing isolates in the broiler production pyramid was not yet investigated. Therefore in 2009-2010 we started an investigation in the Dutch broiler production pyramid to investigate the presence of these isolates at Grandparent and Parent level (**Chapter 4**). **ESBL/AmpC-producing isolates turned out to be present at every level in the broiler production chain.** Vertical transmission, as well as recirculation of these isolates at farms and hatcheries in the broiler production chain seem to play a role in the presence of these isolates. Samples were taken from one-day old chickens and no data was available on the course of the occurrence of these isolates at later age. Therefore, in addition to the former study, two longitudinal studies, one at Grandparent

level and one at broiler farms were conducted to investigate the course of the prevalence at a later age of the animals (**Chapter 4**). A difference was seen between the spread at Grandparent level and at broiler level**. At Grandparent level, the prevalence of one-day old broilers carrying ESBL/AmpC-producing *E. coli* was between 0-70%. At 18 weeks the prevalence was between 0-44%, except when the chickens were treated with penicillins. Amoxicillin and phenoxymethylpenicillin treatment at Grandparent level resulted in a prevalence of 100% ESBL/AmpC-positive chickens. At 31 weeks of age the prevalence of Grandparent chickens carrying ESBL/AmpC-producing isolates varied between 2 and 27%. In contrast, at broiler farms, prevalence increased from 0-20% at one-day to 96-100% at one week of age. Moreover the prevalence remained around 100% until slaughter**.

To detect the ESBL/AmpC genes present in the isolates collected in the studies described above we used a miniaturized microarray to screen for ESBL/AmpC gene families[27]. This microarray was developed by the Veterinary Laboratories Agency (VLA), now named Animal Health and VLA (AHVLA). It is based on probes that recognize uniform parts of DNA per groups of ESBL, AmpC or other antimicrobial resistance genes. Although very useful for research purposes, one of the drawbacks of this microarray when used in clinical settings is the detection of $bla_{TEM-}$ and $bla_{SHV}$-genes as whole families. The array does not distinguish between ESBL- and non-ESBL-genes, which are present within these families[1], www.lahey.org/studies. In clinical settings, screening for ESBL-producing isolates needs to be fast and reliable in order to be able to choose the right antimicrobial treatment and to evaluate whether infection control measures are needed. Therefore it would be better if no subsequent sequencing step would be necessary to determine whether an infection is caused by an ESBL producer or not. In **chapter 5** we describe the development and evaluation of a genotypic assay, which can recognize the most prevalent ESBL genes present in clinical settings. This method is able to distinguish between the most common mutations present in members of the SHV and TEM families, associated with ESBL-production. In addition, it can distinguish between the five CTX-M-groups. Still, application of this method does not characterize ESBL-genes completely, but it predicts to which ESBL group the detected gene will belong which is relevant epidemiological information. **The sensitivity of the microarray was 95% and the specificity 100%.** For epidemiological studies the golden standard will still be sequence analysis of the genes involved. However the array can be used to first screen for the ESBL-group present in an isolate, which can already be sufficient information for clinical settings. For research purposes dedicated sequencing can be a follow-up for the array results. At the moment the array is available in several extended formats, aimed at detection of additional ESBL-, AmpC- and carbapenemase-genes.

For research purposes, the microarray described in **chapter 5** was used to screen for ESBL genes in human clinical isolates from 31 Dutch diagnostic laboratories with a positive ESBL phenotypic screen test during February 2009 until May 2009. A selection of the isolates was further characterized by sequencing, plasmid characterization and multi locus sequence typing (MLST) to compare the genes, plasmids and strains with the ones found in Dutch poultry isolates in 2006 that were described in **chapter 2**. At that time it had become clear that ESBL-epidemiology was driven by the dissemination of specific clones or clonal groups and transfer of epidemic plasmids [28-30] and the poultry industry was seen as a potential reservoir of ESBL-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated meat [16, 31]. Therefore plasmid characterization as well as strain typing is very important to follow this epidemiology. In **chapter 6** ESBL genes, plasmids and *E. coli* strains derived from human clinical samples and broiler samples were characterized and

compared. In that way the proportion of *E. coli* isolates with poultry-associated ESBL genes, plasmids and strains could be determined in a collection of human clinical samples. **Nineteen percent of Dutch human clinical isolates shared similar ESBL-genes and plasmids as found in Dutch broilers and in 11% these genes and plasmids were located in *E. coli* strains with similar sequence types in both reservoirs.** The results are based on the finding that $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ were located on similar subtypes (CC7, CC5, CC3) of IncI1 plasmids in Dutch human and broiler isolates. MLST of *E. coli* isolates displayed three sequence types (ST10, ST58 and ST117) present in both human and broiler samples and two of them (ST10 and ST117) were also found in retail chicken meat samples carrying either $bla_{\text{CTX-M-1}}$ or $bla_{\text{TEM-52}}$. A high percentage (94%) of retail poultry meat was positive for ESBL-producing isolates and $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ were indeed most predominant in these samples. This was later confirmed by others[13, 32]. $bla_{\text{TEM-52}}$ is not among the most predominant genes found in Dutch human clinical isolates, but $bla_{\text{CTX-M-1}}$ is[13, 33], although not in isolates from patients residing in hospitals in the Rotterdam region[34]. Other predominant ESBL-types found in isolates from human blood cultures and from human infections, but not in isolates from broilers are $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$[13, 33, 34], indicating that other sources will play a role in the colonization of humans with ESBL/AmpC-producing isolates. One of the possible sources of which no information was available in the Netherlands was companion animals. Other researchers had already shown that these animals could be carriers of ESBL/AmpC-producing isolates[35, 36] and recently it was shown that having pet animals increases the risk of carrying ESBL producers in the gut of the pet owners by almost seven-fold[37]. The simplest way to obtain more information on possible colonization by ESBL/AmpC-producing isolates in Dutch companion animals was to use an existing collection of pet isolates and select for cephalosporin resistant isolates. Such a collection was present at the veterinary faculty in Utrecht. This resulted in the study described in **chapter 7**. Sixty-five clinical isolates (all *Enterobacteriaceae*) mainly from dogs and cats, suspected for ESBL- or AmpC-production were genetically characterized. Finally in 43 isolates ESBL or plasmid located AmpC genes were found. **Among them $bla_{\text{CTX-M-1}}$ was the most predominant gene, but apart from the types found in broilers, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were also present in isolates from companion animals.** Although in these isolates the plasmid genotypes were not determined, strain genotyping revealed *E. coli* sequence types found in Dutch human (ST117, ST58, ST162) and Dutch broiler (ST117, ST58) isolates described in **chapter 6**, suggesting clonal spread of ESBL-producing isolates among different reservoirs.

### 8.2 ESBL/AmpC producing *E. coli* in the broiler production chain

The presence of ESBL/AmpC-producing isolates in the top of the broiler production chain as shown in **chapter 4**, can result in a global spread affecting the broiler industry in many countries. Broilers are produced in a highly industrialized way. Only a few firms control the majority of the broiler production worldwide. As a result broiler meat, products, eggs and live chickens together with their bacterial flora are shipped all over the world[38]. The source of ESBL/AmpC-producing isolates, or risk factors for breeding chickens to obtain these isolates is not known.

*8.2.1 Measures to control the spread of ESBL/AmpC producing isolates in broiler production*

***1. Prevent transmission from the top of the poultry production chain.***

Monitoring of the breeding flocks is one way to better understand what is happening regarding occurrence and characteristics of ESBL/AmpC-producing organisms in the top of the production chain. Several initiatives from the breeding companies themselves to monitor for ESBL/Amp-producing isolates at Grandparent and Parent level are already installed. However these data are not publically available and until now no information concerning the identification of factors that influence ESBL/AmpC presence in isolates of these chickens were published. Monitoring should start at Pedigree stock and Great-grandparent level and should be organized internationally to assess the progeny of the monitored flocks. If ESBL/AmpC free or low prevalence lines exist, it is important to follow these lines and understand where the chickens become colonized with ESBL/AmpC-producing isolates. Factors that need to be taken into account are transport of chickens and biosecurity measures at hatcheries and farms. Formaldehyde fumation at hatch diminished ESBL/AmpC-producing isolates in the hatching units and reduced exposure to environmental bacterial flora (**chapter 4**), however animal welfare should be taken into account when using such an approach.

***2. Identification of risk factors leading to the presence of ESBL/AmpC producing isolates in the broiler production pyramid.***

Biosecurity levels already have a very high standard in the top of the production chain and except for the ceftiofur usage in hatcheries in the past, antibiotics are used more prudently than at the bottom of the production pyramid. Still it was shown in **chapter 4** that selection factors for ESBL/AmpC-producing isolates at rearing and production farms at Grandparent level must be present in order to explain the moderate prevalence of chickens carrying ESBL/AmpC-producing isolates at these farms. The use of beta-lactam antibiotics was shown to be one of the factors favoring the distribution of AmpC-producing isolates among the flock. If further monitoring of ESBL/AmpC-producing isolates in breeding flocks will not lead to the detection of other risk factors, further research, most preferably conducted in controlled environments, should be performed to test the effect of different intervention strategies, both at breeding and at broiler level. Risk factors linked to a high level of ceftiofur resistance at broiler farms were described by Persoons and co-workers and consisted of: clean hygienic condition of the treatment reservoir, no acidification of drinking water, more than three feed changes per cycle, hatchery of origin, breed, litter material and amoxicillin treatment[22]. Interventions aimed at these factors could be tested in controlled environments to establish their effect on the number of ESBL/AmpC-producing isolates in samples of Grandparents, Parents or broilers.

From the difference in prevalence between the longitudinal study in grandparent chickens compared to broiler chickens (**chapter 4**) other factors could also be taken into account. These factors include the use of coccidiostatics in the feed, differences in feed for grandparents and broilers, the density of animals on farms and hygienic measures. In the mean time maintaining monitoring for ESBL/AmpC-producing isolates throughout the broiler production chain is important in order to search for ESBL/AmpC free flocks. If ESBL/AmpC free flocks, or low prevalent flocks are found at breeding level, it will be important to follow the progeny of

these flocks throughout the hatcheries to the parent or broiler farms. This makes it possible to investigate where the progeny of these flocks becomes (increasingly) positive, which again will create opportunities for interference.

***3. Reduction of the selection pressure imposed by the use of antimicrobials.***

Despite all other factors that may play a role, the prudent use of antibiotics will be the most important measure to reduce the amount of multidrug resistant isolates in the broiler production chain. The use of antibiotics is certainly a factor that will maintain the presence of ESBL/AmpC-producing isolates in broilers and broiler meat. This effect was already seen in a study by Dutil and co-workers [39]. They showed that ending ceftiofur treatment at hatcheries in Canada led to a reduction in the percentage of ceftiofur-resistant *Salmonella* and *E. coli* isolates in broiler retail meat and also that it led to a reduction of the percentage of these isolates found in humans. After reintroduction of ceftiofur use, resistance levels increased again[39]. A similar decreasing trend in cefotaxime resistance in Dutch broiler commensal *E. coli* isolated as part of the Dutch monitoring program on antibiotic resistance in food-producing animals is seen since 2010 (Figure 8.1), probably due to the stop of ceftiofur usage at hatcheries and an overall reduction in the use of antibiotics, which will be discussed below. The influence of the use of beta-lactam antibiotics on selection of ESBL/AmpC-producing isolates was also described in **chapter 4**. The use of beta-lactam antibiotics in Grandparents in this study resulted in the presence of 100% of Grandparents colonized by ESBL/AmpC producing *E. coli*.

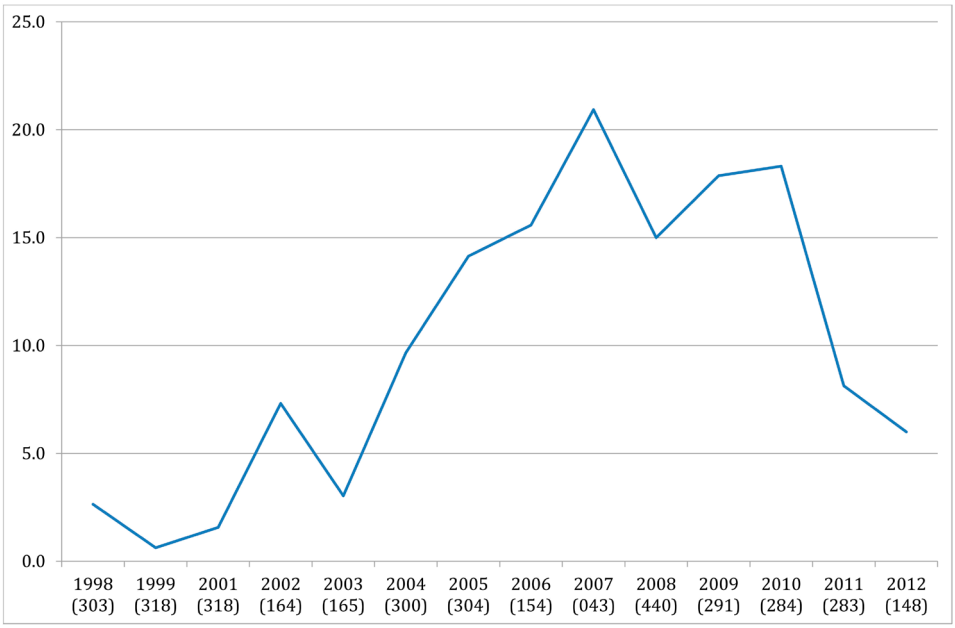


***Figure 8.1.*** *Trends in cefotaxime resistance in commensal* E. coli *isolates derived from Dutch broilers during the Dutch monitoring program on antibiotic resistance in food-producing animals, including preliminary data from 2012.*

### *3a. Reduction of antibiotic use in food-producing animals in the Netherlands.*

In the Netherlands antibiotic usage in food-producing animals is monitored since 1999 in a relative small sample of broiler farms varying from 14 to 29 farms examined each year. A peak in average antibiotic use in these broiler farms was seen in 2008/2009 when 36.5/36.8 animal daily dosages per year were used. After 2008/2009 a decrease to 14.4 animal daily dosages per

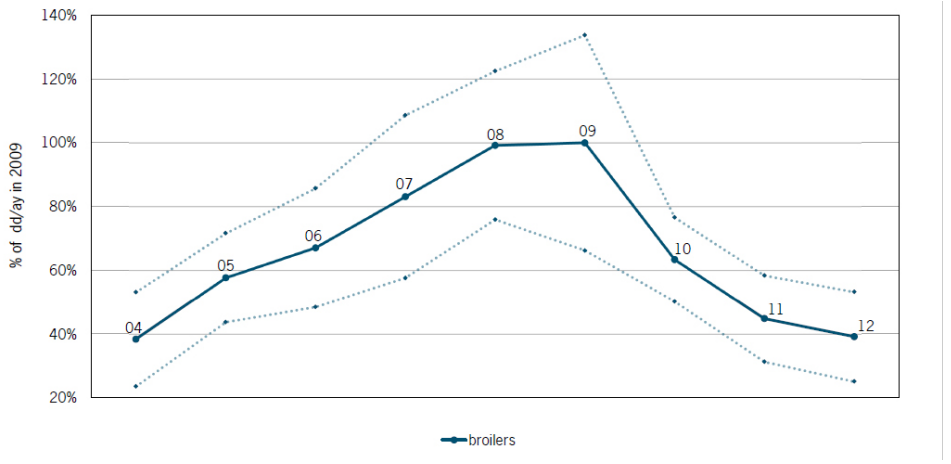


***Figure 8.2*** *Trends in antibiotic use in broilers in the Netherlands from 2004 to 2012. The dashed lines depict the 95% confidence level. (www.maran.wur.nl, last accessed 28 November 2012).*

year in 2012 was reported by the Agricultural Economics Institute (LEI)(Figure 8.2).

This decrease in antibiotic use (which was not only seen in broilers, but also in veal calves and slaughter pigs, www.maran.wur.nl) was a result of measures taken by the food-producing sectors themselves with support and under pressure of the Dutch government. In 2008 the Taskforce on Antibiotic resistance in food-producing animals agreed with all stake-holders in livestock production to work together to achieve a reduction in antimicrobial resistance in isolates derived from food-producing animals, mainly by reducing antibiotic usage. In 2008, the farmer unions of the four most important livestock production sectors in the Netherlands (poultry, pigs, veal calves and cattle) signed agreements, in which they committed themselves to a reduction in antibiotic use in order to contain antimicrobial resistance. In 2010 the Ministry of Agriculture, Nature and Food Quality (now named Ministry of Economic Affairs, Agriculture and Innovation) and the Ministry of Health, Welfare and Sports recommended that the total amount of antibiotics used should be reduced by 20% in 2011 and by 50% in 2013 compared to 2009 as index year.

To control and achieve this reduction an independent foundation was initiated at the end of 2010 (the "Stichting Diergeneesmiddelen Authoriteit" or SDa), which has the task to control the quality and completeness of a central database that collect antibiotic usage information, to report and interpret usage data and to set thresholds for antibiotic use per livestock production sector. Every farm is judged on their antibiotic usage behavior by this system and is able to compare its use to other farmers (benchmarking) and to the threshold levels defined by the SDa. There are three levels of antibiotic use defined by the SDa: the 'target level', in which no action

is needed, the 'signaling level', by which special attention to antibiotic usage at the farm is given and the 'action level' in which antibiotic usage is so high that direct action is needed to reduce the amount of antibiotics used at the farm. Every threshold is set by a certain amount of animal daily dosages per year, which is dependent on the species and category of food-producing animals. For broilers in 2012, the level at which no action is needed is below 15 animal daily dosages per year, the action level is set at more than 30 animal daily dosages per year (SDa, 2012, www.diergeneesmiddelenautoriteit.nl, last accessed 17 November 2012).

A one-to-one relation between farmers and veterinarians must be established. The veterinarian that is linked to the farms is responsible for all antibiotic use at the farm. All these measures seemed to have led to a strong decrease in antibiotic use in broiler production as reported by LEI which has leveled of a bit in 2012 (LEI report November 2012, www.maran.wur.nl, last accessed 28 November 2012). This reduction in antibiotic use (from 36.8 daily dosages per animal year to 14.4 daily dosages per animal year) might have contributed to the slight reduction in antibiotic resistance measured in indicator *E. coli* from broilers in 2011 (Figure 8.3[21]). In addition, illegal ceftiofur usage at hatcheries in the Netherlands was stopped in the spring of 2010[21]. This, together with a possible overall reduction of antibiotic use may have led to a strong reduction in cefotaxime resistance in commensal *E. coli* from broiler samples noticed in the monitoring results from 2011 including preliminary results of 2012 (Figure 8.1).

Although a reduction of 51% in antibiotic usage compared to 2009 at the beginning of 2012 as reported by LEI (LEI report November 2012, www.maran.wur.nl, last accessed 28 November 2012) looks promising, there is still need for concern. Antibiotic resistant isolates can still be found in large quantities[21]. A good evaluation of the effect on resistance of the reduction in antibiotic usage is needed to predict what will happen in the future. Relevant questions are: What are the measures undertaken by the farmers that made it possible to reduce their antibiotic usage? Are there more animal losses due to reductions of antibiotic use at the farms? Are there large differences in management which made this possible? Broiler producers have the difficult task to develop a safe, healthy and animal-friendly production system in which antibiotics are hardly necessary.

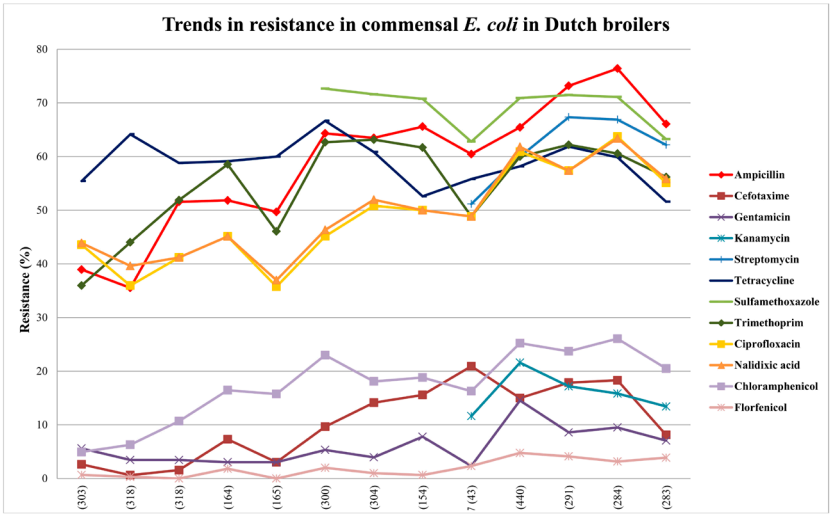


***Figure 8.3*** *Trends in antibiotic resistance in commensal* E. coli *derived from Dutch broilers during 1998 – 2011*[21]

***3b. European data collection of veterinary antibiotic usage.***

Veterinary antibiotic usage is also monitored in other European countries. The European Medicines Agency (EMA) has been asked by the European Commission (EC) to establish a monitoring program for veterinary antibiotic usage in all EU member states. A harmonized data collection protocol is developed within the European Surveillance of Veterinary Antimicrobial Consumption project. The data obtained in this monitoring program is regarded as essential to identify possible risk factors that can lead to the development and spread of antimicrobial resistance in animals. The last report was published in October 2012 which contained data from 19 European countries[40]. Although the protocol needs some adjustments and refinements to make the data obtained with it from all countries comparable it is a good development to collect these data internationally. Usage data together with the data collected in the EU monitoring of antimicrobial resistance in food-producing animals of public health concern (Zoonosis Directive 2003/99/EC) published by the European Food Safety Authority (EFSA) will aid the interpretation of patterns and trends observed in the monitoring of antimicrobial resistance within EU countries for the different livestock producing sectors and it will help to monitor the effects of reductions in antimicrobial use in other European countries.

***4. Prevent local recirculation within subsequent flocks.***

ESBL/AmpC-producing isolates are present in the environment of poultry houses even after cleaning and disinfection of these poultry houses (**chapter 4**). At hatcheries, after hatch ESBL/AmpC-producing *E. coli* are also present in the environment except when formaldehyde fumation was used during hatch (**chapter 4**). After routinely used cleaning and disinfection of the hatcheries ESBL/AmpC-producing isolates could also be found in the environment within the hatchery (personal observation). Although not measured, it is very likely that recirculation of bacterial flora also takes place at Grandparent and Parent farms from a contaminated environment at the farm. Therefore high standards of biosecurity are necessary to prevent recirculation of bacterial flora of the former flock. Hygienic measures at Grandparent and Parent farms are already of high standards, however these should be carefully evaluated to make improvements. At broiler level, hygienic measures can be of lesser quality. Broiler farms try to make as much production rounds per year as possible. This has led to a downtime (period between two production cycles) of sometimes less than a week (**chapter 3**). Such a short period is probably too short to clean and disinfect the poultry house effectively. Ways to improve the effects of cleaning and disinfecting should be developed, to start with a clean place for the chickens.

*8.2.2 Can we predict the effect of measures?*

The most effective way to reduce ESBL/AmpC-producing isolates in the broiler production chain will be the combination of all measures mentioned above. And although I believe this will reduce the number of ESBL/AmpC-producing bacteria, and hopefully other multidrug resistant bacteria, still I am not hopeful that it will be possible to come to a total reduction. Ideally a fresh start with chickens free from antibiotic resistant isolates would be preferred. However to remain free, probably a new, maybe more costly (and hopefully more animal friendly) way of broiler production should be introduced. The industrialized way of broiler production with high production and low costs will also in the future remain conflicting when dealing with upcoming

human and animal health threats. Consumers should be made aware that the low price they pay in the supermarket result in choices made in the broiler production chain, not always in favour of sustainable production and animal welfare. In the current situation, to minimize the threat of transfer ESBL/AmpC-producing isolates from broiler meat to humans, in addition to pre-harvest measurements, as discussed above, post-harvest measurements will be of great importance. These include: prevent cross-contamination in the slaughterhouse, hygienic standards in preparing meat products at retail, decontamination of food and probably the most effective and important measure will be hygienic measures taken in the kitchen by the consumer to prevent (cross-) contamination of foods.

### 8.3 ESBL/AmpC producing isolates in humans

Infections with ESBL/AmpC-producing isolates in humans result in a higher risk of impaired initial treatment and as a result in a higher mortality[41]. Risk factors for the acquirement of these isolates in hospital settings are: a longer length of stay in the hospital, severity of illness, a longer time in the intensive-care unit, intubations and mechanical ventilation, urinary or arterial catheterization and previous exposure to antibiotics (especially cephalosporins)[42]. Carriership of ESBL-producing isolates in humans in the community is estimated to be 5-10% in the Netherlands[13, 20]. Recent studies from other countries describe either similar results: 3% in Sweden[43], 6% in France[44], 7.3% in Tunisia[45] or a much higher percentage of human carriers: 58% in Thailand[46]. In two studies an association between colonization with ESBL producers and a higher risk for developing an infection with an ESBL-producer has been reported[47, 48]. Risk factors for a community onset of infection (mostly urinary tract infections) include repeated urinary tract infections and underlying renal pathology, previous antibiotic use including cephalosporins and fluoroquinolones, previous hospitalization, nursing-home residents, older men and women, diabetes mellitus and underlying liver pathology[42]. A factor which is found to influence ESBL carriage is foreign travel, especially to the Indian subcontinent and Africa, which resulted in a higher risk of ESBL carriage [49].

Transmission in the community due to human-human contact is described in Spain. In this study 70% of index cases of patients with an ESBL-producing strain displayed positive contacts in 16.7% of their household members. Two-third of the isolates from the index-cases was indistinguishable by PFGE from the ones from the household contacts[50]. Overall the prevalence of ESBL-producing isolates in community-dwelling people is increasing[9, 43, 44].

The epidemiology of ESBL/AmpC-producing isolates is very complicated. Similar ESBL-genes, plasmids and bacterial clones, like the spread of the epidemic uropathogenic clone *E. coli* ST131-B2-O25:H4 which contains an IncFII plasmid carrying $bla_{\text{CTX-M-15}}$, has been isolated from humans, animals and foods and is found at different continents[51]. CTX-M-beta-lactamases are now considered the most important types found in human isolates worldwide[52], with $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ as most predominant in Europe[53]. The success of the *E. coli* ST131 clone with an incFII plasmid is partly explained by addiction factors found on the plasmid, which ensures the containment of this multidrug resistant plasmid even in the absence of antibiotic selection [54]. However, IncFII plasmids can also carry other ESBL/AmpC genes found in human isolates ($bla_{\text{CTX-M-1, -3, -9, -14, -24, -27,}}$ $bla_{\text{SHV-2}}$, $_{\text{-5, -12}}$ and $bla_{\text{DHA-1}}$) and can also be present in other *E. coli* lineages that can occur worldwide as well [28, 29]. Next to IncFII plasmids, IncI1 plasmids are among the most predominant plasmids found in human isolates[28]. They are also linked to different ESBL/AmpC genes ($bla_{\text{CTX-M-1, -2, -3, -9, -14, -15, -24}}$, $bla_{\text{CMY-2,-7, -21}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-52}}$)[28].

### 8.4 ESBL/AmpC producing isolates in other sources than humans

ESBL/AmpC producing isolates are now isolated from wildlife (carrying $bla_{\text{TEM-20, -52}}$, $bla_{\text{CTX-M-1, -3, -9, -14, -15, -32,}}$ $bla_{\text{SHV-2, -5, -12}}$ and $bla_{\text{CMY-2}}$)[21, 55], (waste-) water (carrying $bla_{\text{CTX-M-1, -15}}$) [56], fruit and vegetables (carrying $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-12}}$ [12, 31, 57, 58], retail meat ($bla_{\text{CTX-M-1, -2, -14, -15}}$, $bla_{\text{TEM-20, 52}}$, $bla_{\text{SHV-2, -12}}$ and $bla_{\text{CMY-2}}$ [13, 21, 32, 59] as well as in other food-producing animals, like cattle and pigs[21]. As part of the Dutch monitoring program on antimicrobial resistance in food-producing animals in 2011 ESBL/AmpC-producing isolates derived from faecal samples taken at Dutch slaughterhouses from ten (apparently healthy) animals per slaughter batch of animals were characterized. Samples were taken from 100 batches of slaughter pigs, 100 batches of veal calves and 29 batches of broiler chickens. In addition, 100 individual dairy cows were sampled, each representing a different farm. Selective enrichment was used to obtain ESBL/AmpC-producing isolates. One isolate per flock was screened for ESBL/AmpC genes by microarray, PCR and sequencing. The results are displayed in Figure 8.4. From the data displayed in this figure it can be concluded that ESBL/AmpC types found in broilers can also be found in the other food-producing animals, however some types found in veal calves and/or slaughter pigs are not found in broilers ($bla_{\text{CTX-M-3, -14, 15, -32}}$), suggesting a different epidemiology among the different food-producing animals.

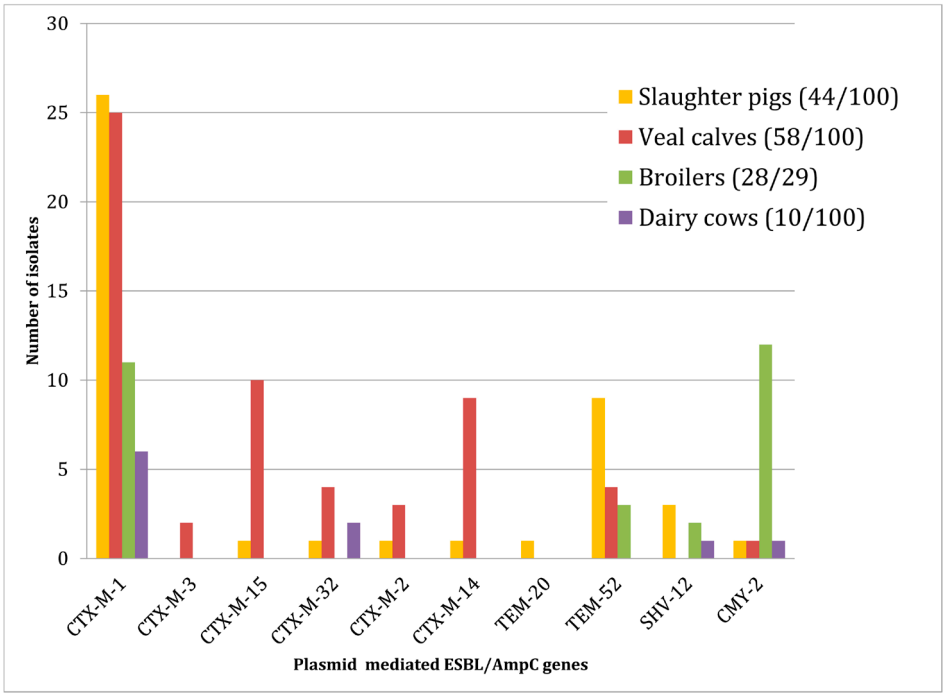


***Figure 8.4*** *Distribution of plasmid mediated ESBL/AmpC genes in faecal samples from Dutch food-producing animals collected in 2011. Percentage of these isolates found in the samples were 44%, 58%, 97% and 10% for respectively slaughter pigs, veal calves, broilers and dairy cows (adapted from[21]).*

To summarize, reservoirs of ESBL/AmpC-producing isolates are omnipresent. Person-to-person contact still seems the most important route for humans to obtain these isolates, as is hospital stay and living in a nursing home. Travelling to the Indian subcontinent and Africa also increases the risk to obtain these isolates and other sources as mentioned above might play a role in transmission to humans, although the relative attribution of each reservoir to this transmission is not known. Where similar ESBL/AmpC genes simultaneously occur in different reservoirs, characterization of the strains and plasmids carrying these genes will help to understand more about the epidemiology and possible routes of transmission of these isolates.

### 8.5 ESBL/AmpC-producing isolates in broilers and impact on human health

As shown in **chapter 6** and confirmed by other publications, a high percentage of broiler meat is contaminated with ESBL/AmpC-producing isolates[13, 32]. So far, the impact and contribution of these ESBL/AmpC-producing isolates to human health is difficult to estimate. Research described in **chapter 6**, is the first in which a quantification is made based on similar ESBL genes and plasmids in both human and broiler isolates. Based on the results described in **chapter 6** we calculated that 6-48 patients per year will suffer from an episode of *E. coli* bacteraemia caused by ESBL producing poultry associated strains in the Netherlands. However, strict evidence that these infections are derived from broilers cannot be obtained. Nevertheless, it is suspected that among all possible sources broilers, through the contamination of broiler meat, can play a significant role in the transmission of the genes, plasmids or isolates to humans[13, 31, 60]. This is especially the case, since reports on ESBL/AmpC-producing isolates present on meat derived from cattle and pigs reveal less contamination[13, 32, 59].

The contrast between antibiotic use in humans and in food-producing animals in the Netherlands is very large. Antibiotic usage in humans in the Netherlands is among the lowest in Europe (Figure 8.5) and in food-producing animals it is among the highest in Europe (Figure 8.6).

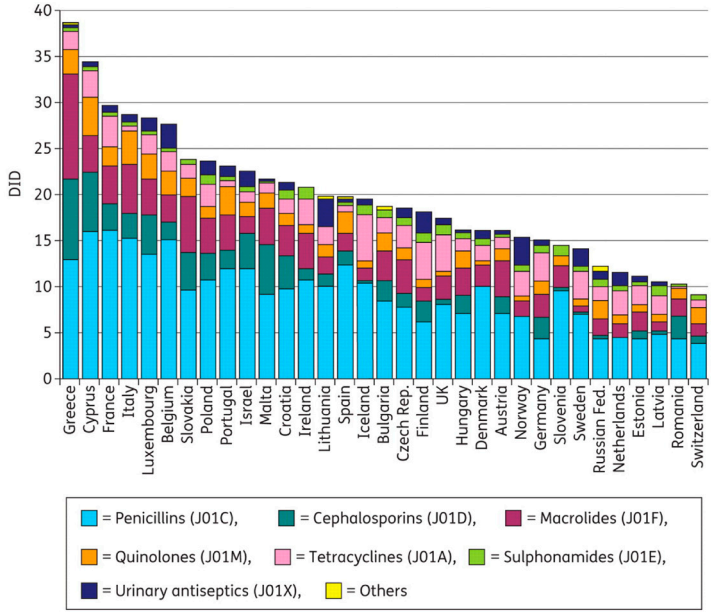


***Figure 8.5*** *Total outpatient antibiotic use in 33 European countries in 2009 in Defined Daily dosages per 1000 inhabitants per day (DID) (2004 data for Switzerland)[61].*

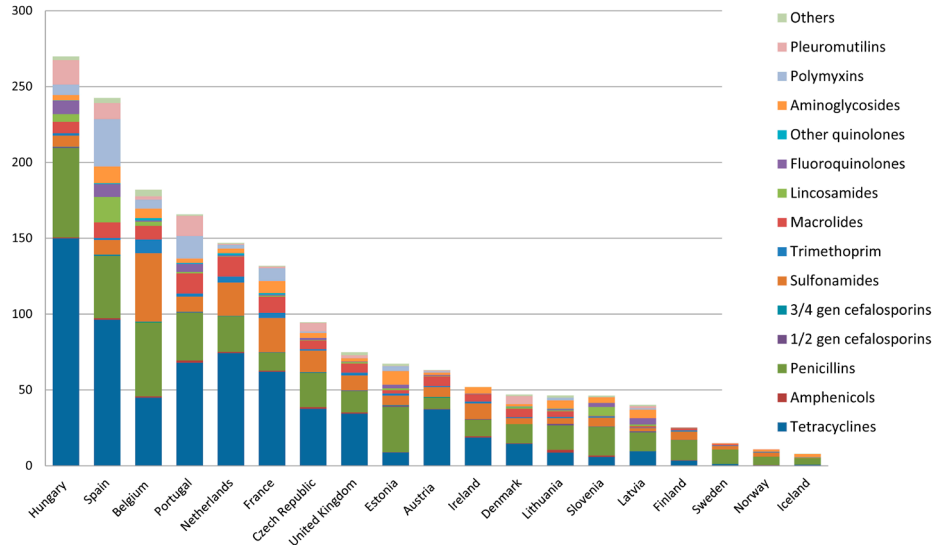


***Figure 8.6*** *Antibiotic sales in 19 EU Member States for food-producing species, including horses shown as milligrams of active ingredient of antibiotics normalized by the population correction unit (PCU) that uses a standardized weight at treatment for each animal category and subtracts imported animals for slaughter or fattening and add exported animals to the country of origin (adapted from the European Medicines Agency, Figure 7[40]) .*

As a consequence antibiotic resistance in human clinical isolates is found to be very low in the Netherlands compared to other countries and very high in isolates derived from food-producing animals [62]. Therefore we can expect that a contribution of infections with multidrug resistant isolates derived from livestock will be noticed earlier in the Netherlands compared to countries in which infections with multidrug resistant isolates are more common. Still the impact of a high prevalence of ESBL/AmpC-producing isolates in broilers on human health must be established.

## 8.6 Conclusions

The research described in this thesis is of particular relevance as an advice for the government and other stakeholders especially on the importance of prudent use of antimicrobials in veterinary medicine. Of course this was already under attention when the spread of MRSA from pigs and calves to humans was described. However, it became clear that the epidemiology of ESBL/AmpC-producing isolates, also driven by antibiotic usage and probably by other factors, is much more complex and therefore probably more threatening than that of MRSA. Compared to MRSA, ESBL/AmpC-producing isolates can spread more easily due to the fact that ESBL/AmpC-producing isolates cover multiple bacterial species, which can be more pathogenic than *Staphylococcus aureus*, and due to the plasmid-mediated features of this resistance. Human health risks due to ESBL/AmpC-producing isolates in food-producing animals or food derived from these animals are difficult to predict. Although in this thesis an extensive overview of what can be found in the broiler production pyramid is given, the potential implications for human health are still not completely clear.

Characterization of ESBL/AmpC genes, plasmids and strains in broilers have shown that overlap between this and other reservoirs exit, however the research described in this thesis does not give an answer to the impact of these isolates present in broilers to human health. Therefore future research should focus on quantifying the risk of ESBL/AmpC-producing isolates or other multidrug resistant isolates in food-producing animals to humans. Thereby taking into account all possible sources that can lead to colonization of the human gut. Also the link between human gut colonization and the chance of becoming infected with such isolates should be addressed. Information gathered in this thesis about characterization of genes, plasmids and strains will facilitate such an approach. Interdisciplinary research where microbiologist work closely together with epidemiologists, mathematical and bioinformatics scientists are needed to conduct a mathematical model in which human health risks can be predicted as well as the attribution through different transmission routes can be calculated.

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# Summary

An increase is seen in bacteria resistant to commonly used antimicrobials in people without a history of antibiotic treatment or hospital stay. Infections with these bacteria can result in impaired treatments. An important and worrying resistance mechanism that is increasingly found worldwide, is the one that confers resistance to the commonly used beta-lactam antibiotics (e.g. penicillins and modern cephalosporins). Bacteria can become resistant to these antibiotics by producing enzymes that inactivate these antibiotics. There are several groups of enzymes that can be produced by the bacterium. The two main groups are the extended spectrum beta-lactamases (ESBLS) and AmpC beta-lactamases. The genes encoding these enzymes are located on mobile genetic elements, named plasmids. These plasmids and the associated resistance genes, can easily be exchanged between bacteria, causing spread of resistance between bacteria of the same species, but also between different bacterial species. Resistance that occurs in bacteria that are part of the normal bacterial flora (commensals), can then be transferred to disease-causing bacteria (pathogens). Although no information is available on the source of these bacteria, consumption of food could be one of the sources. In this thesis the occurrence of these resistant bacteria (also called ESBL/AmpC–producing bacteria) in broilers is described.

From 2003 to 2007 the number of bacteria resistant to the modern cephalosporin cefotaxime increased in bacterial isolates derived from broilers (in contrast to isolates derived from pigs and cattle). In **chapter 2**, the genes encoding these antibiotic-degrading enzymes in two different bacterial species: *Escherichia coli* and *Salmonella* derived from the intestines of broilers were molecularly characterized. Also the plasmids on which the genes were located were typed, because many different plasmid variants exist. It was found that the gene types $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ were commonly present in both *E. coli* and *Salmonella* from broilers and that these genes were mostly located on plasmids of the IncI1-family. Because the same combination of plasmid and resistance genes was found in both bacterial species, we can conclude that it is very likely that exchange of resistance genes in the intestine of broilers will have occurred.

In 2007, 20% of the broiler samples (between 300-400 samples), collected at the slaughterhouse, contained an *E. coli* that was resistant to cefotaxime. One bacterium represented one flock of chickens. In these samples, one bacterium was randomly chosen from a non-selective growth plate. Sensitive and resistant bacteria can both grow on this plate. One colony is chosen to determine the susceptibility to a panel of antibiotics. Because this method is not very sensitive to find resistant bacteria and nothing was known about the level of resistance on broiler farms, it was decided to sample broilers at broiler farms and grow the samples on selective plates. These plates contain 1 mg/L cefotaxime leaving only the resistant (ESBL/AmpC-producing) bacteria to grow. The results of this study are described in **chapter 3**. In this study, performed in 2009, 26 broiler farms were included. Twenty-five to 41 animals were tested. All farms contained broilers with ESBL/AmpC-producing bacteria in their faeces. At 85% of the farms the percentage of broilers that carried ESBL/AmpC-producing bacteria was 80% or more. Possibly this was the result of (non legal) use of the cephalosporin ceftiofur at hatcheries. In addition, the high use of antibiotics on broiler farms might have played a role. Broiler farmers were found to have an increased risk to be carriers of ESBL/AmpC-producing bacteria. These isolates are found in 10% of the general population, but in broiler farmers 33% was carrier.

The broiler industry in the Netherlands is not confined to production farms. The Grandparents and the Parents of the broilers are also present on farms in the Netherlands. It is known that bacteria can spread from parents to their offspring, therefore Grandparent and Parent farms and hatcheries were screened for ESBL/AmpC-producing bacteria. This study took place in 2009-2010 and is described in **chapter 4**. It is clear that at every level in de broiler production pyramid ESBL/

AmpC-producing bacteria were present, however with a lower contamination rate at Grandparent and Parent farms and in one-day old broilers on hatcheries compared to that of broiler farms. Grandparents, after being sampled at two days of age, were also sampled at 18 weeks and 31 weeks of age. The contamination rate varied between 0-70% at two days of age to 0-44% and 2-27% at 18 or 31 weeks of age. On three broiler farms, the broiler flocks were also followed in time. The contamination rate at these farms increased within one week from 0-20% to 96-100%. The cause of the difference between the prevalence of ESBL/AmpC bacteria at broiler and Grandparent farms could not be detected, but will most likely be related to the differences in farming systems, management and antibiotic use.

A new microarray test to accurately and fast determine to which group an ESBL gene in a resistant bacterium belongs is described in **chapter 5**. This microarray is able to detect very small differences (mutations) between the most important groups of ESBL genes in a bacterial culture. An ESBL group can then be identified. This result gives less information, but is faster than the conventional way that identifies the whole genetic make-up of the gene. This microarray has a lot of advantages when, for example in a clinical setting, an outbreak must be traced very quickly. However, for research purposes the slower conventional way is still preferred.

In **chapter 6** it is shown that the resistance genes and plasmids present in ESBL-producing bacteria derived from broilers are similar to the ones found in19% of human infections (mostly urinary tract infections) with ESBL-producing bacteria (in 2009). Furthermore, a study performed in 2010 shows that a high percentage (94%) of broiler meat collected from different stores in the Netherlands contains cephalosporin resistant bacteria. This shows that broiler meat is a possible source for human infections with these bacteria.

According to the study described in **chapter 7** this will not be the only source. ESBL/AmpC-producing bacteria are also found in companion animals and horses. Comparison of the genes present in these bacteria, show similarity to the ones found in human bacteria. Exchange of ESBL/AmpC-producing bacteria between humans, pets and horses is not excluded.

The studies described in this thesis do not give an answer to the question whether and to what extent the presence of ESBL/AmpC-producing bacteria in broilers threatening is to human health. However, because broiler meat can be a possible source of these bacteria, measures to reduce the amount of ESBL/AmpC-producing bacteria in the broiler production chain are desirable. In the time between the research described in this thesis and the publication of this thesis, much effort is made by the food-producing animal sectors and the Dutch government to achieve a reduction in the use of antibiotics (**chapter 8**). In particular a decrease in antibiotic use at broiler farms and stopping ceftiofur administration at hatcheries may have resulted in a decrease in cefotaxime resistant bacteria measured in 2012. This looks promising, but we must realize that resistant bacteria are still abundantly present. The broiler industry has the difficult task to achieve a healthy, safe and animal-friendly product in which antibiotics are hardly necessary.

# Samenvatting

Steeds meer mensen blijken bacteriën bij zich te dragen die resistent zijn tegen de meest gangbare antibiotica, zonder dat zij antibiotica hebben gebruikt of in een ziekenhuis zijn behandeld. Dit kan leiden tot infecties die moeilijk te behandelen zijn. Een belangrijk en zorgwekkend resistentiemechanisme, dat wereldwijd toeneemt, is de resistentie tegen de veel gebruikte beta-lactam antibiotica (o.a. penicillines en moderne cefalosporinen). Bacteriën kunnen resistent worden tegen deze antibiotica door het aanmaken van enzymen, die deze antibiotica afbreken. Er zijn verschillende groepen enzymen, die door de bacterie kunnen worden aangemaakt. De twee belangrijkste groepen zijn de extended spectrum beta-lactamasen (ESBLs) en de AmpC beta-lactamasen. De genen die voor deze enzymen coderen liggen op mobiele stukjes genetisch materiaal, ook wel plasmiden genoemd. Deze plasmiden en de daarop liggende resistentiegenen, kunnen heel makkelijk uitgewisseld worden tussen bacteriën. Hierdoor kan resistentie zich verspreiden tussen bacteriën van dezelfde soort, maar ook tussen verschillende bacteriesoorten. Resistentie die voorkomt bij niet-ziekmakende bacteriën (commensalen), kan daarbij overgaan naar ziekmakende bacteriën (pathogenen). Een bron van deze bacteriën voor de mens is niet bekend, maar zou de consumptie van voedsel kunnen zijn. In dit proefschrift wordt het voorkomen van deze resistente bacteriën (ook wel ESBL/AmpC beta-lactamase producerende bacteriën genoemd), bij vleeskuikens beschreven.

Van 2003 tot 2007 is er in vleeskuikens (in tegenstelling tot varkens en runderen) een toename waargenomen van het aantal bacteriën dat ongevoelig is voor het moderne cefalosporine: cefotaxime. Dit blijkt uit de monitoring van antibioticaresistente bacteriën afkomstig uit voedselproducerende dieren. In **hoofdstuk 2** worden de genen die coderen voor de antibioticumafbrekende enzymen uit twee verschillende bacteriesoorten: *Escherichia coli* en *Salmonella* gevonden in de darmen van vleeskuikens moleculair gekarakteriseerd. Ook de plasmiden, waar deze genen op liggen zijn getypeerd, want er bestaan heel veel verschillende plasmid types. Hierbij bleek dat de gentypes $bla_{\text{CTX-M-1}}$ en $bla_{\text{TEM-52}}$ bij beide bacteriesoorten het meest voorkomen en dat deze grotendeels op plasmiden van de IncI1 familie liggen. Aangezien dezelfde combinatie van plasmide en resistentie-gen teruggevonden werd in zowel *E. coli* als *Salmonella* bacteriën, kunnen we concluderen dat er zeer waarschijnlijk in de darm van de kip uitwisseling van resistentie tussen verschillende bacteriesoorten plaatsvindt.

In 2007 bleek 20% van de vleeskuiken monsters (tussen 300-400 monsters), genomen op het slachthuis, een *E. coli* bacterie te bevatten die resistent was tegen cefotaxime. Eén isolaat staat voor één koppel vleeskuikens. Bij deze monsters wordt één isolaat ad random gekozen van een niet-selectieve groei plaat. Hierop groeien gevoelige en resistente bacteriën doorelkaar. Van één kolonie wordt de gevoeligheid bepaald voor een panel van antibiotica. Omdat deze methode niet erg gevoelig is voor het vinden van resistente bacteriën en er nog niks bekend was over het resistentie niveau op vleeskuikenbedrijven, is er voor gekozen om vleeskuikens op bedrijven te gaan bemonsteren en de monsters te laten groeien op selectieve platen. Deze platen bevatten 1 mg/L cefotaxime, waardoor alleen de resistente (ESBL/AmpC-producerende) bacteriën kunnen groeien. De resultaten hiervan zijn beschreven in **hoofdstuk 3**. Bij dit onderzoek, gedaan in 2009 zijn 26 vleeskuikenbedrijven onderzocht. Per bedrijf zijn 25-41 dieren onderzocht. Op alle bedrijven werden ESBL/AmpC-producerende bacteriën in de mest van vleeskuikens gevonden. Bij meer dan 85% van de bedrijven werden deze bacteriën bij meer dan 80% van de dieren gevonden. Mogelijk is dit het gevolg geweest van (niet legaal) gebruik van het cefalosporine ceftiofur op broederijen. Daarnaast kan het hoge gebruik van antibiotica op de vleeskuikenbedrijven een rol hebben gespeeld. Vleeskuikenhouders bleken een verhoogde kans te hebben om drager te zijn. In de algemene bevolking draagt ongeveer 10% van de mensen ESBL/AmpC-producerende bacteriën bij zich, bij vleeskuikenhouders was dit percentage 33%.

In Nederland worden niet alleen vleeskuikens voor consumptie gehouden, maar zijn er ook bedrijven waar de grootouders en de ouders van vleeskuikens worden gehouden. Bacteriën kunnen doorgegeven worden van de moeder naar het kuiken, daarom is er bij de grootouders en de ouders en op de verschillende broederijen gekeken naar het voorkomen van ESBL/AmpC-producerende *E. coli* bacteriën. Dit onderzoek vond plaats in 2009-2010 en is beschreven in **hoofdstuk 4**. Het is duidelijk, dat in alle lagen van de vleeskuiken-productie-kolom ESBL/AmpC-producerende *E. coli* bacteriën worden gevonden, maar met een lagere besmettingsgraad dan op de vleeskuikenbedrijven. Op grootouderdier niveau zijn groepen dieren, na te zijn bemonsterd op één à twee dagen leeftijd, ook vervolgd in de tijd en bemonsterd op 18 weken en 31 weken leeftijd. De besmettingsgraad varieerde tussen 0-70% op 2 dagen leeftijd, 0-44% op 18 weken en 2-27% op 31 weken leeftijd. Op drie vleeskuikenbedrijven zijn de koppels ook vervolgd in de tijd. Daar steeg de besmettingsgraad binnen één week van 0-20% tot 96-100%. Een oorzaak voor dit verschil in voorkomen van resistente bacteriën bij vleeskuikens en grootouderdieren kon niet worden aangetoond, maar zal verband kunnen houden met de verschillen in houderijsystemen, management en het antibioticumgebruik.

Om vast te stellen tot welke groep een ESBL-gen uit een resistente bacterie behoort, wordt in **hoofdstuk 5** een microarray test beschreven, die snel en accuraat de belangrijkste ESBL-genen in een bacteriecultuur kan detecteren. Deze microarray is in staat om hele kleine verschillen (mutaties) tussen groepen ESBL genen te detecteren. Hierdoor kan bepaald worden tot welke groep het te onderzoeken gen behoord. Dit geeft iets minder informatie, maar gaat sneller dan de conventionele manier, waarbij een heel gen genetisch in kaart wordt gebracht. Voor klinische doeleinden, om bijvoorbeeld een uitbraak in een ziekenhuis snel in kaart te brengen, kan dit veel voordelen geven. Echter voor onderzoeksdoeleinden kan de tragere conventionele manier toch de voorkeur hebben.

In **hoofdstuk 6** is beschreven dat het resistentie-gen en plasmide in ESBL-producerende bacteriën in vleeskuikens overeenkomen met het resistentie-gen en plasmide dat in 19% van met name urineweginfecties bij de mens voorkomt (in 2009). Verder blijkt uit een studie in 2010, dat een hoog percentage (94%) kippenvlees in verschillende winkels in Nederland verzameld, cefalosporine resistente bacteriën bevat. Dit laat zien dat er mogelijk een bron van deze resistente bacteriën voor de mens aanwezig is in vleeskuikens en het vlees daarvan.

Dat dit niet de enige bron is, blijkt uit het onderzoek beschreven in **hoofdstuk 7**. Ook bij gezelschapsdieren en paarden zijn ESBL/AmpC-producerende bacteriën aangetroffen. Hier zijn ook overeenkomsten gevonden tussen de resistentietypes in bacteriën van gezelschapsdieren en die bij de mens. Uitwisseling van ESBL/AmpC-producerende bacteriën tussen mens, gezelschapsdier en paard is niet uitgesloten.

In dit proefschrift wordt geen antwoord gegeven op de vraag òf en in welke mate de aanwezigheid van ESBL/AmpC-producerende bacteriën bij vleeskuikens een gevaar is voor de gezondheid van de mens. Echter door de mogelijke bijdrage vanuit deze bron zijn maatregelen om resistente bacteriën in de vleeskuiken-productieketen te verminderen wenselijk. In de tijd tussen dit onderzoek en het uitkomen van dit proefschrift is er onder druk van de overheid door de verschillende voedselproducerende diersectoren veel inspanning verricht om te komen tot een reductie in het gebruik van antibiotica (**hoofdstuk 8**). Met name een afname in antibioticumgebruik op vleeskuikenbedrijven en het stoppen van ceftiofur toediening op de broederijen, heeft er mogelijk toe bijgedragen dat in 2012 een afname te zien is van bacteriën die ongevoelig zijn voor cefotaxime. Dit lijkt veelbelovend, maar we moeten beseffen dat resistente bacteriën nog steeds overvloedig aanwezig zijn. De vleeskuikensector heeft de moeilijke taak om tot een gezond, veilig en diervriendelijk product te komen, waarbij antibiotica bijna niet meer nodig zijn.

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# About the author

**About the author**

Cindy Dierikx was born on 19 July 1974 in Hoogerheide, the Netherlands. In 1992 she finished her pre-university education (VWO) at comprehensive school "Rijksscholengemeenschap 't Rijks" in Bergen Op Zoom. In 1992 and 1993 she studied Animal Management at Van Hall Institute in Groningen (which is moved now to Leeuwarden). In 1994 she started to study Veterinary Medicine at Utrecht University. During her study she was member of the board of the Foundation Veterinary Medicine in developing countries (Stichting DIO). At the end of 2001 she graduated in veterinary medicine in the direction of small animals and started working as veterinarian for small animals at Animal Hospital "Visdonk" in Roosendaal, the Netherlands.

She gained laboratory and research experience from 2004-2006 as junior scientific researcher at the Department of Gastroenterology and Hepatology at Erasmus MC, University Medical Centre in Rotterdam, the Netherlands. The research topic was immunology and treatment of *Helicobacter pylori.*

Between august 2006 to march 2007 she worked as research assistant in the PIENTER-project at the Laboratory for Infectious Diseases and Screening and Evaluation of the National Vaccination Program at the National Institute for Public Health and the Environment (RIVM), de Bilt, the Netherlands. Here she was responsible for the logistics in order to collect serum to establish a serum bank of a representative sample of the Dutch population to evaluate the national vaccination program.

In 2007 she started her training as veterinary microbiologist at the Central Veterinary Institute part of Wageningen UR in Lelystad (formerly called CIDC-Lelystad). In 2008 she started her PhD with the project 'ESBLs in farm animals' followed by the project 'Prevalence of ESBLs in broilers' in 2009. Both resulted in this thesis. She will continue her work at the Central Veterinary Institute part of Wageningen UR at the reference laboratory on antimicrobial resistance.

**Publications**

*Related tot his thesis:*

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*Other publications*

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