



EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum -lactamases and/or AmpC -lactamases in food and food-producing animals

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SCIENTIFIC OPINION

Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β -lactamases and/or AmpC β -lactamases in food and food-producing animals¹

EFSA Panel on Biological Hazards (BIOHAZ)^{2,3}

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The potential contribution of food-producing animals or foods to public health risks by ESBL and/or AmpC-producing bacteria is related to specific plasmid-mediated ESBL and/or AmpC genes encoded by a number of organisms. The predominant ESBL families encountered are CTX-M, TEM, and SHV; the predominant AmpC-family is CMY. The most common genes associated with this resistance in animals are *bla*_{CTX-M-1} (the most commonly identified ESBL), and *bla*_{CTX-M-14}, followed by *bla*_{TEM-52} and *bla*_{SHV-12}. Among the genes encoding AmpC-type β -lactamases, *bla*_{CMY-2} is the most common. The bacterial species most commonly identified with these genes are *Escherichia coli* and non-typhoidal *Salmonella*. ESBL/AmpC transmission is mainly driven by integrons, insertion sequences, transposons and plasmids, some of which are homologous in isolates from both food-production animals and humans. Cefotaxime is used as the drug of choice for optimum detection of *bla*_{ESBL} and/or *bla*_{AmpC} genes. The preferred method for isolation of ESBL- and/or AmpC-producers is screening on selective agar preceded by selective enrichment in a broth. The establishment of risk factors for occurrence of ESBL/AmpC-producing bacteria is particularly complicated by the data unavailability or lack of its accuracy. The use of antimicrobials is a risk factor for the selection and spread of resistant clones, resistance genes and plasmids. Since most ESBL- and AmpC-producing strains carry additional resistances to other commonly-used veterinary drugs, generic antimicrobial use is a risk factor for ESBL/AmpC and it is not restricted specifically to the use of cephalosporins. An additional risk factor is extensive trade of animals in EU MS. There are no data on the comparative efficiency of individual control options in reducing public health risks caused by ESBL and/or AmpC-producing bacteria related to food-producing animals. Prioritisation is complex, but it is considered that a highly effective control option would be to stop all uses of cephalosporins/systemically active 3rd/4th generation cephalosporins, or to restrict their use (use only allowed under specific circumstances). As co-resistance is an important issue, it is also of high priority to decrease the total antimicrobial use in animal production in the EU.

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KEY WORDS

Resistance, ESBLs, AmpC, occurrence, transmission, control options, public health microbiology.

SUMMARY

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ) was asked to deliver a Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum beta (β)-lactamases (ESBL) and/or AmpC β -lactamases (AmpC) in food and food-producing animals. In particular, the Panel was asked: (i) to propose a definition of the ESBL- and/or AmpC-producing bacterial strains and genes relevant for public health and linked to food-producing animals or food borne transmission; (ii) to review the information on the epidemiology of acquired resistance to broad spectrum cephalosporins including the genes coding for such resistance in food-producing animals and food, ensuring that differentiation was made between transmission of resistant bacterial strains and/or genes to humans by consumption or handling of contaminated food; and transmission of resistant bacterial strains and/or genes to humans through the food animal production environment; (iii) to perform a critical analysis of the methods (phenotypic and genotypic) and the interpretive criteria currently used for detection (isolation and identification) and characterisation of ESBL- and/or AmpC-producing bacterial strains, ESBL- and/or AmpC-encoding genes and associated mobile elements; (iv) to make recommendations for a harmonised monitoring of resistance (phenotypic and genotypic) caused by ESBL- and/or AmpC in food and food-producing animals in the EU; (v) to the extent possible, to identify risk factors contributing to the occurrence, emergence and spread of ESBL- and/or AmpC-producing bacterial strains in food producing animals and food; and finally, (vi) to identify and rank possible control options, taking into account the expected efficiency in reducing public health risk caused by ESBL and/or AmpC-producing bacterial strains transmitted *via* the food chain or *via* food animal production environment, and consider the advantages and disadvantages of different options.

The BIOHAZ panel concluded that ESBLs may be defined as plasmid-encoded enzymes found in the *Enterobacteriaceae*, frequently in *Escherichia coli* and *Klebsiella pneumoniae*, that confer resistance to a variety of β -lactam antibiotics, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams (eg aztreonam), but usually not the carbapenems or the cephamycins (e.g. cefoxitin). In contrast, AmpC β -lactamases are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria, which confer resistance to penicillins, 2nd- and 3rd-generation cephalosporins including β -lactam/inhibitor combinations, cefamycins (cefoxitin), but usually not to 4th-generation cephalosporins (cefepime, ceftazidime) and carbapenems; a growing number of these AmpC enzymes are now plasmid-borne.

The potential contribution of food-producing animals or foods to public health risks by ESBL and/or AmpC-producing bacteria is related to specific plasmid-mediated ESBL and/or AmpC genes encoded by a number of organisms. Although there are a large number of genes which encode ESBL and AmpC enzymes not all are equally prevalent among human and animal bacteria. The predominant ESBL families encountered are CTX-M, TEM, and SHV. The predominant AmpC-family is CMY. The bacterial species most commonly identified with these genes are *Escherichia coli* and non-typhoidal *Salmonella*. Among *E. coli*, the clonal lineages: B2-*E. coli* O25:H4-ST131, D-*E. coli* O25a-ST648 and D-*E. coli*-ST69, -ST393, are being increasingly detected among both humans and animals. Among *Salmonella* the most common serovars are Typhimurium, Newport, and Heidelberg; ESBL/AmpC transmission is mainly driven by integrons, insertion sequences, transposons and plasmids, some of which are homologous in isolates from both food-production animals and humans.

Cefotaxime is used as the drug of choice for optimum detection of *bla*_{ESBL} and/or *bla*_{AmpC} genes in *Salmonella* and *E. coli*. From the results presented in the Community Summary Report it can be concluded that the prevalence of resistance to cefotaxime in food animals varies by country and animal species. High levels are observed in *E. coli* and *Salmonella* from poultry in Spain, Italy, the

Netherlands and Poland. From raw meat from poultry only limited cefotaxime resistance prevalence data are available. Belgium and the Netherlands reported high to moderate cefotaxime resistance prevalences in *Salmonella* and *E. coli* from poultry meat. In pigs and cattle the prevalences were low. Since 2000, the presence of ESBL- and/or AmpC-producing *Salmonella* and *E. coli* in animals and food has been increasingly reported in both Europe and globally. Although these enzymes have been described in bacteria from all major food-producing animals, poultry and poultry products are most frequently reported to carry ESBL and/or AmpC-producing bacteria. The most frequently reported ESBL subtypes in the EU in both *Salmonella* and *E. coli* in food-producing animals and foods are CTX-M-1, CTX-M-14, TEM-52 and SHV-12; the predominant plasmidic AmpC variant described globally to occur in *Salmonella* and *E. coli* from food-producing animals or foods since the mid-1990s is CMY-2. A wide range of additional CTX-M subtypes (CTX-M-1, -2, -3, -8, -9, -14, -15, -17/18, -20, -32, -53) have been detected in food-producing animals and food in European countries. A range of additional TEM (TEM-20, -52, -106, -126) and SHV (SHV-2, -5, -12) variants have similarly been detected in different European countries. Epidemic plasmids belonging to the incompatibility groups F, A/C, N, HI2, I1 and K groups carrying particular ESBL-encoding genes (*bla*_{TEM-52}, *bla*_{CTX-M-1, -9, -14, -32}) or AmpC-encoding genes (*bla*_{CMY-2}) have been detected among farm and companion animals, food products and humans. There are few studies that describe clear evidence of direct transmission of ESBL- or AmpC-producing *E. coli* isolates from food-producing animals or food to humans. Data do exist about common clones of ESBL- and/or AmpC-producing *E. coli* isolates in humans and food-producing animals and foods, which provide indirect evidence about this transmission. Comparison of *E. coli* derived from humans and poultry has shown that antibiotic-resistant *E. coli* isolates from both reservoirs are more frequently genetically related than antibiotic-susceptible isolates. Recent findings indicate transmission of ESBL genes, plasmids and clones from poultry to humans is most likely to occur through the food chain. There is limited evidence for spread of ESBL/AmpC-carrying organisms via direct contact with animals or indirectly via the environment. Nevertheless people working with poultry have a higher risk for intestinal carriage of ESBL/AmpC-producing bacteria.

The preferred method for selective isolation of ESBL- and/or AmpC-producers is using cephalosporin-supplemented agar preceded by selective enrichment in a broth. The preferred method for selective isolation of ESBL- and/or AmpC-producers is chromogenic (e.g. MacConkey agar) with 1 mg/L cefotaxime or ceftriaxone. Using low concentrations will result in optimum sensitivity to detect all relevant beta-lactamase families. Pre-enrichment may be performed in a general broth like Mueller-Hinton, Brain Heart Infusion or Luria-Bertani broth with 1 mg/L cefotaxime or ceftriaxone.

Identification is performed by determination of susceptibility to cefotaxime, ceftazidime and cefoxitin. ESBL producers are resistant to cefotaxime, variably resistant to ceftazidime and susceptible to cefoxitin. Confirmation of ESBLs is performed by testing for synergy with clavulanic acid by combination disks, ESBL-etest or broth micro-dilution including cefotaxime, and ceftazidime as single drugs, and in combination with clavulanic acid. Confirmation of AmpC producers is performed by determination of susceptibility to cefepime. AmpC producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and cefoxitin. To identify ESBL and/or AmpC-suspected *Enterobacteriaceae* by broth micro-dilution susceptibility tests, optimum breakpoints or interpretive criteria need to be used. Although CLSI has recently redefined MIC breakpoints for 3rd- and 4th-generation cephalosporins, the R-breakpoints for ceftazidime, cefoxitin and cefepime are still one to two dilution steps higher than those defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In order to harmonize the interpretation of susceptibility data and for optimum phenotypic detection of ESBL and/or AmpC producers, it is important to use EUCAST clinical breakpoints for interpretation of susceptibility or resistance and EUCAST epidemiological cut-off values (ECOFFs), to determine if an isolate belongs to the wild-type population or not.

All isolates confirmed phenotypically to be either ESBL or AmpC producers may be screened for β -lactamase gene families using micro-array or (multiplex) PCR. The ESBL and/or AmpC subtypes may be identified by dedicated PCRs and sequence analysis of the amplicons. Characterisation of plasmids on which *bla*_{ESBL} and/or *bla*_{AmpC}-genes are located is essential to study the epidemiology of these

genes and plasmids. Since in *Enterobacteriaceae* several different plasmids are often present in each isolate, a structured approach is needed to identify the characteristics of the plasmid on which the β -lactamase genes are located. If the presence of an ESBL and/or AmpC gene in a bacterial isolate is confirmed, plasmid isolation is performed to determine the number and sizes of plasmids present. Subsequently, by conjugation or electroporation, transconjugants or transformants are isolated on selective agar plates with only the plasmid that harbours the β -lactamase gene present. The plasmid can be typed using replicon typing and sub-typed by fingerprinting or plasmid MLST. Ultimately whole plasmid sequence analyses may replace the current typing and subtyping techniques. The choice of the molecular typing method to be used for isolates is determined by epidemiological relatedness of the isolates. Next to phenotypic methods such as serotyping and phage typing, PFGE or MLVA can be used to identify clusters of isolates that are related to a certain 'outbreak' in a restricted time frame. MLST is generally the method of choice to identify relatedness of isolates of the same species from different backgrounds (eg. animal versus human).

The establishment of risk factors for occurrence of ESBL/AmpC-producing bacteria is particularly complicated by the data unavailability or lack of its accuracy. Few studies designed to assess risk factors for ESBL and/or AmpC occurrence in animals are available. The use of antimicrobials is a risk factor for selection and spread of resistant clones, resistance genes and plasmids. Most ESBL- and AmpC-producing strains carry additional resistances such as to sulphonamides and other commonly-used veterinary drugs. Therefore, generic antimicrobial use is a risk factor for ESBL/AmpC and it is not restricted specifically to the use of cephalosporins. Currently there are no pan-European data available on the use of antimicrobials. The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), coordinated by the European Medicines Agency (EMA), is collecting information. An additional risk factor is extensive trade of animals in EU member states (MS), with few countries leading the production and the export, and with a small number of companies producing pure line breeding animals. How widespread ESBL-carrying bacteria are in food-producing animals in the breeding/rearing/fattening sectors is generally unknown, although a few reports suggest that ESBL/AmpC are not uncommon in the top of some production pyramids (breeding). ESBL- and/or AmpC-producing *E. coli* are introduced in poultry production chain through day-old grandparent chickens. Moreover, some data indicate that the occurrence of these organisms in the different levels of the poultry production chain is the result of vertical transmission, local recirculation and selection.

There are no data on the comparative efficiency of individual control options presented in this document in reducing public health risks caused by ESBL and/or AmpC-producing bacteria related to food-producing animals. Prioritisation is complex, and the effectiveness of measures discussed in this Opinion is based on the best available evidence and expert opinion. As such it is considered that a highly effective control option to reduce selection of ESBL/AmpC-producing bacteria at an EU level, would be to stop all uses of cephalosporins/systemically active 3rd/4th generation cephalosporins, or to restrict their use (use only allowed under specific circumstances). Measures intended to minimize off label use should focus on increased compliance with existing legislation. As co-resistance is an important issue, it is also of high priority to decrease the total antimicrobial use in animal production in the EU. Also of importance (more so after the ESBL/AmpC-producing microorganisms have emerged) are the measures to control dissemination, for example by implementing increased farm biosecurity and controls on animal trade (of ESBL/AmpC-carriers), and by improving hygiene throughout the food chain, and implementing other general post-harvest controls for food-borne pathogens. Because most evidence is available for high prevalence of ESBL/AmpC-producing bacteria in the poultry production pyramid, and their consequent involvement in public health, it is of high priority to reduce selection pressure imposed by the use of antimicrobials, to prevent vertical transmission from the top of the poultry production pyramid, and to prevent local recirculation within subsequent flocks.

Recommendations for the harmonised monitoring of resistance caused ESBL- and/or AmpC-producing bacteria have been provided.

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BACKGROUND AS PROVIDED BY EC

A number of documents on public health concern of food-borne or other zoonotic spread of antimicrobial resistance have recently been published by the Commission⁴ and relevant agencies (EFSA⁵, EMEA⁶ and ECDC⁷). One of the new concerns highlighted in all of these documents is the increased occurrence of bacterial strains producing extended-spectrum beta (β)-lactamases (ESBLs) and/or acquired AmpC β -lactamases, thus causing it difficult to treat severe infections in humans, and their possible link to food or food producing animals. Many different kinds of bacteria are able to produce such enzymes, which deteriorate the effects of 3rd- and 4th-generation cephalosporin and monobactam antimicrobials. These antimicrobials are considered to be critically or highly important medicines for humans according to the report of the second World Health Organization (WHO) Expert Meeting⁸. They are also listed as antimicrobials of veterinary importance by the World Organisation for Animal Health (OIE)⁹.

EMEA's reflection paper on the use of 3rd- and 4th-generation cephalosporins in food producing animals in the EU (2009)³ stresses that the rapid emergence of resistance caused by ESBL and/or AmpC in *Enterobacteriaceae* in Europe is a major public health concern, in particular regarding the increasing frequency of community-acquired infections. It is also said that it is possible that spread from animal reservoirs via food or via the environment may contribute to the dissemination of resistance in the community.

It is stated in the EMEA's reflection paper that strains of *E. coli* producing ESBL and/or AmpC have been isolated from many types of food producing animals such as calves, cattle, swine, poultry, broilers, horses and rabbits in different countries in the EU. ESBL and/or AmpC producing *Salmonellas* have been detected in poultry and poultry meat samples. An occurrence of resistance caused by ESBL and/or AmpC has been linked to the use of antimicrobials in food producing animals, in particular 3rd generation cephalosporins, but increase in ESBL and/or AmpC-producing strains of *Salmonella* and *Escherichia coli* have also been detected without any prior use of cephalosporins. According to the report studies have also shown that humans can be exposed to ESBL- and/or AmpC-producing pathogens causing clinical illness *via* food or *via* direct contact with infected animals, or to ESBL- and/or AmpC-encoding genes *via* direct contact, *via* contaminated food or indirectly through the environment. These genes can be transferred to bacteria with potential to cause infections in humans. EMA is proposing monitoring of resistance caused by ESBL and/or AmpC, including commensals in the zoonoses monitoring programmes.

According to the EFSA opinion on food-borne antimicrobial resistance as a biological hazard (2008)², the potential role of food and environmental sources in the epidemiology of transferable resistance genes has gained increased attention in relation to the rapid and recent emergence of resistance to 3rd generation cephalosporins. It is stated in the opinion that genes coding for the production of ESBL and/or AmpC are found in bacteria causing infections in hospitals, but also infections acquired in the community, in *Salmonella* from cases of human infections and food animals and commensal *E. coli*

⁴ Staff working paper of the services of the Commission on antimicrobial resistance SANCO/6876/2009r6

⁵ Scientific Opinion of the Panel on Biological Hazards on a request from the European Food Safety Authority on food-borne antimicrobial resistance as a biological hazard. The EFSA Journal (2008) 765, 1-87

⁶ Revised reflection paper on the use of 3rd and 4th generation cephalosporins in food producing animals in the European Union: Development of resistance and impact on human and animal health EMEA/CVMP/SAGAM/81730/2006-Rev. 1

⁷ Scientific Opinion of the European Centre for Disease Prevention and Control; Scientific Opinion of the Panel on Biological Hazards; Opinion of the Committee for Medicinal Products for Veterinary Use; Scientific Opinion of the Scientific Committee on Emerging and Newly Identified Health Risks: Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic infections. EFSA Journal 2009; 7(11): 1372

⁸ FAO/WHO/OIE 2008. Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials. Report of a meeting held in FAO, Rome, Italy, 26-30 November 2007; third revision 2009.

⁹ OIE list of antimicrobials of veterinary importance. Resolution No. XXVIII, 75th General Session in May 2007.

isolated from animals. The ESBL- and/or AmpC-encoding genes have also been linked to transferable fluoroquinolone resistance and other resistance genes.

According to the Joint opinion of ECDC, EFSA, EMEA and Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) on antimicrobial resistance focused on zoonotic agents (2009)⁴ extra-intestinal and urinary tract infections in humans caused by resistant *E. coli* strains are becoming increasingly common and further work to elucidate the sources of such strains is necessary. In the joint opinion it is proposed in order to improve surveillance activities and risk assessment to further explore of the origin and transmission of ESBL-producing *E. coli* through the food chain. It is also proposed to develop strategies to explore the occurrence of resistance caused by ESBL and/or AmpC in non-pathogenic commensal micro-organisms (e.g. *E. coli*) together with their ability to develop, harbour and transmit resistance genes. Identification and characterisation of those environments that facilitate bacterial ESBL and/or AmpC gene transfer focusing on both zoonotic bacteria and other bacteria such as *E. coli* are also included in the proposals. Common recommendation of the above mentioned papers is to put resistance caused by ESBL and/or AmpC in place as a matter of priority.

The staff working paper of the services of the Commission on antimicrobial resistance (2009)¹, which is meant to serve as a basis of further discussions and needs in combating the issue of antimicrobial resistance (AMR), notices also the importance of ESBL and/or AmpC producing bacteria by reflecting that specific surveys could be carried out for resistance caused by ESBL and AmpC.

The risk of spread of antimicrobial resistance via companion and other animals in direct contact with humans has also been addressed in several opinions of the agencies. This issue should, however, be subject of a separate future request from the Commission.

In view of the above, there is a need to:

- Assess the public health risk posed by ESBL- and/or AmpC-producing bacterial strains in food producing animals and food;
- Assess the need to establish harmonised monitoring and control of ESBL- and/or AmpC-producing bacterial strains in food producing animals and food in the EU.

TERMS OF REFERENCE AS PROVIDED BY EC

EFSA is asked to issue a scientific opinion on the public health risk of ESBL- and AmpC-producing bacterial strains in food and food-producing animals and, in particular:

1. To propose a definition of the ESBL- and/or AmpC-producing bacterial strains and genes relevant for public health and linked to food-producing animals or food borne transmission;
2. To review the information on the epidemiology of acquired resistance to broad spectrum cephalosporins including the genes coding for such resistance in food-producing animals and food. Differentiation should be made between:
 - Transmission of resistant bacterial strains and/or genes to humans by consumption or handling of contaminated food.
 - Transmission of resistant bacterial strains and/or genes to humans through the food animal production environment.
3. To perform a critical analysis of the methods (phenotypic and genotypic) and the interpretive criteria currently used for detection (isolation and identification) and characterisation of ESBL-

and/or AmpC-producing bacterial strains, ESBL- and/or AmpC-encoding genes and associated mobile elements.

4. To make recommendations for a harmonised monitoring of resistance (phenotypic and genotypic) caused by ESBL- and/or AmpC in food and food-producing animals in the EU.
5. To the extent possible to identify risk factors contributing to the occurrence, emergence and spread of ESBL- and/or AmpC-producing bacterial strains in food producing animals and food.
6. To identify and rank possible control options taking into account the expected efficiency in reducing public health risk caused by ESBL- and/or AmpC-producing bacterial strains transmitted via the food chain or via food animal production environment. Advantages and disadvantages of different options should be considered.

ASSESSMENT

1. INTRODUCTION

In the last decade a variety of plasmid-mediated beta (β)-lactamases, have emerged in Gram-negative bacteria, resulting in reduced susceptibility to broad spectrum β -lactams. These β -lactamases included both extended spectrum β -lactamases (ESBLs) and AmpC β -lactamases (AmpC).

More specifically, ESBLs are plasmid-encoded enzymes in *Enterobacteriaceae*, frequently found in *Escherichia coli* and *Klebsiella pneumoniae*, but also present in other members of this bacterial family. ESBLs confer resistance to a variety of β -lactam antibiotics, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams (eg aztreonam), but usually not the carbapenems or the cephamycins (e.g. cefoxitin). ESBL-producing organisms are frequently co-, or multiresistant, exhibiting resistance to other antimicrobial classes such as fluoroquinolones, aminoglycosides and, trimethoprim-sulphamethoxazole due to associated resistance mechanisms, which may be either chromosomally- or plasmid-encoded (Jacoby and Munoz-Price, 2005; Paterson and Bonomo, 2005). The most frequently encountered ESBLs in *Enterobacteriaceae* belong to the TEM, SHV and CTX-M families (Paterson and Bonomo, 2005).

AmpC β -lactamases are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria, including many members of the *Enterobacteriaceae* (but, notably, not in *Klebsiella* or *Salmonella*), and opportunistic pathogens such as *Pseudomonas* and *Acinetobacter*. These enzymes confer resistance to penicillins, 2nd- and 3rd-generation cephalosporins including β -lactam/inhibitor combinations, cefamycins (cefoxitin), but usually not to 4th-generation cephalosporins (cefepime, cefquinome) and carbapenems. It is a serious concern that a growing number of AmpC enzymes have “escaped” on to plasmids. These are the so called ‘acquired’ or ‘plasmidic’ AmpCs.

Over the past decade another type of clinically important β -lactamases, the carbapenemases, have emerged and spread in *Enterobacteriaceae*. *Klebsiella*-producing carbapenemase (KPC) was the first carbapenemase reported in a *Klebsiella pneumoniae* isolate (Nordmann et al., 2009). The emergence and spread of carbapenemase-producing *Enterobacteriaceae* is a matter of great concern for the treatment of human infections, because carbapenemases hydrolyze all β -lactams, including carbapenems and frequently aztreonam, making these organisms extremely drug-resistant. Moreover, all types of carbapenemases that so far have been found in *Enterobacteriaceae* are spreading globally (Nordmann et al., 2009; Struelens et al., 2010; Walsh, 2008). Such carbapenemase-producing strains are usually only susceptible to the polymyxins (e.g. colistin), fosfomycin and variably susceptible to tigecycline, although there are recent reports of colistin-resistant *Enterobacteriaceae* (Endimiani et al., 2008; Nordmann et al., 2009; Zarkotou et al., 2010). These carbapenemases are outside the remit of this report as they have not yet been identified in animal isolates. Nevertheless the recent global emergence of these new resistances deserves particular attention for the near future.

Different classification systems have been suggested for these enzymes. The most commonly used are those introduced by (Bush et al., 1995), based on functional similarities and by Ambler, based on structural similarities (Ambler, 1980), or a combination of both (Bush and Jacoby, 2010). A simplified overview is provided in Table 1.

The current β -lactamase classifications have become extremely complex (Tables 2 and 3), making them less accessible to clinicians, infection control professionals, hospital management and politicians, thus facilitating a debate about the usefulness of using traditional classification schemes (Giske et al., 2009; Livermore, 2008). The term ‘ESBL’ is usually restricted to enzymes belonging to functional class 2be/molecular class A, inhibited by clavulanic acid and showing activity against extended-spectrum cephalosporins. Recently, it has been proposed to expand the definition and to include mutants with borderline ESBL activity (e.g. TEM-12) and acquired β -lactamases with activity

against extended-spectrum cephalosporins and/or carbapenems, which may fit the definition for group 2be (e.g. KPC) or not (e.g. OXA and AmpC type derivatives with increased activity against cefepime). In this classification scheme it is suggested to keep the term 'ESBL', always accompanied by mention of the enzyme's family as, e.g., in 'TEM ESBL' or 'OXA ESBL', not as a sole moniker/designation (Giske et al., 2009). In this document we will follow the “classical” system for classification and nomenclature for ESBLs.

There are some class D β -lactamases (so-called oxacillinases) that might also be considered as “extended-spectrum” enzymes. Indeed, the class D β -lactamase OXA-1 (that is also named OXA-30 in some publications, both enzymes actually having the same sequence) possess the ability to compromise the efficacy of penicillins, but also that of 4th-generation cephalosporins such as cefepime or ceftipime (Aubert et al., 2001). By contrast to “classical” ESBLs, OXA-1 is poorly inhibited by clavulanic acid and tazobactam, and does not include ceftazidime in its hydrolysis spectrum.

1.1. ESBLs

1.1.1. SHV and TEM ESBLs

Before 2000 SHV and TEM types of ESBLs were the predominant variants found in *Klebsiella* and *E. coli* that caused nosocomial infections. These ESBLs developed by mutations of the broad spectrum TEM-1 and SHV-1 and 2 genes were transferred between bacteria by plasmids, which were in turn spread by clonal distribution between hospitals and countries through patient mobility (Baraniak et al., 2005; Damjanova et al., 2007; Jacoby and Munoz-Price, 2005; Paterson and Bonomo, 2005).

1.1.2. CTX-M ESBLs

Since the early 2000s the CTX-M group of genes, named after their ability to produce enzymes capable of hydrolysing cefotaxime, emerged in human isolates. These genes were also located on highly transmissible plasmids, thus facilitating fast and efficient spread of resistance (Bonnet, 2004; Canton and Coque, 2006; Canton et al., 2008; Hunter et al., 2010; Livermore and Hawkey, 2005; Pitout et al., 2005a; Pitout and Laupland, 2008). Bacteria that express CTX-M enzymes are also commonly co-resistant or multiresistant, exhibiting resistance to multiple antimicrobials including fluoroquinolones (Jacoby et al., 2006). In the last decade the epidemiology of ESBLs in humans has changed. Successful international bacterial clones harbouring members of the CTX-M family have emerged and spread globally. As a result the CTX-M- β -lactamases have become the most prevalent ESBLs in human *Enterobacteriaceae* worldwide (Livermore et al., 2007; Pitout et al., 2005a; Pitout et al., 2005b). The epidemiology of bacteria that produce CTX-M enzymes has also changed. Since the early 2000s, *E. coli* producing CTX-M enzymes (specifically CTX-M-15) have increasingly been found in the community in uncomplicated and complicated (including bacteraemias) community-acquired urinary tract infections, as well as in serious intra-abdominal and skin and soft-tissue infections (Canton et al., 2008; Livermore et al., 2007; Peirano et al., 2010; Pitout and Laupland, 2008; Rodriguez-Bano et al., 2008; Rodriguez-Bano et al., 2004; Rodriguez-Bano et al., 2006; Woodford et al., 2004).

TEM, SHV and CTX-M ESBL-producing *Enterobacteriaceae* (mainly *E. coli* and *Salmonella*) have, in the last decade, also been increasingly reported in food-producing animals and food.

1.2. AmpC β -lactamases

The ‘acquired’ or ‘plasmidic’ AmpC enzymes fall into six phylogenetic groups (www.bellinghamresearchinstitute.com), CMY-2 being the most commonly-found enzyme. Resistance due to production of these enzymes is also a significant public health concern. Several of

these plasmidic AmpC enzymes have also been found with increasing frequency in *Enterobacteriaceae* isolated from food-producing animals and food (Jacoby, 2009; Smet et al., 2009).

1.3. Significance and public health threat of human infections with ESBL/AmpC- β lactamase-producing bacteria.

The broad resistance profile in bacteria that follows the production of ESBL/AmpC β -lactamases (and also those that produce carbapenemases), is significant in human infections, and poses an ongoing and worrisome public health threat (Pitout and Laupland, 2008; Rodriguez-Bano et al., 2010). This is primarily because many community infections, and also infections treated or transmitted within hospitals, are caused by bacteria that are no longer sensitive to 2nd, 3rd and 4th generation cephalosporins (Ben-Ami et al., 2006). These antimicrobials are administered many times as first-line therapy for a wide variety of infections found in the community and in the hospital, which include mild to extremely severe infections, ranging from an uncomplicated cellulitis or urinary tract infection, to pyelonephritis, bacteraemia or septic shock (Ben-Ami et al., 2006; Marchaim et al., 2010; Rodriguez-Bano et al., 2008; Rodriguez-Bano and Navarro, 2008; Rodriguez-Bano et al., 2004; Rodriguez-Bano et al., 2006; Rodriguez-Bano et al., 2010).

The multiresistant nature of bacteria that produce ESBLs and AmpCs can affect the selection and timely administration of appropriate antimicrobials for community-acquired and healthcare-associated infections, since many first-line antimicrobials are no longer active against them. Examples are, fluoroquinolones, frequently used in urinary tract infections (UTI) and cephalosporins, as part of an antimicrobial regimen for intra-abdominal infections. Furthermore, infections with such resistant organisms are associated with poorer patient outcomes, increased morbidity, mortality, increased length of stay and increased costs (Anderson et al., 2006; Ben-Ami et al., 2009; Cosgrove et al., 2003; Ibrahim et al., 2000; Lautenbach et al., 2001; Roberts et al., 2009; Schwaber and Carmeli, 2007).

1.4. Incidence of human infection

The total burden of human infection of ESBL-producing bacteria is not entirely known, nor is the prevalence of human faecal carriage. The data on frequency of occurrence in invasive infections in humans in Europe come from the European Antibiotic Resistance Surveillance System (EARS-Net www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx) (formerly EARSS).

EARS-Net reports annual rates of antimicrobial resistance in bloodstream infections (BSI) and infections of cerebrospinal fluid (CSF) from hospitals in Europe. Such reports have demonstrated a steady increase in the rates of invasive *E. coli* and *K. pneumoniae* isolates that are resistant to 3rd generation cephalosporins since 2000. In 2009, 28 countries reported *E. coli* isolates to EARS-Net, of which 7.4% were resistant to 3rd generation cephalosporins (a surrogate marker for presence of an ESBL/AmpC-producing bacteria). Trends over the period 2006-2009 showed a rapid and substantial increase in the proportions of *E. coli* isolates resistant to 3rd-generation cephalosporins in 16 of the 28 countries included in this surveillance system. Third-generation cephalosporin resistance in human clinical *K. pneumoniae* isolates has also increased substantially.

Trends in faecal carriage of ESBL/AmpC-producing *Enterobacteriaceae* (predominantly *E. coli*), in the community are also of relevance, since these resistant organisms have emerged globally as a significant pathogen in the community and in hospitals. Various studies report faecal carriage of ESBL/AmpC-producing *Enterobacteriaceae* to be 3.7-5.5% in non-hospitalized patients (Valverde et al., 2004), or 10.8% in patients admitted to the hospital (Ben-Ami et al., 2006). In a study, 14% of blood-stream infections in non-hospitalised patients have been reported to be caused by *Enterobacteriaceae* resistant to 3rd- generation cephalosporins (Ben-Ami et al., 2006). Another report (Rodriguez-Bano et al., 2008) states that the estimated population-based incidence of community-acquired infection due to ESBL-producing organisms was 2.2 cases per 100,000 population per year.

The risk of human colonisation or infection with ESBL and/or AmpC β -lactamase-producing organisms is a complex matter. From observational studies risk factors for the acquisition of these bacteria were related to human health care and involved prior use of antimicrobial agents, breaches in infection control practices, and mobility of patients, invasive medical devices, lengthy hospital stay and compromised host defences. Overall, prior use of antimicrobials is a known risk factor for patient colonisation or infection with ESBL-producing organisms. Many antimicrobials have been associated with either or both of these, but those most frequently found in various studies are oxyimino β -lactams (cefuroxime, cefotaxime, ceftriaxone, ceftazidime, or aztreonam), fluoroquinolones, β -lactam- β -lactamase inhibitor combinations (Park et al., 2009; Paterson et al., 2004; Rodriguez-Bano and Navarro, 2008; Wener et al., 2010).

1.5. Possible reservoirs of ESBL/AmpC β -lactamase-producing-bacteria.

Although person-to-person spread is recognised as the main method of spread of ESBL/AmpC- β -lactamase-containing *E. coli* both in hospitals and the community, the primary reservoirs of such organisms are contentious. ESBL-producing *E. coli* have been isolated from food animals in many European countries, particularly poultry and cattle, and farm animals are now recognised as important carriers of ESBL/AmpC-producing *E. coli* and *Salmonella* (Carattoli, 2008). Similarly there have been an increasing number of reports of ESBL-producing *E. coli* being isolated from foods of animal origin (Bergenholtz et al., 2009). This raises questions about the possible role of animal- and food-related reservoirs on this phenomenon.

Table 1: Main hydrolytic characteristics of ESBLs and AmpC

β -lactamase	Hydrolysis profile ^a				Inhibited by clavulanic acid
	CAZ/CTX	FOX	FEP	IPM	
ESBL	+	-	+	-	Yes
AmpC	+	+	-	-	No

^aCAZ, ceftazidime; CTX, cefotaxime; FOX, ceftaxitin; FEP, cefepime; IPM, imipenem

Table 2: Classification schemes for bacterial β -lactamases, adapted from (Bush and Jacoby, 2010)

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Representative enzyme(s)
				CA/TZB ^a	EDTA	
1	1	C	Cephalosporins	No	No	<i>E. coli</i> AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	1b	C	Cephalosporins	No	No	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	PC1
2b	2b	A	Penicillins, cephalosporins	Yes	No	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins (ESCs), monobactams	Yes	No	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	TEM-30, SHV-10
2ber	NI	A	ESCs, monobactams	No	No	TEM-50
2c	2c	A	Carbenicillin	Yes	No	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	RTG-4
2d	2d	D	Cloxacillin	Variable	No	OXA-1, OXA-10
2de	NI	D	ESCs	Variable	No	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	OXA-23, OXA-48
2e	2e	A	ESCs	Yes	No	CepA
2f	2f	A	Carbapenems	Variable	No	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	IMP-1, VIM-1, NDM-1, SPM-1
		B (B3)				L1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	CphA, Sfh-1
NI	4	Unknown				

^aCA; clavulanic acid; TZB, tazobactam.

^bNI, not included

Table 3: Major families of β -lactamases of clinical importance, adapted from (Bush and Jacoby, 2010)

Enzyme family ^a	Functional group or subgroup	No. of enzymes ^{b,c}	Representative enzymes
CMY	1, 1e	68	CMY-2, CMY-4
ACT	1, 1e	9	ACT-1
ACC	1, 1e	4	ACC-1
DHA	1, 1e	8	DHA-2
MOX	1, 1e	8	MOX-1
FOX	1, 1e	9	FOX-2
TEM	2b, 2be, 2br, 2ber	172	
	2b	12	TEM-1, TEM-2, TEM-13
	2be	79	TEM-3, TEM-10, TEM-26
	2br	36	TEM-30 (IRT-2), TEM-31 (IRT-1), TEM-163
	2ber	9	TEM-50 (CMT-1), TEM-158 (CMT-9)
SHV	2b, 2be, 2br	127	
	2b	30	SHV-1, SHV-11, SHV-89
	2be	37	SHV-2, SHV-3, SHV-115
	2br	5	SHV-10, SHV-72
CTX-M	2be	90	CTX-M-1, CTX-M-44 (Toho-1) to CTX-M-92
PER	2be	5	PER-1 to PER-5
VEB	2be	7	VEB-1 to VEB-7
GES	2f	15d	GES-2 to GES-7 (IBC-1) to GES-15
KPC	2f	9	KPC-2 to KPC-10
OXA	2d, 2de, 2df	158	
	2d	5	OXA-1, OXA-2, OXA-10
	2de	9	OXA-11, OXA-14, OXA-15
	2df	48e	OXA-23 (ARI-1), OXA-51, OXA-58
IMP	3a	26	IMP-1 to IMP-26
VIM	3a	23	VIM-1 to VIM-23
NDM	3a	3	NDM-1

a Enzyme families include those for which numbers have been assigned based on primary amino acid structures (G. Jacoby and K. Bush, <http://www.lahey.org/Studies/>).

b Compiled through December 2009.

c The sum of the subgroups in each family does not always equal the total number of enzymes in each family, because some enzyme numbers have been withdrawn, and some enzymes have not been assigned a functional designation by the investigators who provided the amino acid sequence.

d GES-1, unlike other members of the GES family, has little detectable interaction with imipenem.

e Nine clusters of OXA carbapenemases with their individual members have been designated.

2. ESBL- AND AMPC-PRODUCING BACTERIAL STRAINS AND GENES RELEVANT FOR PUBLIC HEALTH AND LINKED TO FOOD-PRODUCING ANIMALS OR FOODBORNE TRANSMISSION

2.1. ESBL and/or AmpC encoding resistance genes

A large number of genes encode ESBL and AmpC enzymes conferring reduced susceptibility to broad-spectrum β -lactams (see <http://www.lahey.org/Studies/>, a site that contains additional information and GenBank accession number references for β -lactamases from various functional groups). Despite this, not all of such genes are equally prevalent among human and animal bacteria.

In the last few years some ESBLs relevant to human medicine have been described in isolates from animals. By far the most common genes associated with this resistance have been those encoding CTX-M enzymes (the most commonly identified ESBL), followed by *bla*_{TEM-52} and *bla*_{SHV-12} (Bortolaia et al., 2010b; Chiarretto et al., 2008; Cloeckert et al., 2007; Escudero et al., 2010; Hasman

et al., 2005; Jensen et al., 2006; Machado et al., 2008; Randall et al., 2011; Smet et al., 2008). The ESBL enzymes associated with animals correspond to CTX-M (-1, -2, -3, -8, -9, -14, -15, -17/18, -20, -32, -55), TEM (TEM-20, -52, -106, -126) and SHV (SHV-2, -5 and -12). PER variants have been identified among fish and environmental samples (Carattoli, 2008).

Among the AmpC-type β -lactamases, *bla*_{CMY-2} is the most common, while *bla*_{ACC-1} and *bla*_{DHA-1} have only been scarcely reported (Arlet et al., 2006; Carattoli, 2008; Coque et al., 2008a; Cortes et al., 2010; Dierikx et al., 2010a; Hasman et al., 2005; Rayamajhi et al., 2010; Rayamajhi et al., 2008; Rodriguez et al., 2009). Noteworthy, whereas ESBL producers have been mostly found in Europe, AmpC producers have been mostly encountered in the US, mirroring the trends observed among human isolates.

Broad-spectrum SHV-1-, TEM-1- and OXA-type (e.g. OXA-1) β -lactamases have been frequently recovered from animals and food of animal origin in EU countries. It is important to note that OXA-1-like enzymes may confer reduced susceptibility to 4th-generation cephalosporins, and in addition they confer high level of resistance to β -lactam/ β -lactamase inhibitor combinations. Since the *bla*_{OXA-1} gene is commonly associated with plasmids carrying the *bla*_{CTX-M-15} ESBL gene, carriage of this gene significantly adds to the overall β -lactam resistance pattern of the corresponding producers. In addition *bla*_{OXA-1} has also commonly been identified in association with many other β -lactamase genes among all enterobacterial species.

As mentioned above, OXA-1-like enzymes may confer reduced susceptibility to 4th-generation cephalosporins, and in addition they confer high level of resistance to β -lactam/ β -lactamase inhibitor combinations. Isolates belonging to OXA-1-producing *S. Typhimurium* have been described over several years in Portugal, Spain and the UK as a cause of food-borne infections (Antunes et al., 2010; Herrero et al., 2009; Herrero et al., 2008; Hopkins et al., 2006). In addition, OXA-1 was shown to be responsible for high level resistance to ampicillin in *S. Typhimurium* isolates recovered from fish in India (Ruiz et al., 1999). Recently, a study conducted in Japan showed that among a series of ampicillin-resistant enterobacterial isolates recovered from traditional Egyptian Domiati cheese, some were producing OXA-1 (Hammad et al., 2009).

2.2. ESBL and/or AmpC-producing microorganisms

ESBL- (e.g. TEM, SHV, CTX-M) and AmpC (e.g. CMY, DHA-1, ACT-1)-producing organisms have been detected in a variety of food-producing animals (poultry, swine, bovine, horse, rabbit, ostrich, wild boars), and food of animal origin (Blanc et al., 2006; Carattoli, 2008; Carneiro et al., 2010; Cortes et al., 2010; Dierikx et al., 2010a; Escudero et al., 2010; Hunter et al., 2010; Poeta et al., 2009; Rodriguez et al., 2009; Vo et al., 2007). From these hosts, the species more commonly identified have been *E. coli* and non-typhoidal salmonellae and to a lesser extent, *Klebsiella pneumoniae*, *Citrobacter freundii* or *Enterobacter spp.* (Arlet et al., 2006; Dierikx et al., 2010a; Hasman et al., 2005; Machado et al., 2008; Rayamajhi et al., 2010; Rayamajhi et al., 2008). A wide diversity of *Salmonella* serovars producing ESBLs or AmpCs has been reported. Although the most common serovars are Typhimurium, Newport, and Heidelberg, such enzymes have also been detected in an expanding number of other serovars (Gonzalez-Sanz et al., 2009).

Few data are available from marine aquaculture production systems in Europe. ESBLs (CTX-M, PER) and/or AmpC (CMY-2) enzymes have been detected among potential human and fish pathogens in these settings, such as *Salmonella*, *Aeromonas*, *Vibrio*, and *Edwardsiella ictaluri* (Doublet et al., 2009; Girlich et al., 2010, 2011; McIntosh et al., 2008; Picao et al., 2008; Welch et al., 2009).

Other ESBL- or AmpC-producing *Enterobacteriaceae* (such as *Proteus*, *Morganella*, *Pseudomonas*, or *Acinetobacter*) have not been identified in isolates from food-producing animals.

2.3. Specific ESBL and/or AmpC-producing clones

Although ESBL/AmpC transmission is mainly driven by plasmids, there are specific human pathogenic clones, particularly of *E. coli* and *Salmonella*, which have been linked to food-producing animals and food. This situation is considered to be dynamic and it is likely that in the future new resistant clones could emerge.

Escherichia coli

Among *E. coli*, the clonal lineages: phylogroup B2-*E. coli* O25:H4-ST131, phylogroup D-*E. coli* O25a-ST648 and phylogroup D-*E. coli*-ST69, -ST393, are being increasingly detected among both humans and animals (Cortes et al., 2010; Mora et al., 2011; Vincent et al., 2010). Recently, *E. coli* ST57, ST156, and ST371, all producing ESBLs, have been recovered from chickens and turkeys in different countries in Europe and North America (Bonnedahl et al., 2010; Randall et al., 2011; Simoes et al., 2010) and ESBL-producing, verocytotoxin-producing *E. coli* (VTEC) isolates have also been described¹⁰.

Salmonella

Among *Salmonella*, global spread of multi-drug resistant (MDR) (to chloramphenicol, florfenicol, streptomycin, spectinomycin, sulphonamides, tetracyclines and trimethoprim) clones belonging to serovars Agona with SGI-1 (*Salmonella* Genomic Island 1)-A, or Typhimurium of definitive phage type (DT) 104 carrying SGI-1 have been linked to TEM-52 production (Clockaert et al., 2007). Particular clones of *S. infantis* seem to be widespread among poultry in some EU countries, for example, in France and Belgium as well Japan (Clockaert et al., 2007; Dahshan et al., 2010; Yang et al., 2010). A recent report has also described this serovar among pigs (Dahshan et al., 2010).

A summary of this information is presented in Table 4.

2.4. Mobile genetic elements involved in transmission of ESBL and/or AmpC resistance

2.4.1. Integrons

Integrons are genetic tools that play a role in the acquisition of resistance genes, and also in their expression. Noteworthy, they are not self-mobile genetic platforms, and are usually identified onto transposon and/or plasmid elements that may vehiculate these integrons. Even if integrons are common vehicles for some ESBL determinants (encoding VEB, GES, or BEL enzymes), they do not correspond to those involved in the acquisition of ESBL determinants identified among ESBL-producing animal isolates (namely TEM, SHV, and CTX-M).

ESBL and AmpC genes originally came into the chromosomes of different species of Enterobacteriaceae. Mobilization of these *bla* homologues housekeeping genes from original chromosomal backgrounds is mediated by widespread integrases (class1 integrases), transposases (ISEcp1, IS26, ISCRI), and to a lesser extent phage-related and repeated ReAV elements. Further successful spread of ESBL and AmpC genes occurs by different lateral genetic transfer processes involving insertion sequences, transposons, Class 1 integrons and overall, the transferable plasmids in which they are located (Arduino et al., 2002; Eckert et al., 2006; Girlich et al., 2011; Oliver et al., 2005; Poirel et al., 2008; Toleman et al., 2006). Among animal isolates, studies focused on these genetic features have shown that, overall, these mobile elements were the same as those identified in human isolates (Carattoli, 2009; Clockaert et al., 2007; Leverstein-van Hall et al., 2011). This

¹⁰ <http://www.efsa.europa.eu/en/efsajournal/doc/2274.pdf>

observation has reinforced some of the hypotheses suggestive of direct links between the two bacterial populations.

2.4.2. Insertion sequences and transposons

Over 150 TEM β -lactamases variants have so far been identified, many of them displaying an ESBL phenotype (www.lahey.org/Studies/). All are TEM-1 or TEM-2 derivatives, and most of the structures surrounding the *bla*_{TEM} ESBL genes derive from Tn3-like structures. The Tn3 class II transposon possesses 38-bp inverted repeats and is able to transpose efficiently the *bla*_{TEM} ampicillin resistance gene marker, together with resistance to expanded-spectrum β -lactams when the TEM determinant encodes an ESBL variant.

SHV ESBLs are point mutants of either narrow-spectrum chromosomal SHV-1 or SHV-2 β -lactamases which originate from the chromosome of *K. pneumoniae*. Complete copies of insertion sequence (IS)26 at the boundaries of different chromosomal regions led to the genesis of different putative IS26-composite transposons containing *bla*_{SHV} genes. Thus it is not surprising to identify some DNA fragments originating from the *K. pneumoniae* chromosome in the immediate vicinity of the *bla*_{SHV}-like genes.

Insertion sequences *Ecp1* and *CR1* (part of the so-called *sul1*-type integron structures) are involved in the mobilization of different *bla*_{ESBL} and *bla*_{AmpC} genes by a mechanism named “one ended transposition”. This means that only one copy of the IS element is enough to mobilize right-ended located sequences by contrast to composite transposons that are made of two copies of some given IS elements bracketing the mobilized fragment. Also, by providing some strong promoter sequences, *ISEcp1* and *ISCR1* are involved in the expression of the genes they mobilize (Poirel et al., 2008; Toleman et al., 2006).

Copies of IS26 at the boundaries of different regions led to the genesis of different putative IS26-composite transposons. The high recombinational potential of all these IS26-mosaic structures contributes to the mosaicism of current mobile genetic elements (MGE) encoding antibiotic resistance and facilitates rearrangements between different genetic elements. IS26 has also been associated with the mobilization of CTX-M and AmpC genes (Ford and Avison, 2004).

Phage-related elements are associated with *bla*_{CTX-M} genes (Oliver et al., 2005) and Re1-like sequences have been identified in association with *bla*_{VEB-like} and *bla*_{PER} genes (Girlich et al., 2011).

2.4.3. Plasmids

Several major plasmid families have emerged in MDR *Enterobacteriaceae* isolated worldwide and carrying ESBL and AmpC enzymes. These enzymes have been mainly spread by plasmids of the incompatibility FII, A/C, L/M, N, K, and II groups (Antunes et al., 2010; Carattoli, 2009; Mataseje et al., 2009). IncF, IncI and IncN plasmid families are largely prevalent in commensal faecal flora of healthy animals (51,1%, 17,4% and 10,9%, respectively), regardless of resistance genes (Johnson et al., 2007b), suggesting that these plasmids are naturally occurring in commensal isolates and may occasionally acquire ESBL or AmpC genes by gene exchanges. Highly transmissible IncFII plasmids carrying *bla*_{CTX-M-15} or the IncIII carrying the *bla*_{TEM-52} are of particular interest as they are globally spread among *E. coli* populations from humans and animals (Carattoli, 2009). In contrast, IncA/C, IncL/M and IncK plasmids have not been identified in faecal flora of antibiotic-free humans and in commensals from healthy animals. Their occurrence in resistant bacteria of different origin and sources seems tightly linked to positive selection exerted by antimicrobial use, incrementing their prevalence compared to that observed in bacterial populations that have not been pre-selected for antimicrobial resistance. These plasmids can be considered as “epidemic associated with the emergence of specific ESBL or AmpC gene variants. For instance, the IncA/C plasmids were

associated with the emergence of *bla*_{CMY-2} in the USA and UK (Hopkins et al., 2006; Randall et al., 2011), the IncL/M plasmids were associated to the spread of *bla*_{CTX-M-3} in European Eastern countries (Golebiewski et al., 2007), while IncK plasmids carrying *bla*_{CTX-M-14} have become diffused in Spain and UK (Cottell et al., 2011; Liebana et al., 2006; Valverde et al., 2009).

The knowledge that some plasmid types are prevalent in resistant populations is useful for tracing their global spread among enterobacterial populations from humans, animals and the environment. Further details of plasmid incompatibility groups and the methods for their identification are provided in Section 4.4 below.

A summary of this information is presented in Table 5.

Table 4: Clones associated with the spread of genes encoding ESBL and AmpC resistance

Species	Clones	ESBL/AmpC genes	Animals/Food	Humans ^a	Spread ^b	Reference
<i>E. coli</i>	B2-O25b:H4-ST131 ^c	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CTX-M-15} , <i>bla</i> _{CTX-M-9} , <i>bla</i> _{SHV-12} , <i>bla</i> _{CMY-2}	Poultry, swine	Yes	Global	(Cortes et al., 2010; Mora et al., 2011; Vincent et al., 2010)
	D-O25a-ST648	<i>bla</i> _{CTX-M-32}	Poultry, Swine	Yes	Global	(Cortes et al., 2010; Mora et al., 2011)
	ST69	<i>bla</i> _{CTX-M-14}	YES	Yes	Global	(Cortes et al., 2010)
	ST156	<i>bla</i> _{CTX-M-15}	Poultry, gulls	No	Portugal, UK	(Bonnedahl et al., 2010; Randall et al., 2011; Simoes et al., 2010)
	ST57, ST371	<i>bla</i> _{ESBL}	Poultry	No	Portugal, Germany, UK	(Randall et al., 2011)
Verocytotoxin-producing <i>E. coli</i> (VTEC)	O26	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{CTX-M-18} , <i>bla</i> _{TEM-52}	Food-borne	Yes	Belgium	(Buvens et al., 2010)
	O157	<i>bla</i> _{CTX-M-2}	Food-borne	Yes	Belgium	(Buvens et al., 2010)
<i>Salmonella</i>	Agona	<i>bla</i> _{TEM}	Poultry	Yes	Global	(CloECKaert et al., 2007)
	Typhimurium -DT104	<i>bla</i> _{TEM}	Poultry	Yes	Global	(CloECKaert et al., 2007)
	Infantis	<i>bla</i> _{TEM-52}	Poultry, Swine	Yes	France, Belgium, Japan	(CloECKaert et al., 2007; Dahshan et al., 2010; Yang et al., 2010)
	Virchow	<i>bla</i> _{CTX-M-2} , <i>bla</i> _{CTX-M-9}	Poultry	Yes	Spain, France	(Bertrand et al., 2006; Riano et al., 2009; Riano et al., 2006; Weill et al., 2004).
	Westhampton/Senftenberg	<i>bla</i> _{CTX-M-53}	Cockles	No	French supermarkets	(Doublet et al., 2009)
	Heidelberg	<i>bla</i> _{CMY-2}	Imported live animals, meat consumption, unpasteurized dairy products	Yes	EU, USA	(Aarestrup et al., 2004; Folster et al., 2009).
	Newport lineage II (ST45 <i>bla</i> _{CMY-2} and single locus variant ST116)		cattle, bovine, and horse meat or pets treats	Yes	EU, USA	(Harbottle et al., 2006; Pitout et al., 2003; Sangal et al., 2010). Espié and Weill, 2003; www.eurosurveillance.org/ew/2003/030703.asp#2

a Clones detected in humans

b Geographical areas in which these clones have been reported

c O25b:H4-ST131-B2 clonal group carrying additional virulence factors *ibeA* and capsule K1 is often detected in poultry farms

Table 5: Major plasmid families associated with genes encoding ESBL and AmpC resistance

Plasmid group	ESBL/AMPc genes	Species	Animals	Humans	Reference
IncFII	<i>bla</i> _{CMY-2} , <i>bla</i> _{CTX-M-1-2-3-9-14-15-24-27} , <i>bla</i> _{DHA-1} , <i>bla</i> _{SHV-2-5-12} , <i>bla</i> _{TEM-1} ,	<i>E. coli</i> , <i>Salmonella</i> , <i>E. aerogenes</i> , <i>E. cloacae</i> , <i>Klebsiella pneumoniae</i> , <i>Serratia marcescens</i> , <i>Shigella sonnei</i>	Yes	Yes	(Carattoli, 2009; Marcade et al., 2009; Mnif et al., 2010)
IncI1	<i>bla</i> _{CMY-2-7-21} , <i>bla</i> _{CTX-M-1-2-3-9-14-15-24} , <i>bla</i> _{SHV-12} , <i>bla</i> _{TEM-1-3-52} , <i>bla</i> _{VIM-1} ,	<i>E. coli</i> , <i>K. pneumoniae</i> , <i>Salmonella</i> , <i>S. sonnei</i>	Yes	Yes	(Carattoli, 2009; Cloeckart et al., 2007; Dierikx et al., 2010a; Jensen et al., 2006; Randall et al., 2011)
IncI2	<i>bla</i> _{CMY-2}	<i>Salmonella</i>	Yes	Yes	(Antunes et al., 2010; Mataseje et al., 2009)
IncK	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{CMY-2}	<i>E. coli</i> , <i>K. pneumoniae</i> , <i>Salmonella</i>	Yes	Yes	(Dierikx et al., 2010a; Liebana et al., 2006; Valverde et al., 2009)
IncA/C	<i>bla</i> _{CMY-2-4} , <i>bla</i> _{CTX-M-2-3-14-15-56} , <i>bla</i> _{SHV-2-5-12} , <i>bla</i> _{TEM-3-21-24} , <i>bla</i> _{IMP-4-8-13} , <i>bla</i> _{VIM-4} , <i>bla</i> _{VEB-1} ,	<i>E. coli</i> , <i>Salmonella</i> , <i>Citrobacter freundii</i> , <i>Citrobacter koseri</i> , <i>E. cloacae</i> , <i>Klebsiella oxytoca</i> , <i>K. pneumoniae</i> , <i>Proteus mirabilis</i> , <i>Providencia stuartii</i> , <i>Serratia marcescens</i> , <i>Edwardsiella</i> , <i>Aeromonas</i>	Yes	Yes	(Hopkins et al., 2006; McIntosh et al., 2008; Randall et al., 2011; Welch et al., 2009)
IncL/M	<i>bla</i> _{CTX-M-1-3-15-42} , <i>bla</i> _{TEM-3-10} , <i>bla</i> _{SHV-5} , <i>bla</i> _{IMP-4-8} ,	<i>E. coli</i> , <i>C. amalonaticus</i> , <i>C. freundii</i> , <i>E. aerogenes</i> , <i>E. cloacae</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>M. organii</i> , <i>P. mirabilis</i> , <i>Salmonella</i> , <i>S. flexneri</i> , <i>S. marcescens</i>	No	Yes	(Carattoli, 2009; Golebiewski et al., 2007)
IncHI2	<i>bla</i> _{CTX-M-2-3-9-14} , <i>bla</i> _{SHV-12} , <i>bla</i> _{IMP-4} , <i>bla</i> _{VIM-1} ,	<i>E. coli</i> , <i>S. enterica</i> , <i>K. pneumoniae</i> , <i>C. youngae</i> , <i>E. cloacae</i> ,	Yes	Yes	(Carattoli, 2008, 2009; Novais et al., 2006)
IncN	<i>bla</i> _{KPC-2} , <i>bla</i> _{CTX-M-1-3-15-32-40} , <i>bla</i> _{VIM-1}	<i>E. coli</i> , <i>K. ascorbata</i> , <i>K. pneumoniae</i> , <i>Salmonella</i>	Yes	Yes	(Blanc et al., 2006; Bortolaia et al., 2010b; Cavaco et al., 2008; Girlich et al., 2007; Moodley and Guardabassi, 2009; Randall et al., 2011; Zong et al., 2010)
IncQ	<i>bla</i> _{GES-1}	<i>Aeromonas</i> , <i>Enterobacteriaceae</i>	No*	Yes	(Poirel et al., 2010)

(*) Reported in fish

3. THE EPIDEMIOLOGY OF ACQUIRED RESISTANCE TO BROAD SPECTRUM CEPHALOSPORINS IN FOOD-PRODUCING ANIMALS AND FOOD.

3.1. Occurrence of ESBL and AmpC in food-producing animals and food.

3.1.1. Harmonised monitoring of resistance to third-generation cephalosporins: EFSA Community Summary Report (CSR)¹¹ and National reports.

In order to harmonise monitoring of AMR, the establishment of optimum phenotypic testing systems for sensitive, specific and rapid detection of ESBL- and AmpC-producing organisms has been recognised as a very important component of antimicrobial resistance monitoring programmes (Livermore et al., 2001).

The European Food Safety Authority (EFSA, <http://www.efsa.europa.eu>) has prepared detailed specifications on minimum requirements for harmonised monitoring of antibiotic resistance in food-producing animals to obtain comparable data across the EU. Guidelines for monitoring of AMR in *Salmonella* and *Campylobacter*¹² and also in indicator *E. coli* and enterococci¹³ are available on the EFSA website. To enable the comparison of the occurrence of resistance between different countries the EFSA guidelines request harmonization of (i) protocols on sampling strategies, (ii) the method of susceptibility testing, (iii) the antibiotics to be tested and (iv) the criteria for categorising isolates as susceptible or resistant, as well as quality control and reporting.

The EFSA guidelines state that cefotaxime is a good substrate for what are currently the most common and important ESBLs in humans in Europe, the CTX-M enzymes. This was confirmed in a recent study, in which it was concluded that for detection of ESBL- and AmpC-producing *E. coli* or *Salmonella* harbouring TEM, SHV, CTX-M, and various AmpC gene families, usage of cefotaxime, cefpodoxime or ceftriaxone with epidemiological cut-off values (ECOFFs) > 0.5, > 2 and > 0.125 mg/L, respectively, were the most efficient cephalosporins for detection of these β -lactamase gene families. Ceftazidime, ceftiofur and cefquinome were less efficient (Aarestrup et al., 2010).

In the 2007-2009 Member State (MS) reports, summarised in Tables 13 and 14 (Appendix A), the occurrence of resistance is given, where available, for cefotaxime, ceftazidime and ceftiofur. The recent implementation of EFSA's recommendations by MSs has resulted in a more frequent monitoring of resistance to third-generation cephalosporins, as based on cefotaxime susceptibility patterns, over the period 2007-2009, this is an important improvement to the EU-wide surveillance programme. Since reports cover only phenotypic monitoring, it is not possible to determine the class or exact type of β -lactamase enzyme which is likely to confer the resistance detected to 3rd-generation cephalosporins. Since in the tables per country and animal species, data are included if as a minimum 10 isolates are tested, the results need to be interpreted with care.

¹¹ EFSA, ECDC; European Community Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals, and food in the European Union in 2009

¹² EFSA, 2007. Report of the Task Force of Zoonoses Data Collection including a proposal for a harmonized monitoring scheme of antimicrobial resistance in *Salmonella* in fowl (*Gallus gallus*), turkeys, and pigs and *Campylobacter jejuni* and *C. coli* in broilers, *the EFSA Journal* (2007), 96,1-46. Available at: <http://www.efsa.europa.eu/en/scdocs/scdoc/96r.htm>

¹³ EFSA, 2008. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. *The EFSA Journal* (2008) 141, 1-44. Available at: <http://www.efsa.europa.eu/en/scdocs/scdoc/141r.htm>

Poultry

Many countries did not detect cefotaxime resistance among the isolates of *Salmonella* from poultry tested. The occurrence of cefotaxime resistance among countries reporting such resistance was low for 2009, at 1-3% for France, Italy, Spain and the Czech Republic and moderate for the Netherlands at 12%. These figures can be compared with the figures obtained in 2008, when the Netherlands and Italy reported an occurrence of cefotaxime resistance in *Salmonella* of 13% and 12% respectively, whereas in the remaining countries which detected resistance, occurrences varied between 0% and 1%. In the Netherlands the most predominant serovar in which cefotaxime resistance was observed was *S. Paratyphi B* variant Java, which is common in Dutch broiler farms. It should not be confused with other serovar *Paratyphi B* variant Java isolates exhibiting multiple resistances and associated with tropical fish, which appear highly pathogenic for humans (Threlfall et al., 2005). Other cefotaxime-resistant serovars found in Dutch poultry or poultry meat products include Virchow, Hadar, Infantis, Kottbus, Mbandaka, Agona, Cubana, Rissen, Senftenberg and Heidelberg (MARAN 2008)¹⁴. In poultry raw meat products, Belgium reported a high occurrence of resistance (26%) in 2008¹⁵, while the Netherlands reported a moderate occurrence of resistance (18% for *Salmonella* and 15% for *E. coli*) (MARAN 2008). From 2003 to 2008, a dramatic increase in occurrence of cefotaxime-resistant *E. coli* and *Salmonella* isolates from Dutch broiler chickens has been observed (3% to 18% for *E. coli* randomly isolated from faecal samples using non-selective plates) (MARAN-2009)¹⁶.

In Sweden, since 2008 healthy food animals are screened for ESBL- or AmpC-producing *E. coli* by culture of intestinal content on media supplemented with cefotaxime. In 2010, ESBL and/or AmpC-producing *E. coli* were found in 34% of the samples from broilers (SVARM, 2010)¹⁷.

In 2009 eight MS and two non-MS tested more than 10 isolates of *E. coli* for cefotaxime resistance in poultry (*Gallus gallus*) isolates. Resistance to cefotaxime for the eight reporting MSs was 8.5%, although the occurrence of resistance reported by individual MSs ranged from 0% to 26%. The resistance ranged from low level of around 3% (Austria, Germany and France) to high level of 26% (Spain) with the Netherlands and Poland reporting moderate proportions of 18% and 11%, respectively.

Cattle

Cefotaxime resistance in *Salmonella* isolates from cattle in 2009 was only reported by Germany, where the occurrence of resistance was 1%. Considering all reporting MS, the overall occurrence of cefotaxime resistance was less than 1%. In *E. coli* the prevalence of resistance to cefotaxime was 1.6% for all reporting MSs (10 MSs) and ranged from 0% to 6.5% (Hungary) for individual MSs.

Pigs

In 2009, cefotaxime resistance in *Salmonella* was only reported in Germany and Spain. In these countries the levels were low (2% and 1% respectively). In *E. coli* the prevalence of resistance to cefotaxime was low (2.3%) for all reporting MS and ranged from 0% to 3.8% for individual MS. Cefotaxime resistance was not detected in isolates of animal origin from Denmark, Estonia or Switzerland.

¹⁴ MARAN-2008 Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2008. http://www.cvi.wur.nl/NR/rdonlyres/A906A4C0-A458-423E-B932-28F222385988/105836/MARAN_2008.pdf

¹⁵ The Community Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2008. <http://www.efsa.europa.eu/en/efsajournal/doc/1658.pdf>

¹⁶ MARAN-2009 Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2009. http://www.lei.wur.nl/NR/rdonlyres/4ED137F2-5A0D-4449-B84E-61A3A8AC4A42/135753/MARAN_2009.pdf

¹⁷ SVARM 2010, Swedish Veterinary Antimicrobial Resistance Monitoring. The National Veterinary Institute (SVA), Uppsala, Sweden, 2011. www.sva.se.

In Denmark (DANMAP, 2009)¹⁸, a low occurrence (0.7%-3.3%) of cefotaxime-resistant *E. coli* from poultry meat, pork and beef meat of domestic origin was found, while a high level of resistance (36%) was observed among imported broiler meat.

3.1.2. Data from published studies targeted to detect ESBL and/or AmpC

A large number of studies have been performed in different European countries as well as in countries in other continents, focused on the analysis of the prevalence of ESBL- and AmpC-carrying *Enterobacteriaceae* (especially *E. coli* and non-typhoidal *Salmonella*) in faecal samples from food-producing animals or in food derived from them. Reviews about this topic have been published by different authors (Carattoli, 2008; Li et al., 2007; Smet et al., 2009; Torres and Zarazaga, 2007). Tables 6 and 7 (and Tables 16 and 17 of Appendix B) show most of the reports published about the detection of ESBL- or AmpC-carrying *Enterobacteriaceae* in food-producing animals or foods derived from such animals.

The methodology used in the different studies to detect ESBL-carrying bacteria in food-producing animals or in derived foods is heterogeneous and comparisons are therefore difficult. Some studies have reported the percentage of ESBL-carrying *E. coli* obtained in media not supplemented with antibiotics; others have analysed the carriage of ESBL-positive isolates in faecal samples of food-producing animals or food samples using media supplemented with broad-spectrum cephalosporins (cefotaxime +/- ceftazidime). Additionally some studies have been performed at slaughterhouse level, and others at farm level.

3.1.2.1. Prevalence of ESBL-producing *E. coli* and *Salmonella* in food-producing animals and food

The first report of the detection of ESBLs in food-producing animals was performed in *E. coli* isolates recovered during 2000-2001 from faecal samples of healthy chickens at the slaughterhouse level obtained as part of the Spanish antimicrobial resistance surveillance programme (Brinas et al., 2003b). In that study, 120 *E. coli* isolates were obtained from faecal samples in non-antibiotic supplemented media and CTX-M-14 and SHV-12 were detected in 1.6% of them. An increase in the percentage of ESBL-producing *E. coli* isolates among commensal faecal isolates (8 of 158 isolates, 5%) was found in a second screening performed in 2003, with a higher diversity of ESBLs detected (CTX-M-9, CTX-M-14 and SHV-12) (Brinas et al., 2005).

Prevalence data from other countries are more difficult to compare, because methodologies used have varied. Percentages of samples of food-producing animals or food in which ESBL-carrying *E. coli* isolates were detected varied from 0.2% to 40.1%, according to the country and the methodology used [(Smet et al., 2009); Table 6]. Occurrences in the range of 10-40% were found when healthy poultry or pigs were analysed for the presence of ESBL-positive *E. coli* isolates in Portugal (Costa et al., 2009; Goncalves et al., 2010; Machado et al., 2008), Netherlands (Dierikx et al., 2010a), France (Girlich et al., 2007), and slightly lower percentages were identified in Czech Republic (Kolar et al., 2010). Moreover, ESBL-carrying *E. coli* isolates were found in most of pig and poultry farms tested in Spain (Blanc et al., 2006), and also in five poultry farms tested in Italy (Bortolaia et al., 2010b). ESBL-producing *E. coli* isolates were also detected in two of four flocks of poultry in farms with no previous antibiotic use in Denmark (Bortolaia et al., 2010a). In a study in Belgium (Smet et al., 2008), 45% of 295 ceftiofur-resistant *E. coli* isolates obtained from 489 cloacal samples collected at five different Belgian broiler farms were ESBL producers.

The prevalence of ESBLs among *Salmonella* isolates of food-producing animals or derived food is much lower than that among *E. coli*. In a study in Germany of 22679 *Salmonella* isolates of the

¹⁸ DANMAP 2009 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. www.danmap.org.

National *Salmonella* Laboratory Collection, sixteen isolates produced CTX-M enzymes (1 CTX-M-15; 15 CTX-M-1) and four isolates produced the TEM-52 or TEM-20 enzymes located on IncII plasmids. The ESBL-producers represented only 0.09% of the total number of isolates tested (Rodríguez et al., 2009). In another study in Spain among isolates obtained through the Spanish Veterinary-Antimicrobial-Resistance-Surveillance Network (VAV), 4 of 556 (0.7%) *Salmonella* organisms obtained from faecal samples of healthy food animals (pigs and poultry) at the slaughterhouse level were ESBL producers, representing 0.2% in the case of pigs and 2.5% of all isolates in the case of poultry (Riano et al., 2006). ESBL-producing *Salmonella* prevalences of 0.5-0.6% were detected in Italy, Korea and Japan (Chiaretto et al., 2008; Hur et al., 2010; Matsumoto et al., 2007).

In a pilot study in 2010 on 26 broilers production farms in the Netherlands, the prevalence of ESBL and/or AmpC-producing *E. coli* was determined. Faecal samples were collected from the cloaca from 25 to 41 animals per farm. All farms were found positive. In 85% of the farms, 80% or more of the chickens examined were positive for ESBL- and/or AmpC-producing *E. coli* (Dierikx et al., 2010b).

Imported meat could be an important source of ESBL/AmpC- *E. coli* producers. A recent survey of frozen chicken meat imported into Sweden showed that 92% of meat from South America had ESBL/AmpC *E. coli* producers, compared with 19% for meat from elsewhere in Europe. Moreover, *E. coli* from South American meat were resistant to a wider range of antibiotics and had a higher diversity of ESBL/AmpC genes (Borjesson et al., 2011). Other studies conducted in chicken meat imported into Denmark and United Kingdom from the same geographical region indicated an occurrence of ESBL/AmpC *E. coli* producers in 30-36% of samples analyzed (Bergenholtz et al., 2009; Dhanji et al., 2010).

3.1.2.2. ESBL-subtypes detected in *E. coli* and *Salmonella* from food-producing animals or food

The following ESBL subtypes have been detected in food-producing animals and food in European countries (table 6):

i) CTX-M class: CTX-M-1 (Spain, Portugal, France, Belgium, Denmark, Italy, Netherlands, UK, Germany and Czech Republic), CTX-M-2 (Belgium, Denmark, Netherlands, UK, Ireland), CTX-M-9 (Spain, France, Denmark, UK), CTX-M-14 (Spain, Portugal, France, Belgium, Denmark and UK), CTX-M-15 (France, Belgium, UK, Germany, Denmark), CTX-M-32 (Spain, Portugal, Italy and Greece), and other CTX-M- variants (CTX-M-53 in France, CTX-M-3, -8, -17/18, -20 in UK);

ii) SHV class: SHV-2 (Spain, Portugal, and Netherlands), SHV-5 (Spain), and SHV-12 (Spain, France, Italy, Netherlands, Denmark, Ireland and Czech Republic).

iii) TEM-class: TEM-52 (Spain, Portugal, Belgium, Netherlands, Ireland, Denmark and Germany); TEM-20 (Netherlands, Ireland and Germany), TEM-106 (Belgium) and TEM-126 (France).

According to these results, CTX-M-1 appears to be well disseminated in food-producing animals and food in most of the European countries for which reports exist. On the other hand, CTX-M-14 and CTX-M-32 appear more associated with food-producing animals or food in Mediterranean and Southern European countries (Blanc et al., 2006; Bortolaia et al., 2010a; Bortolaia et al., 2010b; Brinas et al., 2005; Brinas et al., 2003b; Carneiro et al., 2010; Costa et al., 2009; Madec et al., 2008; Politi et al., 2005), although CTX-M-14 was also found in food-producing animals in UK and Belgium (Hopkins et al., 2006; Hunter et al., 2010; Smet et al., 2008; Warren et al., 2008). CTX-M-2 was observed in Central and Northern European countries, including UK and Ireland (Bertrand et al., 2006; Bortolaia et al., 2010a; Boyle et al., 2010; Cloeckeaert et al., 2007; Dhanji et al., 2010; Dierikx et al., 2010a; Hasman et al., 2005; Hopkins et al., 2006; Liebana et al., 2006; Morris et al., 2009; Randall et al., 2011; Smet et al., 2008; Warren et al., 2008). CTX-M-15, the ESBL considered to have spread in a pandemic fashion in humans, was only detected incidentally in food-producing animals or

food and in only in a few countries in *E. coli* (isolated in France from diseased cattle, in Belgium from healthy poultry, and in UK in broiler chickens and turkey) and in *Salmonella* isolates (Germany, one *S. Typhimurium* from horse origin) (Madec et al., 2008; Randall et al., 2011; Rodriguez et al., 2009; Smet et al., 2008). ESBLs of the SHV class were frequently detected throughout the EU, especially SHV-12 and SHV-2 (Blanc et al., 2006; Bortolaia et al., 2010b; Boyle et al., 2010; Brinas et al., 2003a; Brinas et al., 2003b; Chiaretto et al., 2008; Cortes et al., 2010; Costa et al., 2009; Dierikx et al., 2010a; Doi et al., 2009; Escudero et al., 2010; Goncalves et al., 2010; Hasman et al., 2005; Hopkins et al., 2006; Kolar et al., 2010; Machado et al., 2008; Madec et al., 2008; Morris et al., 2009; Riano et al., 2006). In relation to the ESBLs of the TEM class, the most frequently detected throughout the EU was TEM-52 (Blanc et al., 2006; Brinas et al., 2005; Carneiro et al., 2010; Cloeckart et al., 2007; Costa et al., 2009; Dierikx et al., 2010a; Jensen et al., 2006; Machado et al., 2008; Morris et al., 2009; Randall et al., 2011; Rodriguez et al., 2009; Smet et al., 2008). To a lesser extent TEM-20 was detected in countries as Netherlands, Ireland and Germany (Boyle et al., 2010; Dierikx et al., 2010a; Hasman et al., 2005; Hopkins et al., 2006; Rodriguez et al., 2009).

The ESBLs detected in food-producing animals or food in other non-European countries were: China (CTX-M-3, -13, -14, -15, -22, -24, -55, -64, -65; SHV-2, -12; TEM-52), Japan (CTX-M-2 and -14), Korea (CTX-M-15), USA (CTX-M-1, -29, SHV-12), Tunisia (CTX-M-1, CTX-M-8, CTX-M-14; SHV-5; TEM-20) and Senegal (SHV-12) (Ben Slama et al., 2010; Cardinale et al., 2001; Doi et al., 2009; Duan et al., 2006; Jouini et al., 2007; Kojima et al., 2005; Li et al., 2010a; Li et al., 2010b; Lim et al., 2009; Matsumoto et al., 2007; Rayamajhi et al., 2008; Shiraki et al., 2004; Tian et al., 2008; Wittum et al., 2010). These data show that mainly in Asian countries a higher diversity of CTX-M-variants was observed. The presence of CTX-M-15 in *E. coli* of healthy and sick poultry and pigs in China and Korea may reflect a different epidemiology of ESBLs in these countries (Li et al., 2010a; Li et al., 2010b; Lim et al., 2009; Tian et al., 2008).

3.1.2.3. Prevalence and types of AmpC in *E. coli* and *Salmonella* from food-producing animals or food

The following ESBL subtypes have been detected in food-producing animals and food in European countries (table 7):

Poultry origin

In samples of poultry origin, the percentage of AmpC-carrying *E. coli* isolates ranged from 0.8 to 3.3% in different European (Spain, Belgium, Netherlands, Czech Republic) (Brinas et al., 2005; Brinas et al., 2003b; Dierikx et al., 2010a; Smet et al., 2008) and non-European countries (Japan, Taiwan and China) (Kojima et al., 2005; Li et al., 2010b; Yan et al., 2004). For *Salmonella*, percentages of detection in the range of 0.1-10% were identified for poultry or derived food in Ireland, USA, Canada or Japan (Allen and Poppe, 2002; Boyle et al., 2010; Taguchi et al., 2006; Zhao et al., 2008).

Cattle origin

In samples of cattle origin, AmpC β -lactamases were detected in 0.01% of 8426 *Salmonella* isolates in a Canadian study (Allen and Poppe, 2002). In samples of pig origin, the percentages of AmpC-carrying *Salmonella* or *E. coli* isolates ranged from 2.4 to 23% in Canada, Taiwan and México (Kozak et al., 2009; Yan et al., 2004; Zaidi et al., 2007).

The type of AmpC β -lactamase detected in food-producing animals or derived food has been almost always the CMY-2 variant, and the DHA-1 variant was also identified in *K. pneumoniae* of pig origin in Korea (Rayamajhi et al., 2008). ACC-1 has also been reported to occur on unidentified plasmids in *S. Braenderup* (Dierikx et al., 2010a; Hasman et al., 2005).

Table 6: Type of ESBLs detected in food-producing animals or food in studies performed in European and non-European countries

Country	Source	Microorganism	ESBL CTX-M				ESBL TEM	ESBL SHV	References
			G-1	G-2	G-9	Other group			
Spain	H ^a -poultry	<i>E. coli</i>	-1, -32		-9, -14		-52	-2, -5, -12	(Blanc et al., 2006; Brinas et al., 2005; Brinas et al., 2003b; Cortes et al., 2010; Doi et al., 2009)
	H-poultry	<i>Salmonella</i>			-9				(Riano et al., 2006)
	Pigs	<i>E. coli</i>	-1		-9, -14			-12, -5	(Escudero et al., 2010)
	Pigs	<i>Salmonella</i>						-12	(Riano et al., 2006)
	Rabbits	<i>E. coli</i>			-9, -14				(Blanc et al., 2006)
	Food	<i>E. coli</i>	G-1		G-9			-12	(Doi et al., 2009)
	S _b -animals	<i>E. coli</i>	-1, -32		-9, -14		-52	-12	(Brinas et al., 2005; Brinas et al., 2003a)
Portugal	H-poultry/meat	<i>E. coli</i>	-1, -32		-14		-52	-2	(Costa et al., 2009; Machado et al., 2008)
	Pigs	<i>E. coli</i> <i>C. freundii</i>	-1					-12	(Machado et al., 2008) (Goncalves et al., 2010)
	Ostrich	<i>E. coli</i>			-14		-52		(Carneiro et al., 2010)
France	H-poultry	<i>E. coli</i>	-1						(Girlich et al., 2007)
	H-poultry/meat	<i>Salmonella</i>	-1		-9				(CloECKaert et al., 2010; Weill et al., 2004)
	H-Cattle	<i>E. coli</i>	-1		-14			-12	(Madec et al., 2008)
	Cockles	<i>Salmonella</i>	-53						(Doublet et al., 2009)
	S-Cattle	<i>E. coli</i>	-1, -15		-14		-126		(Madec et al., 2008; Meunier et al., 2006)
	S-poultry/pigs	<i>E. coli</i>	-1						(Meunier et al., 2006)
UK	Poultry/meat	<i>E. coli</i>	-1, -3, -15	-2 G-2	-14, -55	-8 G-8	-52		(Dhanji et al., 2010; Liebana et al., 2006; Randall et al., 2011; Warren et al., 2008)
	S-Cattle/bovines	<i>E. coli</i>	-1, -3, -15, -32	-20	-14				(Hopkins et al., 2006; Hunter et al., 2010; Kirchner et al., 2011)
Ireland	Poultry/cattle	<i>Salmonella</i>		-2			-52, -20	-12	(Boyle et al., 2010; Hopkins et al., 2006; Morris et al., 2009)
Belgium	H-poultry	<i>E. coli</i>	-1, -15	-2	-14		-52, -106		(Smet et al., 2008)
	H-poultry/meat	<i>Salmonella</i>		-2			-52		(Bertrand et al., 2006; CloECKaert et al., 2007)
Netherlands	H-poultry	<i>E. coli</i>	-1	-2			-52	-2	(Dierikx et al., 2010a)
	H-poultry/meat	<i>Salmonella</i>	-1	-2			-52, -20	-2, -12	(Dierikx et al., 2010a; Hasman et al., 2005)
Italy	H-poultry	<i>E. coli</i>	-1, -32					-12	(Bortolaia et al., 2010b)
	H-poultry	<i>Salmonella</i>						-12	(Chiaretto et al., 2008)
Denmark	H-poultry	<i>E. coli</i>	G-1	G-2	G-9				(Bortolaia et al., 2010a)

Country	Source	Microorganism	ESBL CTX-M				ESBL TEM	ESBL SHV	References
			G-1	G- 2	G-9	Other group			
	Cattle	<i>E. coli</i>					-52		(Jensen et al., 2006)
	Quail meat	<i>Salmonella</i>			-9				(Aarestrup et al., 2005)
	Pigs	<i>E. coli</i>	-1						(Aarestrup et al., 2006; Wu et al., 2008)
	Poultry meat	<i>E. coli</i>	-1						(Bergenholtz et al., 2009)
Czech Republic	H-poultry	<i>E. coli</i>	-1					-12	(Kolar et al., 2010)
Germany	Livestock animals and food	<i>Salmonella</i>	-1, -15				-52, -20		(Rodriguez et al., 2009)
Greece	Poultry meat	<i>Salmonella</i>	-32						(Politi et al., 2005)
China	H/S-poultry	<i>E. coli</i>	-3, -15		-14, -55, -65, -24	-64*			(Li et al., 2010a; Li et al., 2010b)
	Pigs	<i>E. coli</i>	-3, -15, -22		-14			-2	(Duan et al., 2006; Tian et al., 2008)
	Pigeons	<i>E. coli</i>			-14				(Duan et al., 2006)
Japan	H-poultry	<i>E. coli</i>		-2	-14				(Kojima et al., 2005)
	Poultry meat	<i>Salmonella</i>			-14				(Matsumoto et al., 2007)
	Cattle	<i>Coli</i>		-2					(Shiraki et al., 2004)
Korea	Poultry/meat	<i>Salmonella</i>	CTX-M						(Hur et al., 2010)
	Pigs/ cows	<i>E. coli/ Klebsiella</i>	-15		-14		-20, -52	-28	(Lim et al., 2009; Rayamajhi et al., 2008)
USA	S/H-cattle	<i>Coli</i>	-1, -29						(Wittum et al., 2010)
	Food	<i>E. coli</i>	G-1						(Doi et al., 2009)
Tunisia	Poultry meat	<i>E. coli</i>	-1		-14	-8		-5	(Ben Slama et al., 2010; Jouini et al., 2007)
	Cattle meat	<i>E. coli</i>	-1						(Ben Slama et al., 2010; Jouini et al., 2007)
	Sheep meat	<i>E. coli</i>	-1				-20		(Ben Slama et al., 2010)
Senegal	Poultry meat	<i>Salmonella</i>						-12	(Cardinale et al., 2001)

* CTX-M-64: hybrid between CTX-M-15 and CTX-M-14. ^aHealthy, ^bSick

Table 7: Type of plasmidic AMPc detected in food-producing animals or food in studies performed in European and non-European countries

Country	Source	Microorganism	CMY-2	Other plasmidic AmpC	References
Spain	H ^a -poultry	<i>E. coli</i>	+		(Blanc et al., 2006; Brinas et al., 2005; Brinas et al., 2003b)
	Rabbit	<i>E. coli</i>	+		(Blanc et al., 2006)
	Sick-animals	<i>E. coli</i>	+		(Brinas et al., 2005; Brinas et al., 2003a)
Belgium	H-poultry	<i>E. coli</i>	+		(Smet et al., 2008)
Netherlands	H-poultry	<i>E. coli</i>	+	AAC-1	(Dierikx et al., 2010a; Hasman et al., 2005)
Czech Republic	H-poultry	<i>E. coli</i>	+		(Kolar et al., 2010)
Germany	Various livestock and food samples	<i>Salmonella</i>	+		(Rodriguez et al., 2009)
Ireland	H-poultry	<i>Salmonella</i>	+		(Boyle et al., 2010)
Denmark	Imported poultry meat	<i>E. coli</i>	+		(Bergenholtz et al., 2009)
Japan	H-poultry	<i>E. coli</i>	+		(Kojima et al., 2005)
	Retail meat poultry	<i>Salmonella</i>	+		(Taguchi et al., 2006)
China	H-poultry	<i>E. coli</i>	+		(Li et al., 2010b)
Taiwan	H-poultry	<i>E. coli</i>	+		(Yan et al., 2004)
	Food of chicken and pork origin				
	Pigs	<i>E. coli</i>	+		(Yan et al., 2004)
Korea	S ^b -pigs	<i>K. pneumoniae</i>	+	DHA-1	(Rayamajhi et al., 2008)
USA	Retail meat poultry	<i>Salmonella</i>	+		(Zhao et al., 2008)
	Cattle	<i>E. coli</i>	+		(Donaldson et al., 2006)
	Various food samples	<i>E. coli</i>			(Doi et al., 2009)
	Various livestock and food samples	<i>E. coli</i> and <i>Salmonella</i>	+	CMY-2-like	(Heider et al., 2009)
	Sick-Cattle	<i>Salmonella</i>	+	AmpC	(Frye et al., 2008; Gupta et al., 2003)
	Sick-Cattle and pigs	<i>E. coli</i> , <i>Salmonella</i>	+		(Winokur et al., 2000)
Canada	H-Poultry, cattle	<i>Salmonella</i>	+		(Allen and Poppe, 2002)
	Pigs	<i>E. coli</i>	+		(Kozak et al., 2009)
México	Pigs	<i>Salmonella</i>	+		(Zaidi et al., 2007)

^aHealthy, ^bSick

3.2. Transmission of ESBL/AmpC-resistant bacterial strains and/or resistance genes to humans by consumption or handling of contaminated food.

In order to discuss and evaluate transmission of ESBL/AmpC-producing *Enterobacteriaceae* from consumption or handling of food, it is necessary to examine the many possible reservoirs and reasons for existence of such resistant bacteria within the ecosystem.

The emergence and spread of ESBL/AmpC-producing microorganisms in the environment is a complex process and has mainly been due to point mutations that arise and are selected (Knox, 1995), and to the spread of resistant mobile genetic elements through the environment, especially plasmids between bacteria (Bonnet, 2004; Carattoli, 2001, 2008). The role of antibiotic selection pressure is also important, from agents used in both animal husbandry and human medicine (Blazquez et al., 2000; Jorgensen et al., 2007; Medeiros, 1997; Rice et al., 1996) in the selection of resistance in commensal bacteria in the gut of food animals (van den Bogaard et al., 2001). Resistance can also emerge in bacteria in soil as a result of pressure from antibiotic residues resulting from the use of antimicrobials in animal husbandry; such residues have been found in all components of the ecosystem, including the farm environment (Sayah et al., 2005), with recent reports of ESBL-producing *E. coli* found along with antibiotic residues in animal manure (Furtula et al., 2010).

Evidence for the transmission of MDR *Enterobacteriaceae* resulting from food consumption to humans has been documented and is well established for resistant *Salmonella* and *E. coli* (Gerner-Smidt and Whichard, 2009). Multiple reports exist, showing the direct association of food with outbreaks of multidrug resistant *Salmonella* in humans, some of which are also connected to outbreaks in the source-animals. Outbreaks associated with raw milk (Bezanson et al., 1983; Cody et al., 1999; Villar et al., 1999), and raw, ground beef (Dechet et al., 2006), pork (Molbak et al., 1999), and breakdowns in pasteurization (Walker et al., 2000) have been reported.

With regard to the possibility of bacteria that produce ESBLs and/or AmpC being transmitted to humans, there are reports that provide circumstantial evidence that ESBL-producing *E. coli* can be associated with its transmission from food to humans (Lavilla et al., 2008), studies whose findings suggest transmission of *E. coli* that produce ESBL, from poultry to humans (Leverstein-van Hall et al., 2011), but also evidence of direct association of transmission of *Salmonella* resistant to 3rd-generation cephalosporins during an outbreak in humans (Fey et al., 2000; Zansky et al., 2002).

In a recent study (Leverstein-van Hall et al., 2011), 35% of human clinical ESBL-producing *E. coli* contained ESBL-genes that were also detected in *E. coli* from poultry origin. 19 % human clinical ESBL-producing *E. coli* contained IncI1 plasmids (harbouring ESBLs) genetically indistinguishable by plasmid MLST (pMLST) from those found in *E. coli* of poultry origin. 39 % of ESBL-producing *E. coli* from poultry origin belonged to genotypes (MLST types) also present in human samples. These findings are suggestive of transmission of ESBL genes, plasmids and clones from poultry to humans most likely through the food chain.

As described in chapter 2 the epidemiology of ESBL/AmpC-producing bacteria and/or the genes encoding for such enzymes is complex. The genes themselves are transferable within bacteria by insertion sequences and transposons and between bacteria by plasmid transfer. This process has resulted in the emergence and spread of epidemic plasmids and clones that have been associated with transmission of ESBL/AmpC between food-producing animals/foods and humans. The demonstration of identical genes, clones or plasmids in hospitals and farms is not sufficient to draw conclusions on the epidemiological relatedness in the different environments. For such conclusions to be valid, epidemiological relatedness should be based on the demonstration of genetic relatedness of genes and/or plasmids and/or clones in combination with quantitative information on their prevalence in food-producing animals, foods, the community and health care.

For food-borne pathogens such as *Salmonella* and enteropathogenic strains of *E. coli*, transmission of ESBL/AmpC-producing variants can be expected to follow the same route as for susceptible bacteria. There are still only a few reports in Europe where such spread has been reliably documented. Other reports describe the same clone of bacteria in humans and food although it might not be clearly stated that the food caused the disease. The pattern gets even more complicated in cases where the same genes are found in food and human patients but detected in different kinds of bacteria. In such instances it is of relevance to map the occurrence in humans of resistant commensal bacteria with a similar gene pattern as those in food as these genes might, at a later stage, transfer to human pathogens. Food could thereby be an indirect cause of resistant infections in humans, although the pathogen itself may not be food-borne. Due to possible lag time between human colonisation with the commensal and transfer of genes to a pathogen such indirect links between occurrence in food and disease in humans would be very difficult to prove.

In summary, there are few studies that provide clear evidence of direct transmission of ESBL- and/or AmpC-producing *E. coli* isolates from food-producing animals or food to humans. Nevertheless, data do exist about common clones of ESBL- and/or AmpC-producing *E. coli* isolates in humans and food-producing animals and foods, thus providing indirect evidence about this transmission.

3.2.1. Clonal transmission of resistance

Expansion of *E. coli* clones (producing ESBL) and *Salmonella* (producing ESBL or AmpC) with zoonotic potential have been reported. Recent studies have showed that *E. coli* isolates from poultry and pig farms differ with respect to ESBL and CMY-2 enzymes, phylogenetic group, virulence genes, and serotype (Cortes et al., 2010). It has been suggested that *E. coli* isolates from poultry are genetically-related to human pathogenic *E. coli*. In a study comparing genetic similarities of *E. coli* derived from humans and poultry, antibiotic-resistant *E. coli* isolates from both reservoirs were more frequently genetically related than antibiotic-susceptible isolates (Johnson et al., 2007a; Johnson et al., 2007b; Vincent et al., 2010).

Escherichia coli

E. coli O25b:H4-ST131 is a globally-spread, highly virulent pathogen identified among fluoroquinolone-resistant isolates causing human urinary tract infections (Johnson et al., 2009). This clone is able to acquire different plasmids carrying ESBL genes and it has driven the pandemic spread of *bla*_{CTX-M-15} among hospitalised patients and community-based individuals (Coque et al., 2008b; Livermore et al., 2007; Peirano et al., 2010; Woodford et al., 2004). *E. coli* O25b:H4-ST131, sometimes associated with ESBL production, has recently emerged among avian isolates (retail chicken and sick animals) (Mora et al., 2010; Peirano et al., 2010; Vincent et al., 2010). In Canada, Vincent et al. recovered such isolates, although lacking ESBL genes, from 0.4% of 250 retail chickens (Vincent et al., 2010). Recent studies from Spain have reported the emergence among poultry of a CTX-M-9-producing variant of the *E. coli* O25b:H4-ST131 clonal group isolates containing additional virulence factors, *ibeA* and capsule K1 (Cortes et al., 2010; Mora et al., 2010). Another widespread extra-intestinal *E. coli* clone (O25a-ST648-D) has been identified in poultry farms linked to CTX-M-32 production (Cortes et al., 2010). Both O25b:H4-ST131 and O25a-ST648-D strains from poultry were indistinguishable from those recovered from human infections, highlighting the zoonotic risk of these *E. coli* lineages (Mora et al., 2010; Vincent et al., 2010). Some other extra-intestinal *E. coli* clones have been detected in both human and poultry isolates (Johnson et al., 2007a; Vincent et al., 2010). Although these strains did not contain *bla*_{ESBL} or *bla*_{AmpC} genes, the possibility that they might acquire plasmids carrying these genes, as has been reported for other widespread *E. coli* clones (Cortes et al., 2010; Vincent et al., 2010), should be considered. In addition, *E. coli* ST57 and 371 producing ESBLs have been recovered from chickens in UK, Germany and Canada (Randall et al., 2011) and ESBL-producing, verocytotoxin-producing *E. coli* (VTEC) isolates of serogroups O26 (CTX-M-3, CTX-M-18, TEM-52) and O157 (CTX-M-2) have been described (Buvens et al., 2010).

CTX-M-15-producing *E. coli* ST156 in gulls sampled in southern France, chickens and turkeys in Portugal and the UK has recently been reported (Bonnedahl et al., 2010; Randall et al., 2011; Simoes et al., 2010). CMY producers of major clonal complexes have been reported among poultry, although transmission to humans has not been demonstrated (Cortes et al., 2010).

Salmonella

Antimicrobial-resistant *Salmonella* clones are often associated with the spread of ESBL and AmpC genes among humans and/or food-borne animals. Some examples are the dissemination of CTX-M-2-producing *S. Virchow* isolates with reduced susceptibility to ciprofloxacin in Belgium and France (Bertrand et al., 2006), the spread of the related serovars Westhampton and Senftenberg producing CTX-M-53 among living cockles in French supermarkets (Doublet et al., 2009), the detection of CTX-M-9 *S. Virchow* producers from human and poultry origins with pulsed-field gel electrophoresis (PFGE)-indistinguishable patterns (Riano et al., 2009), or the spread of MDR *S. Infantis* carrying IncI-*bla*_{TEM-52} in Belgium and France (Cloekaert et al., 2007). The spread of MDR clones *Salmonella* serovars. Heidelberg and Newport producing CMY-2, and also of *S. Typhimurium* have similarly been documented (Arlet et al., 2006). Outbreaks of CMY-2 producers due to clonal expansion of *S. Heidelberg* caused by imported live animals, meat consumption and unpasteurized dairy products were described in the EU and the US (Aarestrup et al., 2004; Folster et al., 2009; Zhao et al., 2008). Human infections by *S. Newport* lineage II comprises most of MDR-AmpC clones of this serotype, which are linked to ST45 (and single locus variant ST116) and associated with consumption of cattle, bovine, and horse meat or pets treats (Espie and Weill, 2003; Harbottle et al., 2006; Pitout et al., 2003; Sangal et al., 2010). In 1984, a strain of *S. Newport* with resistance to cephalosporins originating in cattle in the USA, was traced through the food chain to humans (Holmberg et al., 1984). In a US FoodNet case-control study of sporadic multiple-resistant *S. Newport* infections, (Varma et al., 2006) concluded that patients were more likely to have consumed uncooked ground beef or runny scrambled eggs or omelettes prepared in the home.

Salmonella clones belonging to serovars Agona with SGI-1-A, or Typhimurium DT104 carrying SGI-1 have been linked to TEM-52 production (Cloekaert et al., 2007). Particular clones of *S. Infantis* seem to be widespread among poultry in EU countries as France or Belgium as well Japan (Cloekaert et al., 2007; Dahshan et al., 2010; Yang et al., 2010). A recent report has also reported this serovar among pigs (Dahshan et al., 2010). There are no data about the possible transmission of particular clones between swine and humans.

Although the characterization of the clones has not been fully accomplished in many studies, it is of interest to highlight the increasing rates of *bla*_{OXA-1} gene as a sole β -lactamase gene among *E. coli* or *Salmonella* isolates, in which it confers resistance to penicillins and amoxicillin-clavulanate.

3.2.2. Plasmid analysis studies from animals, food, and humans

Epidemic plasmids belonging to incompatibility groups F, A/C, N, HI2, I1 and K carrying particular ESBL (TEM-52, CTX-M-1, -32, -9, -14) or AmpC (CMY-2) genes have been detected among farm and companion animals, food products and humans.

Plasmids of the IncF group are commonly recovered from animals, mainly associated with *E. coli* and *Salmonella* (Carattoli, 2003; Johnson et al., 2007b) although they are also linked to other enterobacterial species (Villa et al., 2010). IncF plasmids greatly differ in the content of the replicons and maintenance systems (Coque et al., 2008b; Mnif et al., 2010; Villa et al., 2010; Woodford et al., 2009), and often carry ESBLs (*bla*_{CTX-M-1-2-3-9-14-15-24-27}, *bla*_{SHV-2-5-12}) or AmpC genes (Carattoli, 2009). Highly transmissible epidemic IncFII plasmids carrying *bla*_{CTX-M-15} are of particular interest as they are globally-spread among *E. coli* and *K. pneumoniae* populations from humans and animals. The presence of multiple addiction systems seems to influence the persistence of IncF plasmids carrying *bla*_{CTX-M-15} or *bla*_{CTX-M-9} in bacterial *E. coli* backgrounds (Marcade et al., 2009; Mnif et al., 2010).

Plasmids of IncI groups (both IncI1 and IncI2) are also common among *E. coli* and *Salmonella* from animals and humans (Johnson et al., 2007b). Those of the I1 incompatibility group have been linked to a diversity of genes coding for ESBLs (TEM-52, CTX-M-1,-2, -3, -9, -14,-15, -24) and CMY-2 while the group IncI2 (also known as Incy) is increasingly associated with *bla*_{CMY-2} (Antunes et al., 2010; Mataseje et al., 2009). The *bla*_{TEM-52} located on a Tn3 transposon is disseminated on epidemic IncI1 plasmids among the *Salmonella* serovars Agona, Derby, Infantis, Paratyphi B variant Java, and Typhimurium from poultry and humans in Belgium and France (Cloeckeaert et al., 2007). TEM-52-producing isolates of seven other *Salmonella* serovars (mainly serovar Blockey), from poultry, poultry meat and humans have been detected in The Netherlands and France, although plasmids were not fully characterized in these cases (Hasman et al., 2005). IncI1 plasmids producing TEM-52C have been recovered from *E. coli* in chicken and turkey meat in Denmark and the UK (Jensen et al., 2006; Randall et al., 2011). An IncI1 plasmid carrying *bla*_{CTX-M-15} and *bla*_{TEM-1} is also associated with the current 2011 outbreak of *E. coli* O104:H4 in Northern Europe.¹⁹

IncK plasmids have facilitated the diffusion of *bla*_{CTX-M-14} among animals and humans from different European countries (Liebana et al., 2006; Valverde et al., 2009). They have recently found in *E. coli* from animals and foods carrying CMY-2 (Bergenholtz et al., 2009; Dierikx et al., 2010a).

Plasmids of the incompatibility group HI2 are able to disseminate ESBLs genes (*bla*_{CTX-M-2-9-14}, *bla*_{SHV-12}) among humans and animals (Carattoli, 2008, 2009; Novais et al., 2006). Both *bla*_{CTX-M-9}, deriving from *Kluyvera georgiana* and *bla*_{CTX-M-2}, deriving from *Kluyvera ascorbata*, were identified embedded in related *sulI*-class 1 integrons bearing *ISCR1*, which are located within mercury-Tn21 platforms (Arduino et al., 2002; Eckert et al., 2006; Novais et al., 2006).

IncA/C plasmids carrying ESBL and CMY genes have been recovered from a diversity of *Enterobacteriaceae* of poultry and human origin and from *Aeromonas* and *Edwardsiella* among fish (Hopkins et al., 2006; McIntosh et al., 2008; Randall et al., 2011). These plasmids have facilitated the widespread dissemination of *bla*_{CMY-2} among poultry and fish in Canada, the USA and some EU countries (Hopkins et al., 2006; McIntosh et al., 2008; Mulvey et al., 2009; Randall et al., 2011; Welch et al., 2007). In the IncA/C2 plasmids, the insertion of the *ISEcp1-bla*_{CMY-2}-*blc-sugE*-*DecnR* fragment occurred within the conjugative transfer locus of the plasmid, impairing their self-conjugative property (Poole et al., 2009). Recently, IncA/C plasmids carrying multiple replicons and virulence genes have been detected (Garcia et al., 2011). IncA/C plasmids carrying *bla*_{CTX-M-2} have been recovered from *E. coli* and *Salmonella* from poultry and human isolates in the UK (Hopkins et al., 2006; Randall et al., 2011).

IncN plasmids are widely distributed among poultry, swine and humans. They have been linked to a diversity of CTX-M (1, 3, -15, -65) and SHV genes (Zong et al., 2010). The *bla*_{CTX-M-1} is widely spread among poultry and swine (Blanc et al., 2006; Bortolaia et al., 2010a; Cavaco et al., 2008; Girlich et al., 2007; Moodley and Guardabassi, 2009; Randall et al., 2011). IncI1 and IncN plasmids are both involved in the transmission of the this gene among poultry and swine strongly suggests an animal reservoir for this ESBL gene variant, since either IncN or IncI1 plasmid types have been demonstrated to be highly prevalent in *E. coli* of the avian faecal flora and in *Salmonella* from retail meat and food-producing animals. These IncI1 and IncN plasmids are highly related to those in the community and hospitals (Girlich et al., 2007; Novais et al., 2006; Randall et al., 2011). IncI1 plasmids have been more frequently recovered from avian isolates while IncN plasmids are more prevalent among swine.

Plasmids belonging to the IncL/M family are mostly responsible for the spread of *bla*_{CTX-M-3} in European eastern countries, France, Belgium and Korea and often the aminoglycoside resistance gene

¹⁹ European Food Safety Authority; Urgent advice on the public health risk of Shiga-toxin producing Escherichia coli in fresh vegetables. EFSA Journal 2011; 9(6):2274 <http://www.efsa.europa.eu/en/efsajournal/doc/2274.pdf>

armA has been co-localized on the same IncL/M plasmid with the *bla*_{CTX-M-3} gene. It should be noted that as yet, IncL/M plasmids have not been recovered from bacteria isolated from animals. The *bla*_{CTX-M-3} gene expression is driven by the *ISEcp1* insertion sequence; the fragment placed directly downstream from *ISEcp1* was identified to be a chromosomal fragment from a *K. ascorbata* strain and contains 343 bp of a *Kluyvera* orf477. The 3'-end of the insert is terminated by an 18-bp sequence which is a part of orf477 that most probably mimicked the right IR of *ISEcp1* in the transposition event that mobilized *bla*_{CTX-M-3} from the *K. ascorbata* chromosome (Golebiewski et al., 2007).

The IncQ plasmid family is currently becoming of increasing epidemiological relevance since some plasmids of this type have acquired resistance genes such as *qnr* and *bla*_{GES-1}. IncQ plasmids carrying resistance genes have been identified in *Aeromonas* in the environment from France and Switzerland, in plasmid DNA from a water waste treatment plant from Germany and in *E. coli* from a human patient from Switzerland (Bonemann et al., 2006; Cattoir et al., 2008; Picao et al., 2008; Poirel et al., 2010). The IncQ plasmids are not self-transmissible by conjugation, but may be mobilized at high frequency in the presence of a helper plasmid (for instance, the IncP plasmids are particularly efficient mobilisers of these plasmids). The IncQ plasmids have been successfully mobilised, not only to a large number of Gram-negative hosts but also to several Gram-positive bacteria, including *Arthrobacter* spp., *Streptomyces lividans* and *Mycobacterium smegmatis*, cyanobacteria and plant and animal cells. They may therefore contribute to broad host range spread of relevant antimicrobial resistances (Lawley et al., 2004).

3.3. Transmission of resistant bacterial strains and/or genes to humans through the food animal production environment.

There is limited evidence for spread of ESBL/AmpC-carrying organisms *via* direct contact with animals or indirectly via the environment. In cases where a similar gene pattern in humans and animals has been documented it is often not clear whether this has been spread through contact or via food (Fey et al., 2000). It seems as if the “one medicine concept” applies which means that an increase in number of ESBL/AmpC carrying organisms in either animals or humans (or in different animal populations) could make resistance emerge in other human or animal populations but the links might be indirect and thus difficult to map.

In general, the ecosystem acts as a reservoir, a “resistome” (Wright, 2007). Resistant bacteria which can be found in faeces and soil in the farm environment (Cobbald et al., 2006; Goncalves et al., 2010), aquatic systems (Machado et al., 2009), plants (Ruimy et al., 2009) can be transferred from these sources to animals and humans through the food chain (Silbergeld et al., 2008).

These bacteria can infect animals or colonise their gut (Alexander et al., 2009; Hinton et al., 1982). A natural consequence of animal gut colonisation is shedding of resistant bacteria into the farm environment. Although the duration of persistence and shedding is not known, in an outbreak in cows caused by ESBL-producing *Salmonella*, shedding continued for 68 days after the start of the outbreak (Lanzas et al., 2009).

Strong evidence supporting the potential for transmission and colonisation of *Enterobacteriaceae* between animals and farm staff has been provided from reports where farmers were colonised with indistinguishable strains of antimicrobial-resistant *E. coli* from food animals on the farm. Such colonisation was initially reported for *E. coli* from poultry and humans (Levy et al., 1976a, 1976b), and followed by many subsequent reports on *E. coli* (Ojeniyi, 1989; van den Bogaard et al., 2001) (Linton et al., 1977; Price et al., 2007). Transmission of CTX-M1 between pigs and pig farmers has also been reported (Moodley and Guardabassi, 2009).

(Dierikx et al., 2010a) have demonstrated that people working with poultry have a higher risk for intestinal carriage of ESBL-producing bacteria. The ESBL prevalence in poultry farmers was higher

than in the general population (around 30% versus 5%, respectively), which suggests that direct transmission from poultry to humans may also be a possible route of transmission.

4. CRITICAL ANALYSIS OF THE METHODS (PHENOTYPIC AND GENOTYPIC), AND THE INTERPRETIVE CRITERIA CURRENTLY USED FOR DETECTION (ISOLATION AND IDENTIFICATION) AND CHARACTERISATION OF ESBL AND/OR AMPC-PRODUCING BACTERIAL STRAINS, ESBL- AND/OR AMPC-ENCODING GENES AND ASSOCIATED MOBILE ELEMENTS

In this chapter the methods for isolation, identification and characterization of bacteria that produce ESBL and or AmpC-genes are described. Isolation from faeces or foods is performed with selective growth media with low concentrations of cefotaxime or ceftriaxone. Suspected isolates are identified and confirmed as ESBL or AmpC by dedicated susceptibility tests using harmonized interpretive criteria. For molecular identification of ESBL and/or AmpC genes (e.g. *bla*_{TEM-52}, *bla*_{SHV-12} or *bla*_{CTX-M-15}), PCR or microarray and sequence analysis is performed. Characterization of plasmids on which ESBL and/or AmpC-genes are located is essential to study the epidemiology of these genes, which are located on transferable plasmids. Purified plasmids can be typed using replicon typing and subtyped by fingerprinting, multi locus sequence typing or whole plasmid sequence analyses. To identify clonal distribution, isolates can be typed by phenotypic methods as serotyping, and phage typing, or genotyping methods such as pulsed field gel electrophoresis (PFGE), multiple loci analysis of variable number of tandem repeats (MLVA) or MLST. All these methods are described in more detail in the following subsections.

4.1. Selective isolation, phenotypic detection and identification of ESBL- and/or AmpC-producing bacteria

Selective isolation of ESBL and/or AmpC-producers can be performed by adding 3rd generation cephalosporins to selective or non-selective growth media as indicated in Table 8. The concentrations advised vary slightly, but given the known diversity of cephalosporin resistance phenotypes, the concentrations should preferably be not too high. Nevertheless, too low concentrations will affect the specificity and lead to possible false positive results, which will greatly affect the workload. Also the usage of certain drugs affects the sensitivity and specificity of the isolation. Cefpodoxime is considered to be not the ideal drug to include in broth or agar plates because of lack of specificity. Ideally, cefotaxime or ceftriaxone should be used separately or in combination with ceftazidime in selection processes.

Several chromogenic selective agars have become available for the purpose of selective isolation of ESBL-producers. The advantage of these media is that ESBL-producers can easily be detected based on specific colours of colonies of different species. The disadvantage of some selective chromogenic agars is that AmpC-producers may fail to grow (Randall et al., 2009).

Selective isolation always needs a confirmation of the presence of an ESBL and/or AmpC gene by either phenotypic or molecular methods. This can be done phenotypically by diffusion or dilution assays for susceptibility using (combinations of) cephalosporins. A synergistic effect of the combination of a 3rd generation cephalosporin with clavulanic acid indicates the presence of an ESBL gene. Resistance against cefoxitin could be associated with the presence of an AmpC gene. Unfortunately, resistant strains can contain several ESBL and/or AmpC genes. This can interfere with the result of the confirmatory phenotypic testing. Therefore, confirmation of the presence of an ESBL and/or AmpC producer often requires genotypic verification (see chapter 4.3).

Table 8: Methods for selective isolation, phenotypic detection and identification of ESBL and/or AmpC-producing bacteria

Method	Specification	Comments	References
Selective Isolation of ESBL and/or AmpC producing bacteria	Mueller-Hinton broth (Cation adjusted) with 4 mg/l cefpodoxime, 1 mg/L cefotaxime, ceftazidime, ceftriaxone, or aztreonam	1. Recommended by CLSI for screening of ESBLs (M100-S20)	1. CLSI M100 – S20
	Selective agar (MacConkey or Levine agar with: 1 – 2 mg/L cefotaxime or 1 – 2 mg/L ceftazidime)	2. Includes ESBL and AmpC-producers	2. (Cavaco et al., 2008; Cohen et al., 2006; Costa et al., 2009; Duan et al., 2006; Girlich et al., 2007; Komatsu et al., 2000)
	Selective chromogenic agars (ChromID, bioMerieux, Brilliance ESBL-agar Oxoid, ESBL-Bx bioMerieux, CHROMagar-CTX CHROMagar microbiology)	3. Some may miss AmpC-producers	3. (Glupczynski et al., 2007; Huang et al., 2010; Randall et al., 2009; Reglier-Poupet et al., 2008; Sharma et al., 2008)
Phenotypic identification (screening)	In <i>E. coli</i> , <i>K. pneumoniae</i> , and <i>Salmonella</i> , reduced susceptibility to cefotaxime, ceftazidime, cefopodoxime, ceftriaxone, aztreonam, cafoxitin /cefepim (AmpC) by disk diffusion, agar/broth dilution or automated systems (eg Vitek)	The use of more than one antimicrobial agent for screening will improve the sensitivity of detection.	CLSI, HPA-BSAC, SRGA, EUCAST
	For detection of organisms with plasmid mediated ESBL genes: <i>E. coli</i> , <i>Klebsiella</i> , <i>Proteus mirabilis</i> , <i>Salmonella</i> , <i>Shigella</i> , use cefotaxime in combination with ceftazidime For plasmid mediated AmpC-producers, use cefoxitin	Cefpodoxime not advised because of insufficient specificity	(Biedenbach et al., 2006; Hope et al., 2007; Kim et al., 2004; Lee et al., 2006)
Phenotypic confirmation	Combination disks, ESBL E-test	VITKEK II ESBL confirmation test is not advised because of limited and conflicting published information	(Livermore and Woodford, 2006)
	Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid to test for synergy with clavulanic acid	Double disk test not advised because of low sensitivity	(Bedenic et al., 2007; Paterson and Bonomo, 2005)
	For cefoxitin resistant organisms, susceptibility to cefepime will confirm the presence of an AmpC. Cefepime is not inactivated by AmpC enzymes, but will be hydrolysed by most ESBLs.	The cefepime confirmation test does not distinguish between plasmidic and chromosomal AmpC-genes.	

The preferred method for selective isolation of ESBL and/or AmpC producers is a chromogenic agar (e.g. MacConkey agar) with 1 mg/L cefotaxime or ceftriaxone. The use of low concentrations will result in optimum sensitivity to detect all relevant β -lactamase families. Pre-enrichment may be performed in a general broth like Mueller-Hinton, Brain Heart Infusion or Luria-Bertani broth with 1 mg/L cefotaxime or ceftriaxone.

Identification is performed by determination of susceptibility to cefotaxime, ceftazidime and cefoxitin. ESBL producers are resistant to cefotaxime, variable to ceftazidime and susceptible to cefoxitin.

Confirmation of ESBL is performed by testing for synergy with clavulanic acid by combination disks, ESBL-etest or broth microdilution including cefotaxime, ceftazidime as single drugs and in combination with clavulanic acid.

Confirmation of AmpC producers is performed by determination of susceptibility to cefepime; AmpC producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and cefoxitin.

4.2. Interpretive criteria and MIC breakpoints

The interpretive criteria described in this document can only be used for *Enterobacteriaceae*, since for other microorganisms such as *Vibrio* or *Aeromonas* no criteria have been established.

In table 9 the MIC breakpoints or epidemiological cut-off (ECOFF) values of the European EUCAST and the Clinical and Laboratory Standards Institute (CLSI) are presented. National organisations like the British Society of Antimicrobial Chemotherapy (BSAC), Swedish Reference Group of Antibiotics (SRGA), Norwegian Working Group on Antibiotics (NWGA) and Societe Francaise de Microbiologie (CASFM) have agreed to follow the recommendations for clinical breakpoints as defined by EUCAST and are therefore not included in table 9. EFSA advocates the usage of ECOFFs for antimicrobial resistance surveillance since their usage will detect acquired resistance with optimum sensitivity.

Table 9: MIC-breakpoints (S/R) as prescribed by EUCAST, and CLSI for cefotaxime, ceftazidime, ceftriaxone, cefoxitin, cefepime and ceftiofur for *Enterobacteriaceae*.

MICs (mg/L)	Cefotaxime	Ceftazidime	Ceftriaxone	Cefoxitin	Cefepime	Ceftiofur	References
EUCAST ^a	$\leq 1 / > 2$	$\leq 1 / > 4$	$\leq 1 / > 2$	NA	$\leq 1 / > 4$	NA	www.eucast.org
EUCAST ECOFF ^c	$\leq 0.5 - 0.064^b$	$\leq 0.125 - 2b$	$\leq 0.125 - 0.5^b$	≤ 8	≤ 0.12	$\leq 1 - 2^b$	www.eucast.org
CLSI ^a	$\leq 1 / \geq 4$	$\leq 4 / \geq 16$	$\leq 1 / \geq 4$	$\leq 8 / \geq 32$	$\leq 8 / \geq 32$	NA	CLSI M100-S20

EUCAST: European Committee on Antimicrobial Testing; CLSI: Clinical and Laboratory Standards Institute

(a) Clinical breakpoints are not primarily intended to detect resistance mechanisms. Cut-offs are adequate for this objective. Values are not provided for all species associated with ESBL or AmpCs

(b) Range comprises values corresponding to different *Enterobacteriaceae* species

(c) ECOFF: This value separates microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to the agent in question. The ECOFF is the lowest possible value for the clinical breakpoint

(d) NA – not available

Although CLSI has recently redefined MIC breakpoints for 3rd- and 4th-generation cephalosporins, the R-breakpoints for ceftazidime, cefoxitin and cefepime are still one to two dilution steps higher than those defined by EUCAST. To harmonize the interpretation of susceptibility data and for optimum phenotypic detection of ESBL and/or AmpC producers we advise to use EUCAST clinical breakpoints for interpretation of susceptibility or resistance and EUCAST ECOFF's to determine if an isolate belongs to the wild-type population or not.

4.3. Molecular methods for detection and identification of ESBL, and/or AmpC genes

Detection of ESBL, and/or AmpC genes can be performed by polymerase chain reaction (PCR), and microarray. The specificity of these methods depends upon the choices of the oligonucleotide primers (PCR), the probes included in a microarray, or used for hybridization. The choice of the primers determines the possibility to detect certain genes. Recently a list of primers was published for the most important β -lactamases that occur in *Enterobacteriaceae* (Dallenne et al., 2010). These β -lactamases include genes encoding the OXA-1-like broad spectrum β -lactamases, ESBLs, plasmid-mediated AmpC β -lactamases and class A, B and D carbapenemases. Several commercial microarrays have been developed for rapid and specific detection of β -lactamase genes. The limitations of these tests are determined by the probes included. Microarrays can detect most β -lactamase families, and some are also able to identify variants within families (see table 3), by means of sophisticated choices of different probes targeted to the detection of certain Single Nucleotide Polymorphisms (SNPs). This is of specific relevance for TEM and SHV-genes where multiple variants exist that need to be differentiated from the commonly present narrow spectrum β -lactamases TEM-1 and SHV-1.

Identification of the subtype of the ESBL and/or AmpC genes detected by PCR or microarray is normally conducted by sequence analysis of PCR fragments. For this purpose the primers used should be able to amplify DNA fragments of sufficient size including all point mutations to be able to identify the genetic variations based on which a subtype is designated.

Table 10: Methods for genotypic detection and identification of ESBL-, and AmpC genes

Method	Specification	Comments	References
Polymerase Chain Reaction (PCR) and sequencing	<p>Specific PCR reactions have been designed for detection of all relevant β-lactamase genes encoding ESBLs and AmpC</p> <p>To identify individual ESBL/AmpC variants, PCR products should be sequenced and compared to previously sequenced genes.</p>	<p>A strain might carry more than one ESBL and/or ampC gene. Therefore, there is a risk of not detecting all ESBL and/or ampC genes present.</p> <p>Also, some species (<i>Citrobacter</i>, <i>Enterobacter</i> and to some extent also <i>E. coli</i>) carry AmpC genes on the chromosome. These might not be detected by the standard PCR protocols.</p>	(Dallenne et al., 2010; Voets et al., 2011)
DNA arrays	Several commercially available DNA arrays have been designed. In these, the most common gene (groups) associated with ESBL and/or AmpC production is detected.	Often only the gene related to the ESBL/AmpC phenotype is detected at the group level. Further analysis using methods like PCR and sequencing (see above) is also required.	<p>Commercial arrays can be purchased from several sources:</p> <p>Identibac array tube system: www.identibac.com</p> <p>Check-points array system: www.check-points.com</p> <p>(Batchelor et al., 2008; Cohen Stuart et al., 2010)</p>

For molecular identification of ESBL and/or AmpC genes, PCR or microarray are used to screen which β -lactamase families are present (e.g. TEM, SHV, or CTX-M). To study the genetic and epidemiological relatedness of genes present in different backgrounds (eg. animal or human bacteria) subtyping is essential. This is performed by dedicated PCRs aimed at the β -lactamases detected by the screening method to obtain an amplicon of sufficient size. Sequence analysis of the amplicon will result in designation of the ESBL subtype present (e.g. TEM-52, SHV-12 or CTX-M-15).

4.4. Detection and classification of plasmids carrying ESBL and/or AmpC-genes

The horizontal transfer of plasmids carrying ESBL and/or AmpC genes is an important contributory factor in the epidemiology of these genes within the bacterial ecosystem.

To be able to associate a certain β -lactamase gene with a specific plasmid several techniques are available, as listed in table 11. Plasmid isolation and further electrophoresis of S1-digested genomic DNA in agarose gels will provide information on number and mass/size of plasmids present in one isolate. The ability to recognize and categorize plasmids in homogeneous groups on the basis of their phylogenetic relatedness can be helpful to analyse their distribution in nature, the relationship to host cells, and to assist into elucidating their evolutionary origins (Francia et al., 2004).

Identification and classification of plasmids should be based on genetic traits that are present and constant. These criteria are best met by traits concerned with plasmid maintenance, especially replication controls (DeNap and Hergenrother, 2005). In 1971 Datta and Hedges proposed a plasmid classification scheme based on the stability of plasmids during conjugation, a phenomenon called plasmid incompatibility (Datta and Hedges, 1971). In 1988 Couturier and colleagues proposed a genetic plasmid typing scheme, based on Southern blot hybridization using cloned replication regions (replicons) as probes (Couturier et al., 1988). Since 2005, a PCR-based replicon typing (PBRT) scheme has been available, targeting the replicons of the major plasmid families occurring in *Enterobacteriaceae* (HI2, HI1, I1- γ , X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, B/O) and also including PCR assays (FrepB and FIAs PCRs) detecting the FII, FIII, FIV, FIV variants and the FII replicon of the *Salmonella* virulence plasmids, respectively (Carattoli et al., 2005). Typing analysis by identification of replicons associated with predominant conjugative plasmids of *Enterobacteriaceae*, and *Acinetobacter* has been extensively used. The PBRT scheme has still several limitations since the classification is currently based on plasmids belonging to the classic *Inc* groups and can fail the identification of divergent or novel replicons.

The most accurate method to characterize a plasmid is based on the determination of the full-length DNA sequence and up to date, more than 800 plasmids from Gamma-proteobacteria have been fully sequenced (<http://www.ncbi.nlm.nih.gov/genomes/>), contributing to the identification of novel plasmid families. Plasmids can also be classified according to the relaxase type since relaxases have been shown to be excellent markers of plasmid backbones by systematic database analysis (Garcillan-Barcia et al., 2009; Smillie et al., 2010). Typing of relaxases can help to further characterise plasmids which contain either more than one replication genes or harbour replication and conjugation genes from different plasmid origins (Antunes et al., 2010; Valverde et al., 2009).

Transfer of plasmids to well identified recipients by conjugation or electroporation will facilitate the typing of individual plasmids carrying β -lactamase genes. Once an association has been identified between a certain replicon/relaxase-type plasmid and an ESBL and/or an AmpC gene by Southern hybridization, fingerprinting of these plasmids can be performed by restriction enzyme analysis. Further characterization of plasmids belonging to the incompatibility groups I, F and HI can be performed by recently developed plasmid MLST schemes (pMLST) (<http://pubmlst.org/plasmid/>). Such schemes permit further analysis of genetic and epidemiological relations among plasmids from different bacterial species, sources or regions.

Table 11: Methods for detection and classification of plasmids carrying ESBL and/or AmpC genes

Method	Specification	Comments	References
Evaluation of plasmid content	1. Crude plasmid DNA extraction followed by separation by vertical agarose gel electrophoresis	1. Provides size and number of plasmids. Needs standard plasmids of known molecular mass	1. (Kado and Liu, 1981)
	2. DNA S1 nuclease treatment followed by Pulse Field Gel Electrophoresis (PFGE). Agarose plugs are prepared according to the PulseNet Protocol (http://www.pulsenet-europe.org)	2. Allows the conversion of circular plasmid DNA to the linear form visualized by PFGE for determination of high molecular weight plasmids. Low range PFGE markers are used as molecular weight markers.	2. (Barton et al., 1995; Guerra et al., 2004)
	3. Alkaline/SDS extraction followed by separation by horizontal agarose gel electrophoresis. Further purification of plasmid DNA is obtained by silica column-based purification kits	3. Rapid method for plasmid extraction but not recommended to generate unrestricted plasmids profiles	3. (Birnboim and Doly, 1979)
Molecular localization of ESBL and AmpC genes on undigested plasmids	S1-digested genomic DNA gels obtained as above are transferred onto membranes and hybridized by Southern blot with labelled probes specific for the different beta-lactamase genes and plasmid replicons	Allows to locate the β -lactamase genes on undigested plasmids profiles	(Guerra et al., 2004; Sambrook et al., 1989; Valverde et al., 2009)
Resistance transfer by conjugation	Broth mating experiments are performed in Luria-Bertani (LB) or TSB (trypticase soy broth) media using one ESBL- or AmpC- producer as donor and a plasmid free, indole-negative <i>E. coli</i> , showing rifampicin, nalidixic acid, kanamycin or azide resistance as recipient. The transconjugants can be selected on Mueller–Hinton (MH), Brain heart infusion (BHI), LB or MacConkey agar media supplemented with	Plasmids encoding the transfer functions can promote their own transfer and mobilise <i>in trans</i> smaller, non-self-transmissible plasmids.	
	1. 100 mg/L ampicillin	1. Indicated for selection of un-characterized β -lactamases.	1. (Garcia et al., 2007)

Method	Specification	Comments	References
	2. 2 mg/L cefotaxime	2. Indicated for selection of CTX-M-carrying plasmids	2. (Rodriguez et al., 2006)
	3. 8.0 mg/L ceftiofur	3. Indicated for selection of CMY –carrying plasmids	3. (Poole et al., 2009)
	<p><i>E. coli</i> recipient resistance markers are usually selected as follows: 32 mg/L nalidixic acid or 25 mg/L rifampicin or 50mg/L kanamycin or 130mg/L azide</p>		
Plasmid DNA transformation by chemical treatment or electroporation	Plasmids obtained by alkaline lyses and column purification kits are transformed into an electrocompetent recipient using a gene pulser or are transferred in chemically competent DH5alpha <i>E. coli</i> cells.	The transformation of in house-made or commercially available competent cells represents the easiest way to transfer a plasmid from the donor cell to the <i>E. coli</i> recipient strain. This procedure becomes necessary when the plasmid lacks the self-transfer system or shows very low levels of conjugation efficiency.	
	Selection of transformants can be done on LB or BHI agar supplemented with	Selection on cefotaxime is suggested for the recovery of CTX-M carrying plasmids	
	1. 1-2 mg/L cefotaxime		1. (Bortolaia et al., 2010a; Dierikx et al., 2010a; Rodriguez et al., 2006)
	2. 100 mg/L ampicillin		2. (Garcia-Fernandez and Carattoli, 2010)
Plasmid typing	1. Incompatibility tests by conjugation. Requires conjugation experiments performed with a reference plasmid for each Inc group.	1. Not recommended for massive screening of strains	1. (Datta and Hedges, 1971)
	2. PCR-Based Replicon Typing (PBRT): 21 replicons for <i>Enterobacteriaceae</i> and 19 for <i>Acinetobacter baumannii</i> are tested by PCR on boiled cultures or total genomic DNA preparations	2. PBRT on 21 replicons is currently considered the reference method for plasmid typing. The modified multiplex array is suggested for massive screenings but may miss replicons	2. (Bertini et al., 2010; Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Johnson et al., 2007b)
	3. Probe-Based Replicon Typing: plasmid gels obtained as above are transferred onto membranes and hybridized with probes for the different replicon types	3. Association of amplicons obtained by PCR with specific plasmids can be done by Southern hybridization using PCR-probes obtained by PBRT.	3. (Couturier et al., 1988)

Method	Specification	Comments	References
Plasmid sub-typing	1. Fingerprinting: for restriction fragment length polymorphism (RFLP) analysis, plasmid DNA is extracted using column-based purification kits. Restriction fragments are separated by agarose gel electrophoresis	1. Different restriction enzymes can be used: <i>Pst</i> I, <i>Hinc</i> II, <i>Eco</i> RV, <i>Bam</i> HI	1. (Garcia-Fernandez et al., 2009; Literak et al., 2009; Moodley and Guardabassi, 2009)
	2. Plasmid Multilocus Sequence Typing (pMLST). It is based on amplification and sequencing of specific plasmid loci. Currently available for IncII, IncHI2, IncHI1, IncF plasmids	2. Allows the assignation of plasmid alleles and Sequence Types (STs) at the http://pubmlst.org/ plasmid/ site (available databases for STs, ESBLs, AmpCs, sources etc.)	2. (Garcia-Fernandez et al., 2008; Garcia-Fernandez et al., 2010; Phan et al., 2009; Villa et al., 2010)
Whole plasmid sequencing	Genome Sequencers can be used for high-throughput sequencing of plasmids. It can be performed on column-purified plasmid DNAs, adapting the manufacturer's library construction and sequencing procedures	Gives a complete analysis of the plasmid structure; requires bioinformatic expertise for annotation. A classification scheme has been proposed for fully sequenced plasmids based on phylogenetic analysis of the mobilization regions.	(Carattoli et al., 2010; Garcillan-Barcia et al., 2009; Smet et al., 2010; Smillie et al., 2010; Woodford et al., 2009)

Characterization of plasmids on which ESBL and/or AmpC genes are located is an essential tool to study the epidemiology of these genes and plasmids. Since in *Enterobacteriaceae* often several different plasmids are present in each isolate, a structured approach is needed to identify the characteristics of the plasmid on which the β -lactamase genes are located. If by phenotypic and molecular tests described in paragraphs 4.1 and 4.3 the presence of an ESBL and/or AmpC gene in a bacteria is confirmed, plasmid isolation may be performed to determine the number and sizes of plasmids present. Subsequently by conjugation or electroporation, transconjugants or transformants are isolated on selective agar plates with only the plasmid that harbours the β -lactamase gene present. The plasmid can be typed using replicon typing and subtyped by fingerprinting or pMLST. Ultimately, whole plasmid sequence analyses may replace the current typing and subtyping techniques.

4.5. Molecular typing of isolates

The purpose of molecular typing of isolates is to determine genetic characteristics of isolates at the subspecies level in order to identify genetic relatedness and/or to allow source tracking/attribution. For these purpose several genotyping methods are available. The principles on which these methods are based on and their discriminatory power are very different, which greatly influences the choice of method according to the intended use. Phenotypic tests are relatively easy to perform, but lack discriminatory power. Within serotypes, and phage types many different genetically-related clusters of isolates can occur that can be identified by genotyping methods. PFGE is a band-based technique with generally a high discriminatory power. It allows identification of lineages or clusters of epidemiologically related isolates within eg. *Salmonella* sero-, or phage types. It is intended for tracing outbreaks in a limited time period and less suitable for performing phylogenetic analyses. MLVA was developed as a sequence-based method with similar discriminatory power as PFGE.

MLST is a sequence-based method targeting 5-7 highly conserved genes. This method has generally less discriminatory power than PFGE or MLVA but is the most reliable method to determine genetic relatedness of epidemiologically-unrelated isolates. As an example, ESBL-producing *E. coli* are currently assigned by MLST to a certain ST, and within those STs diverse clusters can be detected by PFGE. An example is the ‘pandemic’ *E. coli* ST131 which harbours CTX-M-15 on mostly IncF-plasmids. Within ST131, epidemiologically-related clusters can be identified by PFGE.

Table 12: Methods for molecular typing of isolates (identification of clones)

Method	Specification	Comments	References
Serotyping	Serotyping is based on detecting specific surface components of the bacteria with corresponding antibodies. Serotyping schemes has been developed for <i>E. coli</i> , <i>Klebsiella</i> and <i>Salmonella</i> .	Serotyping is most often used for initial characterisation of <i>Salmonella</i> isolates. Reproducibility between laboratories is high.	Kaufmann-White scheme
Phage typing	Phage typing is based on certain bacteriophages ability to lyse (infect and kill) the tested bacteria.	Among the <i>Enterobacteriaceae</i> , phage typing is almost exclusively used to sub-type <i>S. Typhimurium</i> and <i>S. Enteritidis</i>	
Pulsed Field Gel Electrophoresis (PFGE)	PFGE is a band-based method where chromosomal DNA is digested with a restriction enzyme and separated on agarose gels. It as been utilized for outbreak investigations for more than a decade. Especially for <i>E. coli</i> outbreaks, this method has been standardized as part of the Pulsenet network.	The same method is applicable for outbreak investigations in other <i>Enterobacteriaceae</i> such as <i>Salmonella</i> , <i>E. coli</i> , <i>Klebsiella</i> . Reproducibility between laboratories is adequate.	(Ribot et al., 2006; van Belkum et al., 2007)
Multiple Loci VNTR Analysis (MLVA)	MLVA is a method based on detecting the length of variable repeat regions located on the chromosome of many bacterial species. A specific MLVA scheme has been developed for <i>E. coli</i> and <i>Salmonella Typhimurium</i> . In general, several repeat areas are examined in order to increase the discriminatory power of the analysis.	MLVA requires specialized equipment (a sequencer) and software for analysis of results. Reproducibility between laboratories is high as long as the same equipment is used.	(van Belkum et al., 2007)

Method	Specification	Comments	References
Multi Locus Sequence Typing (MLST)	MLST is a sequence based method where 5-8 “household” genes from the chromosome are amplified by PCR and these products are subsequently sequenced. Based on small genetic variations (point mutations) within these household genes, the ST is determined. Strains, which are clonally related often belongs to the same (or closely related) STs.	As MLST is based on sequencing, the reproducibility between laboratories is high, however, the discriminatory power is not as high as e.g. PFGE (strains with the same ST can have different PFGE patterns). MLST typing of <i>E. coli</i> . Several MLST schemes have been developed although two of them are more commonly used (http://mlst.ucc.ie/mlst/dbs/Ecoli and http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html) schemes. Correlation between these two schemes has not been extensively explored and ST designations can vary among studies MLST typing for <i>Klebsiella pneumoniae</i> . (http://www.pasteur.fr/recherche/genopol e/PF8/mlst/Kpneumoniae.html)	(Jaureguy et al., 2008; van Belkum et al., 2007; Wirth et al., 2006)
Full genome sequencing (FGS)	FGS can also be used for bacterial typing studies. Here, the principle is similar to the MLST method (based on SNPs), however, the amount of DNA examined is much higher than in MLST.	The discriminatory power is high. However different sequencing methods exist, which results in artificial variation due to methodological differences.	

The choice of the molecular typing method to be used is determined by epidemiological relatedness of the isolates. Next to phenotypic methods such as serotyping and phage typing, PFGE or MLVA can be used to identify clonal clusters of isolates that are related to a certain ‘outbreak’ in a restricted time frame. MLST is the method of choice to identify relatedness of isolates of the same species from different backgrounds (eg. animal versus human).

5. RECOMMENDATIONS FOR A HARMONISED MONITORING OF RESISTANCE (PHENOTYPIC AND GENOTYPIC) CAUSED BY ESBL AND AMPC IN FOOD AND FOOD PRODUCING ANIMALS IN THE EU

A harmonized monitoring programme will deliver important baseline information on the prevalence of and the trends in resistance. The basic general principles of harmonized monitoring of antimicrobial resistance in *Salmonella* and *E.coli*, as laid down in the EFSA’s recommendations²⁰, also apply to monitoring resistance caused by ESBLs and AmpC. Some additional considerations are important for this type of resistance. The following sections address these specific considerations.

5.1. Purpose of the suggested harmonised monitoring

Harmonised monitoring of resistance caused by ESBLs and AmpC has several objectives:

²⁰ Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. <http://www.efsa.europa.eu/en/efsajournal/pub/141r.htm>. Report of the Task Force on Zoonoses Data Collection including a proposal for a harmonized monitoring scheme of antimicrobial resistance in *Salmonella* in fowl (*Gallus gallus*), turkeys and pigs and *Campylobacter jejuni* and *C. coli* in broilers. <http://www.efsa.europa.eu/en/efsajournal/pub/96r.htm>.

- To determine the prevalence of resistance in bacteria from different animal populations and food, and describe the geographical distribution of resistance prevalence within Europe
- To detect changes in prevalence of resistance in bacteria over time
- To describe genotypic characteristics of ESBLs and AmpCs, and changes of these characteristics over time.
- To detect emergence of new *bla*_{ESBL/AmpC} resistance genes with a potential relevance to public health

These objectives may add to the general aims for monitoring antimicrobial resistance in bacteria of animal origin in the EU. Since the EFSA guidelines cover only phenotypic monitoring, reported data do not enable determination of the class or exact type of β -lactamase enzyme involved. In addition, the sample size recommended in the guidelines was determined for detecting trends in changes of prevalence over time. This sample size is not sufficient to timely detect emergence of new resistance patterns in the case of low prevalence (as observed for ESBLs in many EU countries). Consequently, harmonised monitoring of resistance caused by ESBL and AmpC needs to go beyond the existing recommendations for routine phenotypic surveillance. Specifically, we recommend that genotypic resistance testing should be performed in addition to the phenotypic testing foreseen in the existing recommendations. For surveillance schemes, we also recommend the analysis of isolates deriving from passive surveillance schemes (diagnostic submissions), from systematic sampling, and from targeted surveys.

5.2. Recommendations for targeted surveys

In order to increase the probability of detection of emerging resistance determinants, active surveys can target those animal categories where the risk of development and spread of resistant isolates is highest. For this, information on risk factors contributing to a high prevalence of resistance caused by ESBL and AmpC is important. For countries where detailed data on antimicrobial consumption are available, those animal species and farms with the highest consumption of third- and fourth-generation cephalosporins should be included in the survey. For countries where these data are not available, risk factors which are a proxy for a high consumption of antimicrobials such as type of production, health status or holding size could be used for a targeted selection of samples instead. For animal categories where trade is suspected to be a risk factor, farms can be targeted according to the origin of the animals received.

5.3. Recommendations for passive monitoring

Enterobacteriaceae isolated at veterinary diagnostic laboratories that are classified resistant to third- or fourth-generation cephalosporins (ceftiofur, cefoperazone, cefquinome, cefovecin) should be subjected to phenotypic and molecular identification and characterization of the plasmid-mediated β -lactamase genes present. If possible, isolates should be accompanied by information on the location of the farm of origin, data of sample collection, animal species and category, and rationale for isolation (e.g. type of disease and requested diagnostics). Diagnostic submissions are likely to originate from farms with a health problem which have often been treated with antimicrobials prior to sample collection. These samples are therefore well suited for early detection of emerging *bla*_{ESBL/AmpC} resistance genes.

5.4. Recommendations for methods for selective enrichment and genotypic surveillance

The preferred method for selective isolation of ESBL and/or AmpC producers is selective isolation on agar preceded by selective enrichment in a broth. The preferred method for selective isolation of

ESBL and/or AmpC-producers is chromogenic (e.g. MacConkey agar) with 1 mg/L cefotaxime or ceftriaxone. Using low concentrations will result in optimum sensitivity to detect all relevant beta-lactamase families. Pre-enrichment may be performed in a general purpose broth such as Mueller-Hinton, Brain Heart Infusion or Luria-Bertani broth supplemented with 1 mg/L cefotaxime or ceftriaxone.

Identification is performed by determination of susceptibility to cefotaxime, ceftazidime and cefoxitin. ESBL producers are resistant to cefotaxime, variably resistant to ceftazidime and susceptible to cefoxitin. Confirmation of ESBLs is performed by testing for synergy with clavulanic acid by combination disks, ESBL-etest or broth microdilution including cefotaxime, ceftazidime as single drugs and in combination with clavulanic acid. Confirmation of AmpC producers is performed by determination of susceptibility to cefepime. AmpC-producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and cefoxitin. To identify ESBL and/or AmpC suspected *Enterobacteriaceae* by broth microdilution susceptibility tests, optimum interpretive criteria need to be used. Although CLSI has recently redefined MIC breakpoints for 3rd and 4th generation cephalosporins, the R-breakpoints for ceftazidime, cefoxitin and cefepime are still one to two dilution steps higher than those defined by EUCAST. To harmonize the interpretation of susceptibility data and for optimum phenotypic detection of ESBL and/or AmpC producers we advise to use EUCAST clinical breakpoints for interpretation of susceptibility or resistance and EUCAST ECOFF's to determine if an isolate belongs to the wild-type population or not.

All isolates phenotypically confirmed to be either ESBL or AmpC producers may be screened for β -lactamase gene families using micro-array or (multiplex) PCR. The ESBL and/or AmpC subtypes may be identified by dedicated PCRs and sequence analysis of the amplicons.

Characterization of plasmids on which ESBL and/or AmpC genes are located is an essential tool to study the epidemiology of these genes and plasmids. Since in *Enterobacteriaceae* often several different plasmids are present in each isolate, a structured approach is needed to identify the characteristics of the plasmid on which the β -lactamase genes are located. If by phenotypic and molecular tests described in paragraphs 4.1 and 4.3 the presence of an ESBL and/or AmpC gene in a bacterial sample is confirmed, plasmid isolation is performed to determine the number and sizes of plasmids present. Subsequently by conjugation or electroporation, transconjugants or transformants are isolated on selective agar plates with only the plasmid that harbours the β -lactamase gene present. The plasmid can be typed using replicon typing and subtyped by fingerprinting or MLST. Ultimately whole plasmid sequence analyses may replace the current typing and subtyping techniques.

The choice of the molecular typing method to be used for isolates is determined by epidemiological relatedness of the isolates. Next to phenotypic methods such as sero-, and phage typing, PFGE or MLVA can be used to identify clusters of isolates (with the same fingerprint) in a restricted time frame. This cluster analysis complements traditional epidemiological outbreak investigations. MLST is the method of choice to identify relatedness of isolates of the same species from different backgrounds (eg. animal versus human).

6. RISK FACTORS CONTRIBUTING TO THE OCCURRENCE, EMERGENCE AND SPREAD OF ESBL- AND/OR AMPC-PRODUCING BACTERIAL STRAINS IN FOOD PRODUCING ANIMALS AND FOOD

The establishment of risk factors for occurrence of ESBL/AmpC-producing bacteria is particularly complicated by the data unavailability or lack of its accuracy. Few studies designed to assess risk factors for ESBL and/or AmpC occurrence in animals are available. The text below briefly summarises present knowledge in general terms.

6.1. Farm management

ESBL- and AmpC-producing bacteria may enter and proliferate in a farm through the stocking of new animals, exposure to contaminated air, through water or feed, insect or rodent vectors, human-to-animal and animal-to-animal transmission. The farm therefore has a significant role as an amplifier of resistance.

A cross-sectional study on 32 different Belgian broiler farms identified risk factors for ceftiofur resistance in *Escherichia coli*, besides on-farm antimicrobial therapy, seven management factors were found to be associated with the occurrence of ESBL- and AmpC-producing *E. coli* (Persoons et al., 2010; Smet et al., 2008). In contrast with the expected effect of biosecurity conditions for infection prevention (hygiene and sanitation), a clean environment was a risk factor for isolates producing these enzymes in broilers. The greater diversity of the intestinal microbiota, that is thought to occur in animals of dirty environments, could prevent the establishment or reduce the incidence of these resistant bacteria. Better sanitation measures were also associated with a higher rate of tetracycline-resistant, lactose-positive coliforms in fattening pigs in Belgium (Dewulf et al., 2007). The other management factors that were found to be associated with the occurrence of ESBL/AmpC producers in *E. coli* from broilers included no acidification of drinking water, more than three feed changes during the production cycle, breed, and litter material used (Persoons et al., 2010; Smet et al., 2008).

A variety of farm management factors may be involved in the occurrence of ESBL/AmpC-producing bacteria. Field surveys and experimental research will be needed to further elaborate the list of risk factors and their solutions.

6.2. Use of antimicrobials

The use of antimicrobials is a risk factor for emergence and spread of AMR clones. Most ESBL and AmpC-producing strains carry additional resistances such as to sulphonamides and other commonly-used veterinary drugs. Generic antimicrobial use is therefore a risk factor for ESBL/AmpC and it is not restricted specifically to the use of cephalosporins.

6.2.1. β -lactams authorised for animals in the EU

β -lactams are among the most important group of antimicrobial agents in veterinary medicine. Penicillins, aminopenicillins (with or without clavulanic acid) and first- to fourth-generation cephalosporins are subgroups of β -lactam-authorised antimicrobials.

Penicillin G (often formulated as a slow release injectable), Penicillin V (for oral use in poultry), and aminopenicillins (with and without clavulanic acid) are approved for use in both food producing and companion animal species in most European countries. As authorisations of these “old molecules” are at national level, the distribution of available formulations and their indications and indicated doses varies between EU countries. There are ongoing procedures aiming at harmonisation of marketing authorisations at EU level but numerous products do not yet have harmonised product information. Formulations are available both as injectables, as oral formulations and as intramammaries for local treatment of mastitis.

Systemically-active cephalosporins have been authorised in the EU for use in animals via decentralised or centralised procedures (the former covers one or several EU countries, whereas the latter always covers all EU and EES countries although certain products might not be actively marketed everywhere). Ceftiofur (free acid) is centrally authorised (trade name: Naxcel see EPAR at www.ema.europa.eu) for subcutaneous administration in pigs for treatment of respiratory tract infections, septicaemia, polyarthritis and polyserositis caused by defined pathogens, and for cattle the indication is interdigital necrobacillosis (footrot). Ceftiofur (free acid) has an extended dosage interval (‘long acting’) in contrast with ceftiofur hydrochloride that is authorised in most EU countries

for intramuscular administration in cattle and pigs and horses with indications for treatment of respiratory diseases, and in cattle also for interdigital necrobacillosis and puerperal metritis. In some MS, ceftiofur was previously authorized for injection of day-old chickens for prevention of septicaemia (Bertrand et al., 2006). Cephalosporins have been used world-wide in poultry production since the 1990s, although precise information on usage in different countries is lacking. There are currently no cephalosporin-containing products authorized for poultry species in the EU.

Cefquinome (a fourth-generation cephalosporin) is available in some MS for systemic use in cattle, pigs and horses. The indications for use are mainly respiratory infections, interdigital necrobacillosis in cattle, septicaemia caused by *E. coli* in calves and foals, and streptococcal infections in horses. Cefquinome can also be used for the treatment of mastitis metritis agalaxia syndrome in sows, exudative epidermitis, and meningitis. Cefquinome is also approved in many EU countries for local use following intramammary application to treat bovine mastitis and for dry-cow treatment.

First generation cephalosporins (e.g. cephapirin, cephalexin, cephazolin), are also available mainly for local intramammary use. Cefalexin is also available for injection. In addition a few EU countries have cephalosporins approved for oral administration in milk replacers to calves.

6.2.2. Use of β -lactams in EU outside approved indication

The use of antimicrobials outside the approved use according to the label is regulated in Directive 2001/82/EC as amended. According to articles 10 and 11 of this directive, use outside approved indication should only be considered by way of exception, under the veterinarian's direct personal responsibility and in particular to avoid causing unacceptable suffering. In addition all treatment of food producing species must comply with MRL (maximum residue limits) regulations and only drugs that have set MRL (or are listed as substances for which there is no need to establish MRLs) are allowed to be used in food producing species.

Use outside approved indication, as intended, is very restrictive as all routine treatment of groups of animals is excluded. Due to poor compliance with this regulation and possibly also differences at the level of national implementation, such use is probably much more comprehensive than foreseen in legislation. For instance, ceftiofur has been used prophylactically in 1-day-old piglets to prevent arthritis, meningitis, septicemia, and diarrhoea (Jorgensen et al., 2007), DANMAP²¹. There are also indications of widespread, off-label use of ceftiofur in poultry (e.g. in ovo use, or use as spray or by subcutaneous injection in hatcheries). FDA conducted inspections at USA poultry hatcheries in 2001 and examined records relating to the hatcheries' antimicrobial use during the 30-day period prior to inspection. FDA found that six of the eight hatcheries inspected that used ceftiofur during that period were doing so in an extra-label manner. For example, ceftiofur was being administered at unapproved dosing levels or by unapproved methods of administration. In particular, ceftiofur was being administered by egg injection, rather than by the approved method of administering the drug to day-old chicks (<http://edocket.access.gpo.gov/2008/E8-15052.htm>) (Dutil et al., 2010).

6.2.3. Illegal use

Besides off-label use of approved products unintended in legislation, there is the possibility of use of illegally-produced and/or imported antimicrobials. Namely, for ceftiofur, mentions of its unnecessary or off-label use in the poultry industry occur worldwide and are linked to cephalosporin resistance (Collignon and Aarestrup, 2007; Webster, 2009). This market has grown with the access to internet and there is today no data on the extent of illegal use. Notably, such use might be even more problematic than misuse of approved products as counterfeit drugs might vary in content and quality.

²¹ DANMAP 2007. http://www.danmap.org/pdfFiles/Danmap_2007.pdf

6.2.4. Quantity of use of cephalosporins for animals in the EU

A project entitled: “The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC)” was set up by the European Medicines Agency (EMA) at the end of 2009 to collect information on how antimicrobial medicines are used in animals across the EU. The project is collecting harmonised data from 2010. Historical data covering ten European countries have been published, of which eight are MS which have surveillance programs on the usage of veterinary antibacterial agents in place (Grave et al., 2010).

The usage of antibacterial agents in the ten countries was reported as overall national sales, in weight of active substance, compiled for all animal species, including dogs and cats. Usage of cephalosporins cannot be identified for two of the countries as only total use of β -lactams is given and sales figures of 3rd and 4th generation of cephalosporins cannot be identified in any country. For those countries that report cephalosporin use in animals the percentage of such use varies between 1 to 7% of the total use. What this represents in daily doses is not known as daily doses are not defined in veterinary medicine. The species and the age of the animal will determine the amount needed to treat a certain number of individuals. Generally it can be assumed that 3rd- and 4th-generation cephalosporins would represent a group of antimicrobials where the number of doses are high in relation to the amount sold as they are given by injection (and not orally) and this is highly potent molecules. Overall the sales figures should be interpreted with great care as the number/biomass of animals at risk for treatment with antibacterial agents varies tremendously between the different countries. It is therefore not possible to compile comparable and relevant data on the use of cephalosporins of different generations in the MSs at the present time. There is some evidence pointing at large differences in antimicrobial use pattern between some European countries (Czech Republic, Sweden and Switzerland) that cannot be explained by differences in the disease situation (Greko et al., 2010).

Available data on use of all cephalosporins in some MSs reveals that a significant part of cephalosporins used in these countries is for pets (55% to 98%) (DANMAP 2009²², FINRES-vet 2005-2006²³, SVARM 2006²⁴, AFSSA 2005²⁵). In addition some countries have products for intramammary use on their market and this may have large impact on the sales figures²⁶.

6.2.5. Cephalosporin use and evolution of genes encoding β -lactamases

The use of 3rd- and 4th-generation cephalosporins can influence emergence of ESBLs and/or AmpC enzymes either by selecting for emerging mutants, or by giving selective advantage to bacteria producing these enzymes.

Human clinical evidence supports the impact of the selective pressure exerted by use of oximino- β -lactams on the evolution of ESBLs (Gniadkowski, 2008). Recently, (Novais et al., 2010) experimentally demonstrated that exposure of bacterial populations carrying CTX-M enzymes to challenges with ceftazidime and cefotaxime seems to have played a critical role in the diversification of these enzymes. In addition, constant pressure by the use of various β -lactams, including penicillins and first-generation cephalosporins, could have contributed to the *in vivo* evolution of ESBLs (Blazquez et al., 2000). As a result of use, mutations occurring in AmpC-encoding genes can extend their spectrum of activity to fourth generation cephalosporins, giving rise to the Extended Spectrum Cephalosporinases (ESCs) (Ahmed and Shimamoto, 2008; Mammeri et al., 2008; Mammeri et al., 2007; Wachino et al., 2006).

²² DANMAP 2009. http://www.danmap.org/pdfFiles/Danmap_2009.pdf.

²³ FINRES-vet 2005-2006. <http://www.evira.fi/uploads/WebShopFiles/1198141211941.pdf>.

²⁴ SVARM. <http://www.sva.se/en/Target-navigation/Animal-health/Antibiotic-Resistance/Monitoring-of-antimicrobial-resistance/SVARM-reports/>.

²⁵ AFSSA. Suivi des ventes de médicaments vétérinaires contenant des antibiotiques en France en 2005. <http://www.afssa.fr>

²⁶ EMA(EMEA/CVMP/SAGAM/81730/2006 rev 1).

6.2.6. Impact of cephalosporin use on selection and amplification of ESBL and/or AmpC

The use of cephalosporins and/or aminoglycosides was considered a risk factor for ESBL producing bacteria in human infections until the end of the 1990's when ESBLs of TEM and SHV-types were predominant. In the last few years, with the increasing observation of CTX-M enzymes prior fluoroquinolone use was identified as a new risk factor (Canton et al., 2008; Zahar et al., 2009).

A strong correlation between reduction in ceftiofur-resistant *S. Heidelberg* and ceftiofur-resistant *E. coli* (both producing AmpC) from both human infections and retail poultry in different regions of Canada and withdrawal of ceftiofur for disease prophylaxis in hatcheries was reported (Dutil et al., 2010). This use of ceftiofur is extra-label and involves injection of hatching eggs.

Some evidence of correlation between the use of cephalosporin antibiotics and ESBLs or AmpC-producing bacteria taken from treated animals is available. Studies conducted in Denmark demonstrated a correlation between the increased occurrence of *E. coli* with CTX-M-1 or AmpC and ceftiofur treatment in piglets (Jorgensen et al., 2007). (Dolejska et al., 2011) in a study conducted on a conventional dairy cattle farm with high consumption of parenteral and intramammary cephalosporins and on an organic dairy farm with no cephalosporin use, verified a correlation between cephalosporin use and prevalence of ESBL-producing *E. coli*.

Using an experimental study (Cavaco et al., 2008) demonstrated that ceftiofur and cefquinome exerted larger selective effects than amoxicillin in pigs colonized and/or inoculated with CTX-M-1-producing coliforms. Significantly higher counts of cefotaxime-resistant coliforms were observed in all treated groups compared with controls for up to 22 days after the end of treatment.

Experimentally administration of ceftiofur to feedlot cattle was also associated with an increase in the population of ceftiofur-resistant isolates (Lowrance et al., 2007). It has been argued that active concentrations of ceftiofur and cefquinome in the intestines of treated animals are very low, as they are mainly excreted in urine with a limited portion, excreted in faeces (Hornish and Kotarski, 2002); see also MRL summary reports available at www.ema.europa.eu). In the intestines ceftiofur is rapidly metabolised by the intestinal microbiota (Hornish and Kotarski, 2002). Undesirably the available information does not permit estimation of the exposure of the gastrointestinal microbiota to these drugs or active metabolites. Nevertheless, correlation between the use of ceftiofur and the occurrence of resistance at herd level has been documented (Jorgensen et al., 2007; Tragesser et al., 2006), showing that the concentrations are sufficient namely to select for *E. coli* and *Salmonella* with resistance to 3rd generation cephalosporins.

Cephalosporins are widely used as lactating or dry cow therapy in many EU MSs. Intramammary use of cephalosporins is generally considered to have less impact on the selection of resistant bacteria as a lesser exposure of the normal microbiota of the target animal occur. Nevertheless, besides the possibility of selection of resistance in target bacteria, the use of recently treated cows to feed calves before the withdrawal time has elapsed could also impose a selective pressure to the intestinal microbiota of the latter.

6.2.7. Other antimicrobials and selection and amplification of ESBL and/or AmpC

ESBL- and AmpC-producing bacteria often present resistance to multiple agents used in veterinary medicine, e.g. amoxicillin, amoxicillin-clavulanate, tetracycline, trimethoprim, sulphonamides, quinolones, phenicols, and streptomycin (Blanc et al., 2006; Carattoli et al., 2005; Machado et al., 2008; Smet et al., 2008). This frequent occurrence of resistance to several antibiotics implies that once ESBL- or AmpC-producing isolates have entered a production unit, a broad range of antimicrobials can favour their selection and spread between animals. Amoxicillin treatment proved to be a significant risk factor for emergence of ESBL- or AmpC-producing *E. coli* in broilers in Belgium (Persoons et al., 2010; Smet et al., 2008). Cross-resistance to these compounds is known, i.e.

amoxicillin and amoxicillin–clavulanic acid may select for isolates producing ESBLs or AmpC β -lactamases.

Resistance to several non- β lactams have been associated with isolates producing ESBLs or AmpC, being more frequently observed to tetracycline, streptomycin, trimethoprim, sulfamethoxazole and nalidixic acid. Genetic linkage between some resistance determinants (e.g. *dfrA* and *sul* genes conferring resistance to trimethoprim and sulphamethoxazole, respectively) and ESBLs/AmpC resistance genes favours the occurrence of ESBL/AmpC producers (Blanc et al., 2006; Machado et al., 2008; Persoons et al., 2010; Smet et al., 2008).

In addition to antimicrobials used in veterinary medicine, other chemicals used in animal production, as antiseptics, disinfectants and metals, might contribute to the selection of such resistant isolates (Aarestrup and Hasman, 2004; Cavaco et al., 2010; Hasman and Aarestrup, 2002).

6.3. Trade and movement of animals

An extensive trade of animals occurs in EU MS, with few countries leading the production and the export, and with a reduced number of companies producing pure line grandparent stock. How widespread are ESBL-carrying bacteria in food-producing animals in the breeding/rearing/fattening sectors is generally unknown, although few reports suggest that ESBL/AmpC are not uncommon in the top of some production pyramids (breeding) as presented in the sections below.

During the post-harvest phase including manufacturing and retail there is a constant flow of products in all directions in EU. This may have a significant impact in the dissemination of bacterial flora associated with food. Recent EFSA Opinions²⁷ have highlighted the importance of these factors in the spread of food-borne pathogens. Because of the complexity of this information, we will not further consider the trade of food in this document.

6.3.1. Swine

Pig farming is divided into two parts, farrowing sows, and their rearing (growing and finishing fattening pigs for slaughtering). The majority of smaller farms producing meat for household consumption and the local market are situated in the “new” EU countries, whereas overall in Europe 75 % of fattening pigs are reared by just 1.5 % of the largest production units²⁸. Most transports between EU countries of live pigs are between neighbouring countries with Germany being the major importer and Denmark the main exporter of weaners. The Netherlands is a substantial exporter of finishing pigs for slaughter.

6.3.2. Poultry

Broiler farming is built upon a pyramidal hierarchy with a very narrow top²⁹. It starts with the breeding company at which from pure line grandparent stock is produced and sold as day old chicks to rearing farms which produces the parent generation to the broilers. There are few breeding organisations worldwide and two companies have more than 85 % of the European market.

²⁷ EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on a quantitative estimation of the public health impact of setting a new target for the reduction of *Salmonella* in broilers. EFSA Journal 2011;9(7):2106. www.efsa.europa.eu/en/efsajournal/pub/2106.htm

²⁸ Eurostat8/2010: Pig farming in the EU, a changing sector. http://epp.eurostat.ec.europa.eu/cache/ITY_OFFPUB/KS-SF-10-008/EN/KS-SF-10-008-EN.PDF

²⁹ Development of new integrated strategies for prevention, control and monitoring of epizootic poultry diseases) Commission of the European Communities, Project no.: SSPE-CT-2004-513737: Chapter 4 Trade in live poultry within the European Union.

Farming of layers follows a similar structure. Two companies in the Netherlands and France supply more than 90% of the layer stock in Europe.

The main flow of trade of fattening broilers is between the three neighbouring countries Belgium, the Netherlands and Germany, whereas France is biggest in slaughter of chicken. The main transport routes for breeding poultry are from the Netherlands to Germany, from Czech Republic to Slovakia and Poland and from France to Spain. The trade can be either day-old chicks for broilers farms or day-old chicks of parent- or grandparent stock.

To understand more about the spread of ESBL- and/or AmpC-producing *E. coli* in the Dutch broiler industry, different levels in the broiler production chain were examined for the presence and prevalence of these bacteria. ESBL- and/or AmpC-producing *E. coli* were present at every level that was examined, including imported day old chickens from great grand parent stock, sampled on the day of arrival in the Netherlands. Prevalence seemed lower in day-old parents and day-old broiler chickens and higher in day-old grandparent chickens, which are derived from outside the Netherlands (UK and USA). The low prevalence in day-old broiler chickens is unexpected as previous research showed that these animals at 6 weeks of age are almost all positive for ESBL and/or AmpC-producing *E. coli* (Dierikx et al., 2010a), (MARAN-2009)³⁰.

Grandparents at weeks 18 and 31 of age were found positive at moderate level, which indicates that especially on Dutch broiler production farms optimum circumstances are present to multiply ESBL- and/or AmpC-producing bacteria. Preliminary data on genetic analysis of these isolates show that throughout the production chain *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{CMY} are the predominant β -lactamase families present (MARAN-2009).

The data from this study indicate that ESBL- and/or AmpC-producing *E. coli* are introduced in the Dutch poultry production chain through imported day-old grandparent chickens. Moreover the data indicate that the occurrence of these organisms in the different levels layers of the Dutch poultry production chain is the result of vertical transmission, local recirculation and selection. Further research is currently being conducted to understand more about the driving forces that have led to a rapid spread of ESBL- and/or AmpC-producing *E. coli* in broilers at the broiler production farms (MARAN-2009).

In Sweden cephalosporins are not used in broilers; thus, occurrence of *E. coli* of CTX-M-1 and CMY-2 genotypes is not associated with a selection pressure through use of such antimicrobials in that country. Instead, transmission of resistant bacteria from breeding stock was suspected. This was supported by findings of *E. coli* carrying genes coding for CMY-2 or CTX-M-1 in environmental samples from hatcheries hatching production animals or breeding-stock (parent animals) of both hybrids produced in Sweden, collected in May to June 2010. Moreover, *E. coli* carrying *bla*_{CMY-2} were found in intestinal content of day-old chickens imported as breeding stock (grand parents) in July to December 2010 and sampled on arrival to Sweden. These findings indicate a spread of cephalosporin-resistant *E. coli* from imported breeding stock into Swedish broiler production. The temporal variation in proportion of CMY-2 or CTX-M-1 genotypes among Swedish broilers might reflect dissemination of different lineages of resistant bacteria from different batches of imported breeding stock (SVARM 2010)³¹.

³⁰ MARAN-2009 Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2009. http://www.lei.wur.nl/NR/rdonlyres/4ED137F2-5A0D-4449-B84E-61A3A8AC4A42/135753/MARAN_2009.pdf

³¹ SVARM 2010, Swedish Veterinary Antimicrobial Resistance Monitoring. The National Veterinary Institute (SVA), Uppsala, Sweden, 2011. www.sva.se.

(Weill et al., 2004) have described CTX-M-producing *S. Virchow* in six chicken farms and one hatchery supplying these farms, as well as from poultry meat. (Persoons et al., 2010) also associated the presence in poultry of ESBL-producing *E. coli* with the hatchery supplying these farms.

6.3.3. Cattle

Cattle production is divided in dairy and beef. Trade of live animals occurs for all categories of animals but is likely to be most pronounced for male calves from dairy farms which are sold to veal production.

6.4. Post-harvest risk factors

Since bacteria that could carry ESBL or AmpC genes are known as common inhabitants of the intestinal tract of animals, it is expected that they contaminate carcasses during the slaughtering process. Any measure by which microbial contamination is reduced at slaughter, or during further processing and retailing will also indirectly help to contain the spread of ESBL/AmpC-producing bacteria to humans.

6.4.1. Food handling

Contamination of meat products, with resistant bacteria from human or animal reservoirs may contribute to further spread within human population. Although it may be assumed that AMR bacteria will follow the same principles as the corresponding antimicrobial-sensitive strain, there may be additional risks related to resistant bacteria. This is because the resistance genes may transfer from food-borne commensals to non-food-borne human pathogens. Thus commensals are a part of the overall risk pattern. This has been shown to be particularly applicable to ESBLs (Lavilla et al., 2008; Mesa et al., 2006)

6.5. Risk factors associated with food of plant origin

Contamination of fresh food of plant origin have been increasingly recognized in many parts of the world as a source of pathogens (Lynch et al., 2009). Moreover, these food products may serve as a vehicle of ESBLs, as demonstrated by the recent description of *Enterobacteriaceae* carrying CTX-M genes in spinach, parsnip, bean sprouts and radish (Raphael et al., 2011; Reuland et al., 2011a) and SHV-2 in ready-to-eat salads (Campos et al., 2011).

ESBLs have been detected in Dutch vegetables. Out of 79 analyzed samples, four yielded ESBL-producing *Enterobacteriaceae* (5%). ESBLs were found in parsnip, bean sprouts and radish; this means that three (17,6%) of the vegetable types were contaminated with ESBLs. Of the four positive samples, three were from vegetables of organic origin. The ESBL-producing strains were *Enterobacter cloacae* (in two samples), *Citrobacter braakii* (in one sample) and *Klebsiella pneumoniae* (in one sample). Three strains carried a *bla*_{CTX-M-1}, and one a *bla*_{SHV} gene (Reuland et al., 2011b).

Recently a large outbreak produced by CTX-M-15-producing *E. coli* O104:H4, and linked to the consumption of contaminated sprouts has occurred in Northern Europe (http://ecdc.europa.eu/en/healthtopics/escherichia_coli/epidemiological_data/Pages/Epidemiological_updates.aspx).

The possibility of human acquisition of bacteria producing ESBL or AmpC, may also be influenced by the increasing consumption of fresh produce (Lynch et al., 2009). Nevertheless, as yet few studies have addressed the impact of these factors.

7. POSSIBLE CONTROL OPTIONS (IDENTIFICATION AND RANKING) TO REDUCE THE PUBLIC HEALTH RISK CAUSED BY ESBL- AND/OR AMPC-PRODUCING BACTERIAL STRAINS TRANSMITTED VIA THE FOOD CHAIN OR VIA FOOD ANIMAL PRODUCTION ENVIRONMENT

The assessment of the efficiency of control options to reduce the public health risk caused by ESBL/AmpC-producing food-borne bacteria is particularly complicated by data unavailability. The section below summarises the present knowledge in general terms, and based on best available evidence and expert opinion.

During recent years a number of different bodies have discussed and proposed control options to minimise public health risks related to AMR. For instance OIE have, in their terrestrial code, published a chapter 6.9 entitled “responsible and prudent use of antimicrobial agents in veterinary medicine (<http://www.oie.int/international-standard-setting/terrestrial-code/>) and Codex Alimentarius are about to finalise their taskforce of antimicrobial resistance (http://www.codexalimentarius.net/web/index_en.jsp) where a list of possible control options will be presented. Recently the BIOHAZ panel has described the need to control the spread of antimicrobial resistant organisms and resistance genes (<http://www.efsa.europa.eu/en/efsajournal/doc/765.pdf>). In the above report control options for spread of AMR bacteria and/or resistance genes from animals, plants and derived foods were described, and prudent use policies for antimicrobial usage were discussed in general terms.

The effect of reducing the prevalence of ESBL- and/or AmpC-producing bacteria in animals/food on public health risks is difficult to assess. Although there is evidence of the contribution of AMR microorganisms transmitted via food-producing animals/food to public health risks, it is currently difficult to quantify that risk. Moreover the magnitude of the contribution from animals/foods to public health risks and the perception of the risks for humans will be greatly influenced by the local epidemiology of ESBL- and/or AmpC-producing organisms in health care and in the community. This epidemiology may vary greatly by country. In countries where human associated ESBL- and/or AmpC-producing clones have spread endemically in health care and the community, contaminated animals/foods will have a minor relative importance compared to countries where these organisms occur only incidentally in health care and the community.

In the present report these control options are more explicitly aimed at control of the **emergence** and **spread** of ESBL and/or AmpC-producing bacteria in animals and foods and their transmission to humans are discussed. Public health risks caused by ESBL- and/or AmpC-producing bacteria are determined by the frequency of the occurrence (prevalence) and the quantity of these organisms in food-producing animals and food, the genetic characteristics of the β -lactamase genes involved, and the transmission from animals/foods to humans. Mitigation measures should therefore aim to reduce the prevalence in animals, and food and to reduce transmission from contaminated animals/foods to humans.

The prevalence of resistant organisms in a certain niche (animals or foods) is determined by two basic mechanisms: **selection** and **dissemination**.

7.1. Selection

Selection of resistant bacteria and/or resistance determinants is primarily caused by usage of antibiotics. The usage of 3rd- and 4th-generation cephalosporins in animals will specifically select for ESBL- and or AmpC-producing *Enterobacteriaceae* in the gastrointestinal tract. Due to the multiresistant nature of these organisms many other drugs also indirectly co-select for them. Such drugs include (fluoro)quinolones, aminopenicillins, trimethoprim, sulphonamides, aminoglycosides and tetracyclines. These drug classes include the vast majority of the antibiotics used in food-producing animals, demonstrating the complexity of the problem once ESBL- and/or AmpC-producing bacterial populations have been established in animal production sectors. Selection may

also occur through disinfectants (e.g. quaternary ammonium derivatives), or metals (e.g. mercury and copper), because genes encoding for resistance to these compounds are known to occur in multiresistant organisms. As yet, the *in vivo* effects of these compounds on these bacterial populations are unknown.

The current systems for marketing authorisation approval of antimicrobials in the EU include a microbial safety assessment and instruction for restrictive and appropriate usage is included in the summary of product characteristics (SPC) and on the label. Once a product is licensed for usage in food animals the control over its usage, according to the label instructions, is limited as legislation allows for use outside approved indications in certain cases (see 6.2.2 above) and it can be that the use in practice is much more extensive than what would be expected from the conditions of the authorisation. For this reason full transparency of antimicrobial usage in food-producing animals, together with increased control of that usage are crucial aspects of any policy to improve the quality of usage and decrease the exposure of animals and animal flora to antimicrobials.

In order to reduce the selection pressure there is a need to reduce the overall antimicrobial burden, with a special focus on restriction of use of systemically active 3rd- and 4th-generation cephalosporins and restriction of all use of antimicrobials that are not strictly needed for animal health.

7.1.1. Restrictive usage of systemically active 3rd- and 4th-generation cephalosporins in animals

In 2007, recommendations on the use of 3rd- and 4th-generation cephalosporins in food producing animals (EMEA/CVMP/SAGAM/81730/2006) were presented by EMA (www.ema.europa.eu), following a consultation a revised document was published in 2009 (EMEA/CVMP/SAGAM/81730/2006 rev 1). The recommendations covered systemic use and it was concluded that systemic 3rd- and 4th-generation cephalosporins are to be reserved for the treatment of clinical conditions in animals which have responded poorly, or are expected to respond poorly, to more narrow spectrum antimicrobials. In addition it was recommended that prophylactic use of systemically administered cephalosporins should always be limited to specific circumstances. Oral use for group medication in food-producing animals and off label use was strongly discouraged. The recommendations are implemented as specific texts in the SPCs for centrally-approved products but not yet for national products in all member states. Notably there are approved indications such as metritis and interdigital necrobacillosis, which are normally mixed infections where the causative agents (and its resistance pattern) are rarely known. In practice the recommendation given are not applicable for such indications.

In some MSs there are national recommendations/regulations for the use of cephalosporins. National legislation in Finland prohibits the use of 3rd- and 4th-generation cephalosporins for animals (with the exception of use according to label where there is a market authorisation or a special licence). In Denmark and France pig producers have in 2010 agreed to a two year moratorium on the use of cephalosporin antibiotics.

Possible control options on EU level would be to **stop** all uses of cephalosporins/systemically active cephalosporins/systemically active 3rd- 4th-generation cephalosporins in animals, **or** to **restrict** their use (i.e., use only allowed under specific circumstances). The more comprehensive the restriction, the more prominent the effect on selection pressure would be, although a very restrictive policy might have unintended consequences on animal health and welfare if effective antimicrobials are not available for treatment.

Examples (partly overlapping and not in a priority order):

- Systemic usage (oral or by injection):
 - Restriction of systemic use of 3rd-4th generation cephalosporins for treatment of individual animals if according to appropriate diagnostics performed, no alternative antimicrobials are available.

Advantages and disadvantages:

- Effective implementation would result in substantial reduction of the exposure of animals and their bacterial flora and less opportunities for selection of ESBL/AmpC producers.
- If the use of cephalosporins is replaced with use of other antimicrobials the effect on selection pressure might be negligible, as there might be co-resistance.

Comments:

- As long as there are alternatives available in veterinary medicine the negative impact on animal health is likely to be minor. There is today no infectious disease where cephalosporins are approved for use and where they are the sole available treatment.
 - Recommendations for this measure have already been given by EMA/CVMP. Implementation has proven to be difficult as there is to day no means to measure compliance.
- Prohibition of systemic usage for prevention or metaphylaxis.

Advantages and disadvantages:

- This would result in reduced selection of resistant organisms or genes and would *not interfere with therapeutic efficacy during treatment*
- If use of cephalosporins is replaced with use of other antimicrobials the effect on selection pressure might be negligible, as there might be co-resistance.

Comments:

- There is currently no definition for metaphylaxis. The expression includes both strategic treatment and unnecessary routine use.
 - As long as there are alternatives available in veterinary medicine, the negative impact on animal health and welfare is likely to be minor.
 - Of special concern are the long-acting formulations where one injection is a full course of treatment, they can be more frequently used for convenience reasons, and exert a long lasting selective pressure in bacterial flora.
- Measures to discourage/prohibit use as group, or flock medication

Advantages and disadvantages:

- Group or flock medication of cephalosporins will select for ESBL/AmpC producers or ESBL/AmpC-genes.

Comments:

- This kind of use is not approved in EU today and is only allowed according to the cascade (see section 6.2.2 above) by way of exception if there are no approved alternatives available. It is believed that mass medication with cephalosporins is quite common in piglets and day-old chickens.
- Restriction of use of products with prolonged elimination half-life for limited indications in individual animals and where no alternatives are available.

Advantages and disadvantages:

- Application of products with prolonged elimination half life will result in prolonged exposure of bacterial flora in animals.

Comments:

- There are few (if any) occasions where no alternatives are available.
- Currently, there is at least one such product approved but its approved use includes a warning sentence to restrict its use in accordance with the above. There are anecdotal data that the compliance with this restriction is poor.
- Topical or local administration (e.g. intra-uterine, intramammary)
 - Restriction of use for clinical cases of mastitis, and for dry cow treatment in individual animals if according to appropriate diagnostics performed no alternative antimicrobials are available.

Comment:

- Local application of cephalosporins in the udder may have less selective effect on the microbial flora of animals outside the udder, than after systemic or oral administration.
- It is believed that restrictions in topical use will, to some degree, reduce exposure of bacterial flora in the farm environment to these antimicrobials.

- Off-label usage
 - Implement control measures covering all off-label usage of 3rd and 4th generation cephalosporins in food-producing animals, including all types of usage at hatcheries (*in ovo*, injection, spray)

Advantages and disadvantages:

- Effective implementation would result in substantial reduction of the exposure of animals and their bacterial flora and less opportunities for selection of ESBL/AmpC producers (and MRSA) or ESBL/AmpC-genes.

Comment:

- There is some off-label use in minor species (e.g. multiresistant infections of foal septicaemia), which is justified.
- It is likely that most off-label use in EU today is not in accordance with current legislation (if strictly interpreted) and this includes all uses to poultry.
- Interpretation of the cascade rule is problematic.

7.1.2. Restrictive usage of all antimicrobials in food-producing animals

With the exception of some products for companion animals, all antimicrobials in EU are prescription only. Some countries (e.g. Denmark and Sweden) have special legislation to separate prescription and sales of antimicrobials to avoid economical incentives for prescription.

Prudent use guidelines have been available since the early 1990s, but their implementation and adoption has not been consistent. In general there is a need to take measures to increase compliance with appropriate prudent use policies in EU-MSs (e.g. OIE, see above). Many prudent use policies have been documented. Implementation is complex and control on its effectiveness is limited. To apply such control there is a need for improvement of transparency in antimicrobial usage by the development and implementation of antimicrobial usage registration systems at the national and EU-level.

An essential part of the implementation process is full transparency in antibiotic prescription and usage data. This would allow the identification of frequent prescribers or users and targeted intervention measures. These systems could e.g. aim at:

- quantify the exposure of food-animal species to antibiotics
- identify users (veterinarians or farms) with a high consumption patterns
- determine and report trends in usage by food-animal species and country
- identify inappropriate, off-label and/or illegal usage

Measures to increase awareness about AMR related risks and to promote the use of appropriate diagnostics could in some settings reduce the total use of antimicrobials.

Comment:

- There are numerous different possible measures to promote implementation of prudent use principles, to increase awareness about AMR-related risks and to promote the use of vaccines and appropriate diagnostics to reduce the total use of antimicrobials

7.1.3. Improved control on antimicrobial usage in animals in MS

Control on antibiotic prescription and usage is complex and mostly limited to the inspection services of national authorities, and aimed at illegal practices. There are also differences in interpretation of what is legal or appropriate according to prudent use policies. There is no comprehensive information on usage practices in the EU, which may encourage propagation of uncontrolled use. Countries that have implemented such a strict control system have lower usage of antimicrobials. As an example, in order to increase compliance with their national treatment guidelines, Denmark has recently introduced a “yellow card system” (Government Order No. 1319 of December 1st 2010 on special provisions for the reduction of the consumption of antibiotics in pig holdings) where farmers sign a contract and allow inspections on their use of antimicrobials.

The review of the legal framework on authorisation and use of veterinary medicinal products is ongoing. Antimicrobial resistance is one of the issues addressed and the impact of different policy options to tackle it are assessed. The summary of the comments received in public consultation and the impact assessment will be published in 2011 and proposals for legislation can be expected in latter half of 2012.

Examples of control activities could be:

- Setting of targets for quantity and quality of antibiotic usage allowed in food-producing animals
- Analyse and report of usage data on farms and by veterinarians
- Identification high users and/or prescribers
- Suggestion of strategies to improve usage/prescription practices
- Identification of illegal, off-label and inappropriate use practices
- Independent drug authorities could define clear targets for appropriate usage, and target strategies for improvement to high users or prescribers.

7.2. Dissemination

Dissemination of ESBL- and/or AmpC-producing bacteria occurs within the gut of animals, and by cross-contamination with faecal material between animals. In the modern intensive animal production systems the possibilities for transmission of enteric organisms between animals is virtually unlimited. Moreover, in all major food-animal production systems massive movement of young animals from reproduction farms to fattening farms occur within and between countries, facilitating continuous transfer of organisms between farms and countries. Trade of food products is a global industry and also contributes to global distribution of AMR organisms and resistance genes.

Because ESBLs are found in certain animals/animal products where antimicrobials are not used in the species in question, measures need to consider both selection and dissemination to be effective.

7.2.1. *Prevention of dissemination of antimicrobial resistance*

- Within farms
 - Optimize biosecurity and farm management to prevent dissemination of resistant bacteria and/or resistance genes by considering:
 - Numbers of animals per square meter
 - Movements of animals within farms
 - Hygiene at farms
 - Avoid mix of animals of different age
 - Use of “all in–all out” systems

Advantages and disadvantages:

- Such measures could generally improve animal health and welfare.

Comments

- The lower the density of the animal population, the fewer the opportunities for contacts between groups of animals, therefore resulting in reduced spread of AMR.

- Much could be achieved by including biosecurity considerations already when buildings and production units are planned.
- Between farms and regions/countries
 - Stimulation of more local, or closed production systems.
 - Improved control of presence of antimicrobial resistant bacteria (like ESBL and/or AmpC producers) at breeder and reproduction farms, and hatcheries to prevent transmission in production pyramids.
 - Definition of acceptable quantitative limits for the presence of resistance genes and/or resistant bacteria in young animals at breeder/reproduction farms.
 - Introduction of systems to certify animals/farms which are documented free from ESBL- and AmpC-producing micro-organisms.

Advantages and disadvantages:

- These measures are likely to be important in the long term.

Comment:

- As a starting point it may be valuable to have a voluntary system for trade of AMR-free animals.

- Into the environment
 - Optimized management of farm waste and prevention of contamination of arable crops by faecally-contaminated water

Comment:

- Due to the complexity of the issue there are little data that would allow estimation of the magnitude of the related risk.

7.2.2. *Post-harvest measures*

Improved hygiene at all steps of the food chain is effective in reducing the number of micro-organisms in food. This will also reduce the numbers of micro-organisms that are resistant to antimicrobials including ESBL- and/or AmpC-producing microorganism. The sustained application of good hygienic practices throughout the food chain provides a varying degree of assurance against the introduction of AMR bacteria onto or into food³². There are no indications that AMR strains behave differently in the food chain compared to their sensitive counterparts. Hence, effect estimates of interventions along the food chain for antimicrobial-sensitive and antimicrobial-resistant strains will be similar.

Advantages:

- Post-harvest measures would be effective without delay in case of rapid alerts nationally, and when appropriate internationally through the EU Rapid Alert System for Food and Feed (RASFF).

³² Foodborne antimicrobial resistance as a biological hazard-Scientific Opinion of the Panel on Biological Hazards. <http://www.efsa.europa.eu/en/efsajournal/pub/765.htm>

- These measures are expected to have a more immediate impact (short-term) in helping to control the spread of AMR microorganisms and resistance genes.

Disadvantage:

- Improved hygiene as a sole measure will not mitigate the selection at source (primary production) of AMR microorganisms/resistance genes.

7.2.3. Measures to reduce trade of ESBL/AmpC-contaminated food-producing animals and derived food

- Introduction of trade restrictions (imports/exports) of animals and food carrying bacteria that harbour ESBL/AmpC-encoding genes.
 - Screening of imported foods and food animals for defined organisms and resistance traits

7.3. Prioritisation of measures

There are no data on the comparative efficiency of the individual control options presented in this document in reducing public health risks caused by ESBL and/or AmpC-producing bacteria related to food-producing animals. Prioritisation is complex. The effectiveness of measures discussed here are based on the best available evidence and expert opinion.

Cephalosporins (especially 3rd- and 4th-generation) specifically select for ESBLs. It is considered that a control option that is likely to be **highly** effective in reducing selection of ESBL/AmpC-producing bacteria at an EU level is stopping/reducing the use of cephalosporins in food animals. Provided adequate compliance, the measure would be more effective the more comprehensive the restrictions. The restrictions could range from **stopping all uses** of cephalosporins/systemically active 3rd/4th generation cephalosporins, to more or less strict **restriction of their use**, allowing use only under specific circumstances.

Off label use of veterinary medicinal products, including cephalosporins, is restricted according to articles 10 and 11 of Directive 2001/82/EC as amended. Such use should be limited to use by way of exception, under the veterinarian's direct personal responsibility and in particular to avoid causing unacceptable suffering. Therefore, the use of cephalosporins could be reduced by measures intended to minimize such use should focus on **increase the compliance with existing legislation** e.g. by:

- Inspections to investigate use of cephalosporins aiming at minimizing unnecessary and illegal use.
- Inspection of status of national competent authorities' implementation of aiming at ensuring compliance with the original intention of the legislator.
- Reinforcement by adding a specific piece of legislation to stop off-label cephalosporin use in group or flock medication.

As co-resistance is an important issue, it is unlikely that restriction of cephalosporin use will be completely effective in reducing public health risks caused by ESBL and/or AmpC-producing bacteria related to food-producing animals. Therefore, risk managers would need to consider uses of all antimicrobials. The most radical measure is to stop all uses. As an alternative, there are other measures likely to be effective to **decrease the total antimicrobial use in animal production in the EU**, for example:

- Implementation of systems to monitor and control antimicrobial usage at MS and EU level.
- Implementation of measures to ensure transparency in antimicrobial usage (at farm and prescriber level).
- Promotion of more tailored treatments by implementation of adequate diagnostic tools.
- Information campaigns on prudent use principles targeting farmers and responsible veterinarians.
- Stopping antimicrobial use at hatcheries.

Also of importance (especially after the ESBLs/AmpC-producing microorganisms have emerged) are **measures to control dissemination**, by for example:

- Promotion of closed production systems with high biosecurity standards.
- Introduction of EU monitoring systems to control trade of ESBL/AmpC-contaminated food-producing animals in production pyramids.
- Improved hygiene throughout the food chain.
- Other general post-harvest controls for food-borne pathogens.

Because most evidence is available for high prevalence of ESBL/AmpC-producing bacteria in the **poultry production pyramid**, and their consequent involvement in public health, it is of high priority:

- To reduce selection pressure imposed by the use of antimicrobials.
- To prevent vertical transmission from the top of the poultry production pyramid.
- To prevent local recirculation within subsequent flocks.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

ESBL- and AmpC-producing bacterial strains and genes relevant for public health and linked to food producing animals or food borne transmission

- The potential contribution of food-producing animals or foods to public health risks by ESBL and/or AmpC-producing bacteria is related to specific plasmid-mediated ESBL and/or AmpC genes encoded by a number of organisms.
- The predominant ESBL families encountered are CTX-M, TEM, and SHV. The predominant AmpC-family is CMY. By far the most common genes associated with this resistance in animals are *bla*_{CTX-M-1} (the most commonly identified ESBL), and *bla*_{CTX-M-14}, followed by *bla*_{TEM-52} and *bla*_{SHV-12}. Among the genes encoding AmpC-type β -lactamases, *bla*_{CMY-2} is the most common.
- The bacterial species most commonly identified with these genes are *Escherichia coli* and non-typhoidal *Salmonella*.
- Among *E. coli*, the clonal lineages: phylogroup B2-*E. coli* O25:H4-ST131, phylogroup D-*E. coli* O25a-ST648 and phylogroup D- *E. coli*-ST69, -ST393, are being increasingly detected among both humans and animals. Among *Salmonella* the most common serovars are Typhimurium, Newport, and Heidelberg.
- ESBL/AmpC transmission is mainly driven by integrons, insertion sequences, transposons and plasmids, some of which are homologous in isolates from both food-producing animals and humans. While ESBL genes are mainly spread by plasmids of IncFII, IncA/C, IncL/M, IncN,

IncK, and IncII groups, AmpC genes are mainly associated with IncA/C, IncII or IncI2 plasmids.

The epidemiology of acquired resistance to broad spectrum cephalosporins in food-producing animals and food

- Cefotaxime is used as the drug of choice for optimum detection of *bla*_{ESBL} and/or *bla*_{AmpC} genes in *Salmonella* and *E. coli*. From the results presented in the Community Summary Report it can be concluded that the prevalence of resistance to cefotaxime in food-producing animals varies by country and animal species. High prevalences are observed in *E. coli* and *Salmonella* in poultry in Spain, Italy, the Netherlands and Poland. In raw meat from poultry only limited cefotaxime resistance prevalence data are available. Belgium and the Netherlands reported high to moderate cefotaxime resistance prevalences in *Salmonella* and *E. coli* from poultry meat. In pigs and cattle the prevalences were low.
- Since 2000, the presence of ESBL- and/or AmpC-producing *Salmonella* and *E. coli* in animals and food has been increasingly reported in both Europe and globally. Although these enzymes have been described in bacteria from all major food-producing animals, poultry and poultry products are most frequently reported to carry ESBL and/or AmpC-producing bacteria.
- The most frequently reported ESBL subtypes in the EU in both *Salmonella* and *E. coli* in food-producing animals and foods are CTX-M-1, CTX-M-14, TEM-52 and SHV-12; the predominant plasmidic AmpC variant described globally to occur in *Salmonella* and *E. coli* from food-producing animals or foods since the mid-1990s is CMY-2.
- A wide range of additional CTX-M subtypes (CTX-M-1, -2, -3, -8, -9, -14, -15, -17/18, -20, -32, -53) have been detected in food-producing animals and food in European countries.
- A range of additional TEM (TEM-20, -52, -106, -126) and SHV (SHV-2, -5, -12) variants have similarly been detected in different European countries.
- Epidemic plasmids belonging to the incompatibility groups F, A/C, N, HI2, II and K groups carrying particular ESBL-encoding genes (*bla*_{TEM-52}, *bla*_{CTX-M-1, -9, -14, -32}) or AmpC-encoding genes (*bla*_{CMY-2}) have been detected among farm and companion animals, food products and humans.
- There are few studies that describe clear evidence of direct transmission of ESBL or AmpC-producing *E. coli* isolates from food-producing animals or food to humans. Data do exist about common clones of ESBL- and/or AmpC-producing *E. coli* isolates in humans and food-producing animals and foods, which provide indirect evidence about this transmission.
- Comparison of *E. coli* derived from humans and poultry has shown that antibiotic-resistant *E. coli* isolates from both reservoirs are more frequently genetically related than antibiotic-susceptible isolates. Recent findings indicate transmission of ESBL genes, plasmids and clones from poultry to humans is most likely to occur through the food chain.
- There is limited evidence for spread of ESBL/AmpC-carrying organisms *via* direct contact with animals or indirectly via the environment. Nevertheless people working with poultry have a higher risk for intestinal carriage of ESBL/AmpC-producing bacteria.

Methods for detection (isolation and identification) and characterisation of ESBL and/or AmpC-producing bacteria, encoding genes and associated mobile elements

- The preferred method for selective isolation of ESBL- and/or AmpC producers is selective isolation on agar preceded by selective enrichment in a broth. The preferred method for

selective isolation of ESBL- and/or AmpC producers is chromogenic (e.g. MacConkey agar) with 1 mg/L cefotaxime or ceftriaxone. Using low concentrations will result in optimum sensitivity to detect all relevant β -lactamase families. Pre-enrichment may be performed in a general broth like Mueller-Hinton, Brain Heart Infusion or Luria-Bertani broth with 1 mg/L cefotaxime or ceftriaxone.

- Identification is performed by determination of susceptibility to cefotaxime, ceftazidime and ceftioxin. ESBL-producers are resistant to cefotaxime, variably resistant to ceftazidime and susceptible to ceftioxin. Confirmation of ESBLs is performed by testing for synergy with clavulanic acid by combination disks, ESBL-etest or broth micro-dilution including cefotaxime, and ceftazidime as single drugs, and in combination with clavulanic acid. Confirmation of AmpC producers is performed by determination of susceptibility to cefepime. AmpC producers are susceptible to cefepime and resistant to cefotaxime, ceftriaxone and ceftioxin.
- To identify ESBL and/or AmpC-suspected *Enterobacteriaceae* by broth micro-dilution susceptibility tests, optimum breakpoints or interpretive criteria need to be used. Although CLSI has recently redefined MIC breakpoints for 3rd- and 4th-generation cephalosporins, the R-breakpoints for ceftazidime, ceftioxin and cefepime are still one to two dilution steps higher than those defined by EUCAST. In order to harmonize the interpretation of susceptibility data and for optimum phenotypic detection of ESBL and/or AmpC producers, it is important to use EUCAST clinical breakpoints for interpretation of susceptibility or resistance and EUCAST ECOFFs to determine if an isolate belongs to the wild-type population or not.
- All isolates phenotypically confirmed to be either ESBL or AmpC-producers may be screened for β -lactamase gene families using micro-array or (multiplex) PCR. The ESBL and/or AmpC subtypes may be identified by dedicated PCRs and sequence analysis of the amplicons.
- Characterization of plasmids on which *bla*_{ESBL} and/or *bla*_{AmpC}-genes are located is essential to study the epidemiology of these genes and plasmids. Since in *Enterobacteriaceae* several different plasmids are often present in each isolate, a structured approach is needed to identify the characteristics of the plasmid on which the β -lactamase genes are located. If the presence of an ESBL and/or AmpC gene in a bacterial isolate is confirmed, plasmid isolation is performed to determine the number and sizes of plasmids present. Subsequently, by conjugation or electroporation, tranconjugants or transformants are isolated on selective agar plates with only the plasmid that harbours the β -lactamase gene present. The plasmid can be typed using replicon typing and sub-typed by fingerprinting or MLST. Ultimately whole plasmid sequence analyses may replace the current typing and subtyping techniques.
- The choice of the molecular typing method to be used for isolates is determined by epidemiological relatedness of the isolates. Next to phenotypic methods such as serotyping, and phage typing, PFGE or MLVA can be used to identify clusters of isolates that are related to a certain 'outbreak' in a restricted time frame. MLST is generally the method of choice to identify relatedness of isolates of the same species from different backgrounds (eg. animal versus human).

Risk factors contributing to the occurrence, emergence and spread of ESBL- and/or AmpC-producing bacteria

- The establishment of risk factors for occurrence of ESBL/AmpC-producing bacteria is particularly complicated by the data unavailability or lack of its accuracy. Few studies designed to assess risk factors for ESBL and/or AmpC occurrence in animals are available.

- The use of antimicrobials is a risk factor for emergence and spread of resistant clones. Most ESBL- and AmpC-producing strains carry additional resistances such as to sulphonamides and other commonly-used veterinary drugs. Therefore, generic antimicrobial use is a risk factor for ESBL/AmpC and it is not restricted specifically to the use of cephalosporins.
- Currently there are no pan-European data available on the use of antimicrobials. The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), coordinated by EMA, is collecting information.
- An extensive trade of animals occurs in EU MS, with few countries leading the production and the export, and with a small number of companies producing pure line grandparent stock. How widespread are ESBL-carrying bacteria in food-producing animals in the breeding/rearing/fattening sectors is generally unknown, although few reports suggest that ESBL/AmpC are not uncommon in the top of some production pyramids (breeding).
- ESBL- and/or AmpC-producing *E. coli* are disseminated in the poultry production chain through day-old grandparent chickens. Moreover, some data indicate that the occurrence of these organisms in the different levels of the poultry production chain is the result of vertical transmission, local recirculation and selection.

Identification and ranking of possible control options taking into account the expected efficiency in reducing public health risk caused by ESBL and/or AmpC-producing bacterial strains transmitted via the food chain or via food animal production environment

There are no data on the comparative efficiency of individual control options presented in this document in reducing public health risks caused by ESBL and/or AmpC-producing bacteria related to food-producing animals. Prioritisation is complex, and the effectiveness of measures discussed in this Opinion are based on the best available evidence and expert opinion.

- It is considered that a highly effective control option to reduce selection of ESBL/AmpC-producing bacteria at an EU level, would be to stop all uses of cephalosporins/systemically active 3rd-4th generation cephalosporins, or to restrict their use (use only allowed under specific circumstances).
- It is important to implement control measures covering all off-label usage of cephalosporins in food-producing animals.
- Measures intended to minimize off label use of antimicrobials should focus on increased compliance with existing legislation.
- As co-resistance is an important issue, it is of high priority to decrease the total antimicrobial use in animal production in the EU.
- Also of importance (more so after the ESBL/AmpC-producing microorganisms have emerged) are the measures to control dissemination, for example, by implementing increased farm biosecurity and controls on animal trade (of ESBL/AmpC carriers), by improving hygiene throughout the food chain, and by implementing other general post-harvest controls for food-borne pathogens.
- Because most evidence is available for high prevalence of ESBL/AmpC-producing bacteria in the poultry production pyramid, and their consequent involvement in public health, it is of high priority:
 - To reduce selection pressure imposed by the use of antimicrobials.

- To prevent vertical transmission from the top of the poultry production pyramid.
- To prevent local recirculation within subsequent flocks.

RECOMMENDATIONS

Harmonised monitoring of resistance caused ESBL- and/or AmpC-producing bacteria

- Harmonised monitoring of resistance caused by ESBLs and AmpC will need to go beyond the existing recommendations for routine phenotypic surveillance. Specifically, genotypic resistance testing should be performed in addition to the phenotypic testing foreseen in the existing recommendations.
- For surveillance schemes, the analysis of isolates deriving from passive surveillance schemes (diagnostic submissions), from systematic sampling, and from targeted surveys, using selective isolation methods and pre-enrichment of samples, should be undertaken.

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APPENDICES

A. RESISTANCE TO CEPHALOSPORINS AMONG *SALMONELLA* AND *E. COLI* IN THE EU

Table 13: Resistance (%) to cefotaxime among *Salmonella* spp. isolates from poultry, pigs and cattle, and meat thereof in 2007, 2008 and 2009, using harmonised epidemiological cut-off values

N is the number of isolates tested

Ceftiofur and ceftazidime susceptibility test results are additionally mentioned in blue and in red, respectively, in the table.

Green shading: resistance detected to cefotaxime was not detected to ceftazidime / Red shading: resistance detected to ceftazidime was not detected to cefotaxime

Country	Method	<i>Gallus gallus</i>						Pigs						Cattle						Broiler meat						Meat from pigs								
		2007		2008		2009		2007		2008		2009		2007		2008		2009		2007		2008		2009		2007		2008		2009				
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%					
Austria	Dil			96*	0	230	0			15*	0																							
Belgium	Dil			148*	1																													
Czech Republic	Dil	234	0	106*	0	508	3			61*	0	26	0		16*	0	17	0	24	0	42*	0	20	0										
Denmark	Dil, Dil	20	0					1150	0	525**	0	393**	0	26	0	31**	0	18**	0						71	0	126**	0.8	100**	0				
Estonia	Dil							19	5.3	18	0	26	0																					
Finland	Dil	10	0			48	0					18	0	18	0	11	0																	
France	Dil			334*	1	360	1			183*	1																							
Germany	Dil, Dil			364	1	581	0.2					343	2												199	4	214	4		166	1			
Greece	Dil					31	0																											
Ireland	Dil			40*	0			66*	1.5	255*	0	25	0														79	5		44	0			
Italy	Dil	136*	2.9	144*	12	414	2	140*	0.7	384*	1	76	0	20*	0	21*	0	22	0						45*	2	18	0	231*	0	219*	0	42	0
Latvia	Dil, Dil			73	0	26	0																									12	0	
Netherlands	Dil	164*	13.4	423*	13	302	12	144*	0	332*	0	87	0	78*	0	57*	0	82	0									26	4			11	0	
Poland	Dil			195*	0	234	0.4			32*	0																							
Portugal	Dil			104	0	34	0			27	4																							
Slovakia	Dil	137*	0	191*	0	109	0	77*	0	44*	0	20	0	17*	0					10	0	10*	0											
Slovenia	DD, Dil	33	0	25*	0	49	0	22	0	14*	0																							
Spain	Dil	295*	7.8	252*	1	287	2	167*	0.6	384*	2	112	1	30	0	47*	0	29	0															
Sweden	Dil	15	0	14	0			29	0	23	0	10	0	20	0	39	0	43	0															
United Kingdom	Dil			55	0	326	0																											
Total (cefotaxime) 2009		-	-	2217	4	3539	2	-	-	-	-	1136	1	-	-	-	-	457	0.4	-	-	-	-	533	5	-	-	-	-	575	2			
Switzerland	Dil									46	2									25	0	10	0											

*also ceftazidime tested

**also ceftiofur tested

Dil: Dilution method

DD: Disk diffusion method

Table 14: Resistance (%) to cefotaxime among indicator *E. coli* isolates from poultry, pigs and cattle, and meat thereof in 2007, 2008 and 2009, using harmonised epidemiological cut-off values

N is the number of isolates tested

Ceftiofur and ceftazidime susceptibility test results are additionally mentioned in blue and in red, respectively, in the table.

Green shading: resistance detected to cefotaxime was not detected to ceftazidime / Red shading: resistance detected to ceftazidime was not detected to cefotaxime

Country	Method	<i>Gallus gallus</i>						Pigs						Cattle						Broiler Meat		Turkey Meat		Pig Meat				Bovine Meat		
		2007		2008		2009		2007		2008		2009		2007		2008		2009		2009		2009		2008		2009		2009		
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Austria	Dil	43	0	170	2.9	170	2.4	46	0	170	0.6	162	0.6	43	0	170	0	168	0											
Denmark	Dil, Dil	114	1.8	114**	0	152**	0	150	0.7	151**	0	150**	0	98	0	97**	0	94**	0	143**	0			66**	0	106**	0.9	32**	0	
Estonia	Dil							19**	0	10**	0	17**	0	25**	4	10**	0	23**	4.3											
Finland	Dil			388	1.3			135	0									272	0											
France	Dil	101*/**	2	146*	6.2	189*	2.6	126*/**	0.8	137*	0.7	155*	1.3	103*/**	1	118*	0.8			151*	3.3	155*	1.9	102*	1	100*	5			
Germany	Dil, Dil					514*	2.5									76	11.8	361*	1.4	194*	6.2	203*	1			46*	2.2	51*	0	
Hungary	DD (bp: 27)					176	4.5					317**	3.8					340	6.5											
Italy	Dil	54*	11.1					149*	0.7																					
Netherlands	Dil	43*	20.9	440*	15	291	17.9	169*	1.2	296*	1	296	3.7	432*	2.3	301*	1.7	307	1.6					17*	0					
Poland	Dil					356	11.2					184	3.8					173	0.6											
Slovenia	DD, DD (bp: 14)							34	3					22	0							18	0							
Spain	Dil			113*	30.1	197*	26.4	229	0.9	168*	0.6	278*	1.1	158	0	167*	0	256*	0.4											
Sweden	Dil	296**	1							349	0							223	0							19	0			
Total (cefotaxime) 2009		-	-	-	-	2045	8.5	-	-	-	-	1559	2.3	-	-	-	-	2217	1.6	-	-	-	-	-	-	-	-	-	-	
Norway	Dil					162	0	198**	0.5	258	0																			
Switzerland	Dil	284	1.1	149	0	136*	1.5	98	0			181*	0			80	0	132*	0											

*also ceftazidime tested
 **also ceftiofur tested
 Dil: Dilution method
 DD: Disk diffusion method

B. DETECTION OF ESBLs AND AMPc IN *ENTEROBACTERIACEAE* RECOVERED FROM FOOD-PRODUCING ANIMALS OR FOOD OF ANIMAL ORIGIN

Table 15: Detection of ESBLs in Enterobacteriaceae recovered from food-producing animals or food of animal origin

Animal	Type of ESBL	Species	Year of isolation	Country	Reference
Healthy animals or food of animal origins					
Poultry					
	CTX-M-14 , SHV-12	<i>E. coli</i>	2000-2001	Spain	(Brinas et al., 2003b)
	CTX-M-2, CTX-M-14	<i>E. coli</i>	1999-2002	Japan	(Kojima et al., 2005)
	CTX-M-14, SHV-12, CTX-M-9	<i>E. coli</i>	2003	Spain	(Brinas et al., 2005)
	TEM-52	<i>E. coli</i>	2005	Portugal	(Machado et al., 2008)
	CTX-M-1, -9, -14, -32 SHV-2, -5, -12 TEM-52	<i>E. coli</i>	2003	Spain	(Blanc et al., 2006)
	TEM-52, CTX-M-14, CTX-M-32	<i>E. coli</i>	2004	Portugal	(Costa et al., 2009)
	CTX-M-1	<i>E. coli</i>	2005	France	(Girlich et al., 2007)
	TEM-52, TEM-106, CTX-M-1, CTX-M-2, CTX-M-14, CTX-M-15	<i>E. coli</i>	2007	Belgium	(Smet et al., 2008)
	CTX-M groups -1, -2 and -9	<i>E. coli</i>	2009	Denmark	(Bortolaia et al., 2010a)
	CTX-M-1, CTX-M-32, SHV-12	<i>E. coli</i>	2007	Italy	(Bortolaia et al., 2010b)
	CTX-M-1, -2, TEM-52 and SHV-2	<i>E. coli</i>	2006	Netherlands	(Dierikx et al., 2010a)
	CTX-M-14, CTX-M-65, CTX-M-55, CTX-M-24, CTX-M-3, CTX-M-15 (1 strain), CTX-M-64.	<i>E. coli</i>	2006-2007	China	(Li et al., 2010a)
	CTX-M-1 and SHV-12	<i>E. coli</i>	2008	Czech Republic	(Kolar et al., 2010)
	TEM-52	<i>Salmonella</i> .	2001-2005	Belgium	(Clockaert et al., 2007)
	SHV-12	<i>Salmonella</i> .	2005-2006	Italy	(Chiarretto et al., 2008)
	CTX-M-1	<i>Salmonella</i>	2006	France	(Clockaert et al., 2010)
	CTX-M-1, -2, TEM-52 and TEM-20	<i>Salmonella</i>	2006	Netherlands	(Dierikx et al., 2010a)
Broiler and turkey	CTX-M-1, -3, -14, -15, -55, TEM-52	<i>E. coli</i>	2006-2009	Great Britain	(Randall et al., 2011)

Animal	Type of ESBL	Species	Year of isolation	Country	Reference
Broiler and lay hens	CTX-M-9	<i>Salmonella</i>	2003-2004	Spain	(Riano et al., 2006)
Poultry meat	TEM-52, CTX-M-1, SHV-2	<i>E. coli</i> , <i>K. pneumoniae</i>	2003-2005	Portugal	(Machado et al., 2008)
Poultry and poultry meat	CTX-M-2, TEM-52, TEM-20, SHV-2, SHV-12	<i>Salmonella</i>	2001-2002	The Netherlands	(Hasman et al., 2005)
Poultry and poultry meat	CTX-M-2	<i>Salmonella</i>	2000-03	Belgium	(Bertrand et al., 2006)
Poultry and poultry meat	CTX-M-9	<i>Salmonella</i>	2003	France	(Weill et al., 2004)
Poultry and poultry meat	CTX-M	<i>Salmonella</i>	2004-2008	Korea	(Hur et al., 2010)
Sick and healthy poultry	CTX-M-14, CTX-M-15, TEM-52, SHV-12, CTX-M-65, and CTX-M-3 (CTX-M: 75%)	<i>E. coli</i>	1970-2007 0 (1970-2003) 18.5 (2004-2007)	China	(Li et al., 2010a)
Poultry meat	CTX-M-8, CTX-M-14, SHV-5	<i>E. coli</i>	2006	Tunisia	(Jouini et al., 2007)
Poultry meat	CTX-M-1	<i>E. coli</i>	2007	Tunisia	(Ben Slama et al., 2010)
Poultry meat	CTX-M-14 (recovered in 2004)	<i>Salmonella</i>	1999-2005	Japan	(Matsumoto et al., 2007)
Poultry meat	CTX-M-1 CTX-M-2 CTX-M-14 and CTX-M-8	<i>E. coli</i>	2006	UK (imported and not imported meat)	(Warren et al., 2008)
Poultry meat	Group 2 CTX-M Group 8 CTX-M	<i>E. coli</i>	2008	raw chicken imported to UK	(Dhanji et al., 2010)
poultry	SHV-12	<i>Salmonella</i>	2008-2009	Ireland	(Boyle et al., 2010)
Poultry meat	SHV-12	<i>Salmonella</i>	2000	Senegal	(Cardinale et al., 2001)
Poultry meat	CTX-M-32	<i>Salmonella</i>	2001	Greece	(Politi et al., 2005)
Quail meat	CTX-M-9	<i>Salmonella</i>	2003	Denmark	(Aarestrup et al., 2005)
Cattle					
	CTX-M-1, CTX-M-14, SHV-12	<i>E. coli</i>	2006	France	(Madec et al., 2008)
	CTX-M-1	<i>E. coli</i>	2006	Tunisia	(Jouini et al., 2007)
	CTX-M-2	<i>E. coli</i>	2000-2001	Japan	(Shiraki et al., 2004)
	CTX-M ^a	<i>E. coli</i>	2004	United Kingdom	(Liebana et al., 2006)
	CTX-M-1	<i>E. coli</i>	2007	Tunisia	(Ben Slama et al., 2010)
	CTX-M-2	<i>E. coli</i>	2000-2001	Japan	(Shiraki et al., 2004)
	CTX-M-13	<i>E. coli</i>	2002	Hong Kong	(Duan et al., 2006)

Animal	Type of ESBL	Species	Year of isolation	Country	Reference
	TEM-52	<i>E. coli</i>	2004	Denmark	(Jensen et al., 2006)
	CTX-M-1 CTX-M-29	<i>E. coli</i>	2009	USA	(Wittum et al., 2010)
	SHV-1, -25, -26, -110, -111 CTX-M-14	<i>Enterobacteria</i>		Egypt - Japan	(Hammad et al., 2009)
	CTX-M-1	<i>E. coli</i>	2009	USA	(Wittum et al., 2010)
	CTX-M-15	<i>E. coli</i>	2006-2007	UK	(Kirchner et al., 2011)
Pigs					
	CTX-M-15, CTX-M-22, SHV-2	<i>E. coli</i>	2002-2007	China	(Tian et al., 2008)
	CTX-M-1, CTX-M-9, CTX-M-14, SHV-12	<i>E. coli</i>	2004	Spain	(Escudero et al., 2010)
	CTX-M-1 SHV-5, -12	<i>E. coli</i>	2003	Spain	(Blanc et al., 2006)
	CTX-M-1	<i>E. coli</i>	2007	Portugal	(Goncalves et al., 2010)
	CTX-M-1	<i>E. coli</i>	2005-2006	Denmark	(Wu et al., 2008)
	SHV-12	<i>Salmonella</i>	2003	Spain	(Riano et al., 2006)
	SHV-12	<i>Citrobacter freundii</i>	2004	Portugal	(Machado et al., 2008)
	CTX-M-3, -14	<i>E. coli</i>	2002	Hong-Kong	(Duan et al., 2006)
Other species					
Sheep-meat	CTX-M-1, TEM-20	<i>E. coli</i>	2007	Tunisia	(Ben Slama et al., 2010)
Pigeon	CTX-M-14	<i>E. coli</i>	2002	Hong Kong	(Duan et al., 2006)
Rabbit	CTX-M-9, -14	<i>E. coli</i>	2003	Spain	(Blanc et al., 2006)
Ostrich	CTX-M-14, TEM-52	<i>E. coli</i>	Not specified	Portugal	(Carneiro et al., 2010)
Cockles	CTX-M-53 (Asp240Gly as CTX-M-15)	<i>Salmonella</i>	France	2004	(Doublet et al., 2009)
Various food samples	CTX-M-1 and -9 groups SHV-12	<i>E. coli</i>	Non specified	Spain	(Doi et al., 2009)
Various food samples	CTX-M-1 group	<i>E. coli</i>	Non specified	USA	(Doi et al., 2009)
Various livestock and food samples	CTX-M-1, CTX-M-15, TM-52, TEM-20	<i>Salmonella</i>	2003-2007	Germany	(Rodriguez et al., 2009)

Animal	Type of ESBL	Species	Year of isolation	Country	Reference
Sick animals					
Various species	SHV-12	<i>E. coli</i>	1997-2001	Spain	(Brinas et al., 2003a)
Various species	CTX-M-14, -9, -32, -1, TEM-52, SHV-12	<i>E. coli</i>	2003	Spain	(Brinas et al., 2005)
Cattle	CTX-M-1, CTX-M-14, CTX-M-15, TEM-126	<i>E. coli</i>	2006	France	(Madec et al., 2008)
Pigs	TEM-20, TEM-52, SHV-28, SHV-33	<i>E. coli</i> , <i>K. pneumoniae</i>	1999-2006	Korea	(Rayamajhi et al., 2008)
Poultry	CTX-M-1	<i>E. coli</i>	2003-2004	France	(Meunier et al., 2006)
Pigs	CTX-M-1	<i>E. coli</i>	2000-2004	France	(Meunier et al., 2006)
Cattle	CTX-M-1, -15	<i>E. coli</i>	2004	France	(Meunier et al., 2006)
Pigs	CTX-M-1	<i>E. coli</i>	2005	Denmark	(Aarestrup et al., 2006)
Cattle	CTX-M-14	<i>E. coli</i>	2004-2005	UK	(Hopkins et al., 2006)
Poultry	CTX-M-2	<i>Salmonella</i>	unknown	Ireland	(Hopkins et al., 2006)
Poultry	TEM-52	<i>Salmonella</i>	2003	Ireland	(Morris et al., 2009)
Cattle	TEM-20	<i>Salmonella</i>	2001	Ireland	(Morris et al., 2009)
Horse	SHV-12	<i>Salmonella</i>	2003	USA	(Rankin et al., 2005)
Cows and pigs	CTX-M 14 CTX-M-15	<i>E. coli</i>	2003-2006	Korea	(Lim et al., 2009)
Bovine	CTX-M-1, -3, -14, -15, -20, -32	<i>ESBL producers</i>	2006-2008	UK	(Hunter et al., 2010)

Table 16: Detection of plasmidic AmpC beta-lactamases in *Enterobacteriaceae* recovered from food-producing animals or food of animal origin

Animal	Type of AmpC	Species	Year of isolation	Country	Reference
Healthy animals or food of animal origin					
Poultry					
	CMY-2	<i>E. coli</i>	2000-2001	Spain	(Brinas et al., 2003b)
	CMY-2	<i>E. coli</i>	1999-2002	Japan	(Kojima et al., 2005)
	CMY-2	<i>E. coli</i>	2003	Spain	(Brinas et al., 2005)
	CMY-2	<i>E. coli</i>	2003	Spain	(Blanc et al., 2006)
	CMY-2	<i>E. coli</i>	2007	Belgium	(Smet et al., 2008)
	CMY-2	<i>E. coli</i>	Not specified	Taiwan	(Yan et al., 2004)
	CMY-2	<i>E. coli</i>	(2004-2007)	China	(Li et al., 2010b)
	CMY-2	<i>E. coli</i>	2006	Netherlands	(Dierikx et al., 2010a)
	CMY-2	<i>E. coli</i>	2008	Czech Republic	(Kolar et al., 2010)
	CMY-2	<i>Salmonella</i>	1994-1999	Canada	(Allen and Poppe, 2002)
Retail meat (poultry)	CMY ^a	<i>Salmonella</i>	2002-2006	United States	(Zhao et al., 2008)
	CMY-2	<i>Salmonella</i>	2004-2005	Japan	(Taguchi et al., 2006)
Poultry	CMY-2	<i>Salmonella</i>	2008-2009	Ireland	(Boyle et al., 2010)
Poultry (and humans)	ACC-1	<i>Salmonella</i>	2002/2006	Netherlands	(Dierikx et al., 2010a; Hasman et al., 2005)
Cattle					
	CMY-2	<i>E. coli</i>	2003	United States	(Donaldson et al., 2006)
	CMY-2	<i>Salmonella</i>	1994-1999	Canada	(Allen and Poppe, 2002)
Pigs					
	CMY-2	<i>E. coli</i>	Not specified	Canada	(Kozak et al., 2009)
	CMY-2	<i>E. coli</i>	Not specified	Taiwan	(Yan et al., 2004)
	CMY-2	<i>Salmonella</i>	2000-2005	Mexico	(Zaidi et al., 2007)
Rabbit	CMY-2	<i>E. coli</i>	2003	Spain	(Blanc et al., 2006)
Various food samples	CMY-2	<i>E. coli</i>	Non specified	USA	(Doi et al., 2009)
Various livestock and food samples	CMY-2	<i>Salmonella</i>	2003-2007	Germany	(Rodriguez et al., 2009)
Various livestock and food samples	CMY-2 and CMY-2-like	<i>E. coli</i> and <i>Salmonella</i>	None specified	USA	(Heider et al., 2009)
Food of chicken and pork origin	CMY-2	<i>E. coli</i>	None specified	Taiwan	(Yan et al., 2004)

Animal	Type of AmpC	Species	Year of isolation	Country	Reference
Sick animals					
Various species	CMY-2	<i>E. coli</i>	1997-2001	Spain	(Brinas et al., 2003a)
Various species	CMY-2	<i>E. coli</i>	2003	Spain	(Brinas et al., 2005)
Pigs	CMY-2, DHA-1	<i>K. pneumoniae</i>	1999-2006	Korea	(Rayamajhi et al., 2008)
Cattle	CMY-2	<i>Salmonella</i>	2000-2004	United States	(Frye et al., 2008)
Cattle	AmpC ^a	<i>Salmonella</i>	1999-2001	United States	(Gupta et al., 2003)
Cattle & pigs	CMY-2	<i>E. coli</i> , <i>Salmonella</i>	1998-1999	United States	(Winokur et al., 2000)

^aNot genotypically characterized