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Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

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List of abbreviations

AmpC - AmpC Beta-Lactamases

BIOHAZ - EFSA Panel on Biological Hazards

CI - Credibility Interval

DAFC - Danish Agriculture & Food Council

DANMAP - Danish Surveillance Programme for Antibiotic Use and Antibiotic Resistance

DTU - Technical University of Denmark

***E. coli* -** *Escherichia coli*

EARS-net - European Antimicrobial Resistance Surveillance Network

EARSS - European Antimicrobial Resistance Surveillance System

ECDC - European Centre for Disease Prevention and Control

EFSA - European Food Safety Authority

ESBL - Extended-Spectrum Beta-Lactamases

ESBL-PB - Extended-Spectrum Beta-Lactamases-producing bacteria

ESBL-PEC - Extended-Spectrum Beta-Lactamases-producing *Escherichia coli*

ESBL/AmpC-PB - Extended-Spectrum Beta-Lactamases/AmpC Beta-Lactamases-producing bacteria

ESBL/AmpC-PEC - Extended-Spectrum Beta-Lactamases/AmpC Beta-Lactamases -producing *Escherichia coli*

ESVAC - European Surveillance of Veterinary Antimicrobial Consumption

EU - European Union

ICBAS - Abel Salazar Biomedical Sciences Institute

IMTA - Integrated Multi-Trophic Aquaculture

Kg - Kilograms

NOSOVE - Nordic Society for Veterinary Epidemiology

OIE - World Organization for Animal Health

SAGAM - Scientific Advisory Group on Antimicrobials

SSI - State Serum Institute in Denmark

SVARM - Swedish Veterinary Antimicrobial Resistance Monitoring

SVEPM - Society for Veterinary Epidemiology and Preventive Medicine

WHO - World Health Organization

Foreword

For a better understanding of this study and the way it is structured, its story should be told. This project was undertaken during a 4-month period and it acts as my final thesis. I was an ERASMUS student at the University of Copenhagen, working in collaboration with the Danish Agriculture & Food Council (DAFC).

When we first started to plan this study, we aimed to perform a full risk assessment including a source attribution. We intended to make use the so-called "OIE approach" which considers four major steps for a risk assessment: release assessment, exposure assessment, consequence assessment and risk estimation. Moreover, we were planning to estimate the number of human urinary tract infections and septicaemia cases caused by Extended-Spectrum Beta-Lactamases/AmpC Beta Lactamases-producing bacteria (ESBL/AmpC-PB) that could be attributed to each possible source per year in Denmark.

However, this idea was abandoned due to lack of data that would enable us to perform an accurate risk assessment. Thus, we decided to select a different approach, using an exposure assessment to evaluate the relative role of meat for human ESBL/AmpC-producing *E. coli* (ESBL/AmpC-PEC) infections.

This internship enabled me to be enrolled in several activities, which are listed in the Appendix III, and greatly contributed to my personal and academic development. From all of them, I would like to highlight the various presentations of the project I did. And also the granting of a bursary to attend the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM) 2013 congress in Madrid, where I won a Poster Prize Award (Appendix IV).

We also submitted the abstract of this project for oral presentation at the 10th International Conference on the Epidemiology & Control of Biological, Chemical and Physical Hazards in Pigs and Pork (Safe Pork 2013) this September in Portland (USA) and it will also be submitted to a peer-reviewed journal in the coming months.

The thesis is composed by three main parts. The first part is the project addressing the exposure assessment of ESBL/AmpC-PEC in meat in Denmark. Few changes will be made to the version that will be submitted for peer-review. The second part is a consideration on the potential sources of ESBL/AmpC-PB. This part gives the reader a wider perspective on ESBL/AmpC and reflects on the state-of-art regarding the possible sources of this hazard and their potential implications for human health. The last part is a general conclusion of the entire project, focusing on future perspectives.

This was a very challenging but rewarding internship, where I had the opportunity to work with great specialists in the Epidemiology/ Public Health field and perform a very interesting and actual study.

Summary

Extended-Spectrum Beta-Lactamases (ESBL) and AmpC Beta-Lactamases (AmpC) are of great concern because of their ability to cause antimicrobial resistance in *Enterobacteriaceae* hampering the effect of treatment with beta-lactam antibiotics.

The main objective of this study was to assess the relative importance of different types of meat for the exposure of consumers to ESBL/AmpC and their potential relevance for human cases in Denmark.

This was assessed by weighting the prevalence of each genotype of ESBL/AmpC in imported and nationally produced broiler meat, pork and beef with the meat consumption patterns in Denmark. Data originated from the Danish surveillance programme for antibiotic use and antibiotic resistance (DANMAP) for 2009 to 2011. Data about human ESBL cases in 2011 were also collected to assess a possible genotype overlap.

Uncertainty was assessed by inspecting beta distributions of the genotype prevalences in each type of meat.

Broiler meat represented the largest part of the estimated ESBL/AmpC contaminated pool of meat role (84.9%) compared to pork (11.5%) and beef (3.6%). CMY-2 was the genotype with the highest relative importance for human exposure (58.0%), and it was mainly isolated from broiler meat of either Danish or imported origin. However, CMY-2 is rarely found in humans in Denmark.

In general the overlap between ESBL/AmpC genotypes in meat and those found in human *E. coli* infections isolates was limited. CTX-M-1 had a relative importance of 28.9% for human exposure through meat. Moreover, the prevalence of CTX-M-1 in humans was 7.3% of *E. coli* urinary tract infections and 8.0% of *E. coli* bloodstream infections. Hence, the genotype CTX-M-1 was considered the most relevant genotype found in meat when referring to human exposure.

This suggests that meat might constitute a less important source of ESBL/AmpC exposure of humans in Denmark than previously thought. More detailed surveillance data are required to determine the contribution of meat compared to other sources such as nosocomial infections, travelling, contact with animals, human-to-human transmission and the environment.

Keywords: ESBL, AmpC, *E. coli*, meat, exposure assessment.

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To Liza Nielsen, for all the patient and support, for always finding time to listen to me. Since the beginning your kindness was amazing and I am very grateful for providing me the opportunity to work with you.

To Lis Alban for all the energy, great ideas for the project and for giving me the opportunity to be enrolled in several activities. It was extremely nice of you to welcome me at your place and present me to your family.

I had high expectations for this internship but they were completely exceeded thanks to both of you. It was a pleasure to listen to all your knowledge. You are professional examples to me and persons for whom I feel great esteem. Thank you so much for all you have done for me and for all your helpful advices on my future.

To Paulo Martins da Costa who was, since we first met, a reference for me. I am very grateful for all the help and for all the advice, which was of great relevance and conducted me to this experience.

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I acknowledge the Danish Agriculture & Food Council (DAFC) for funding my participation at the NOSOVE course and for all the activities I could experience due to their collaboration. A special thanks to Jan Dahl and Marianne Sandberg for their precious help. It was a pleasure to meet you and have the opportunity to listen to you.

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To all the scientist who made themselves available to participate in the ESBL seminar on March 1st 2013. Your help was of great value to our project. A special thanks to Anette Hammerum and Luca Guardabassi for their availability to answer to my questions.

Thanks to the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM) for the bursary award and the opportunity to go to their conference in Madrid.

I would like to thank Thea, William and Robert for welcoming me at their place and for making everything that was possible to make me feel comfortable and at home. You were great!

I am also grateful to Sara Pires for her availability to help me during the time I was in Copenhagen and for all her advices.

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To Carla Gomes and Filipa Baptista, for their availability to help me before I come to Copenhagen.

I would also like to thanks to all the Epidemiology department of the University of Lisbon for their collaboration.

I also acknowledge the European Union (EU), which supported my stay and my internship through the ERASMUS grant.

This project is the culmination of 6 years of study and I would like to express my gratitude for those who were important during this period of my life.

I am very proud to complete my studies at the Abel Salazar Biomedical Sciences Institute (ICBAS). With all its virtues and faults, it provided me an education of excellence and I am grateful to everyone who contributed for it.

To all my friends, especially to Afonso Porfírio, Bernardo Archer, Bruno Lopes, Bruno Silva, Fernando Almeida, Hugo Santos, Joana Devesa, João Madeira and João Vieira. For all the study group motivation during the “Red Bull nights” at ICBAS and “Coffee mornings” in Vairão’s gas station, for the dinners, football games and vacations together, for all the memorable moments we will not forget, but most of all for your friendship.

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A special thanks to Ana for being my best friend and support during the last 6 years. You made this pathway much easier and I am everlastingly grateful for always being there when I need. Your love and affection will not be forgotten.

And for the “last but not least” cliché, many thanks to my family for all the love and for being my Maecenas all these years. To Matosinhos’ grandparents, Penafiel’s grandmother, Padrinho, Fred and Cristina, for their affection and support. To my father, my mother and Sara, for their effort to provide me the best education possible and for their unconditional love.

I have an eternal debt of gratitude to all of you.

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Exposure assessment of Extended-Spectrum Beta-Lactamases/AmpC Beta-Lactamases-producing *E. coli* in meat in Denmark

1. Introduction

Extended-Spectrum Beta-Lactamases (ESBL) were defined by the EFSA Panel on Biological Hazards (BIOHAZ) as plasmid-encoded enzymes found in the bacterial family *Enterobacteriaceae*, commonly in *Escherichia coli* and *Klebsiella pneumonia*. ESBL confer resistance to a variety of Beta-lactam antibiotics, including penicillins, 2nd, 3rd and 4th generation cephalosporins and monobactams (EFSA, 2011). The BIOHAZ panel also stated that AmpC Beta-Lactamases (AmpC) are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria. However, the number of AmpC enzymes that are plasmid-borne is increasing (EFSA, 2011). An example of such plasmidic AmpC is CMY-2.

1.1. Burden of disease

The World Health Organization (WHO) calculated 25,000 deaths each year in the European Union (EU) due to infections with antibiotic resistant bacteria (WHO, 2011a). The increase of ESBL/AmpC is particularly worrying due to their resistance to 3rd and 4th generation cephalosporins, which have been considered critically important to human medicine by WHO (WHO, 2011b).

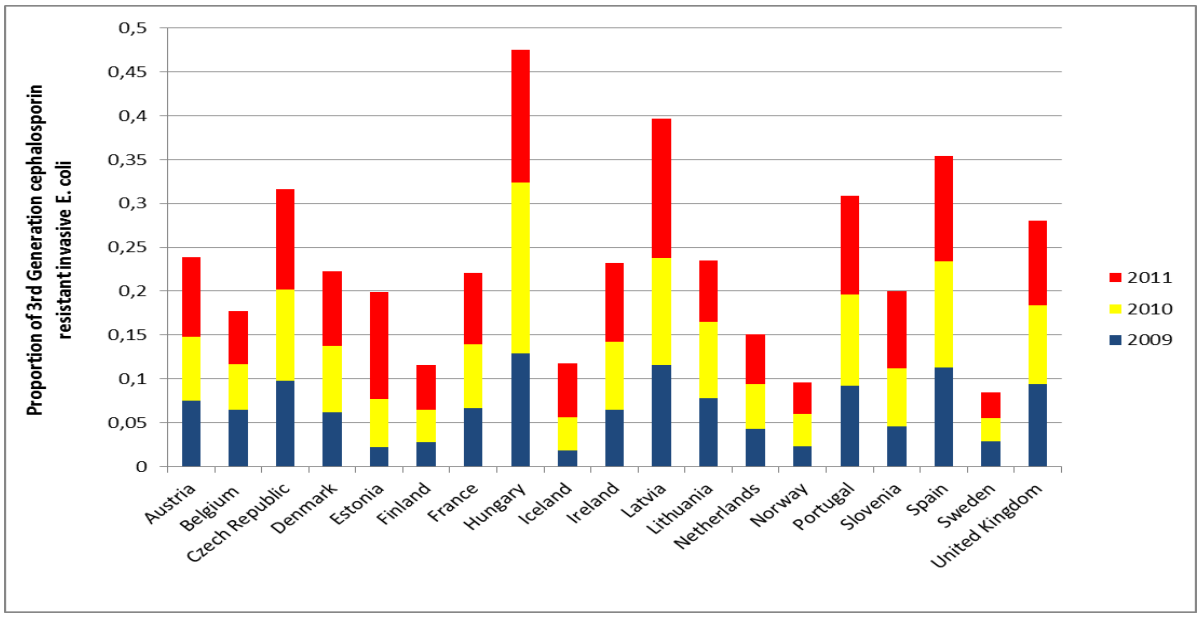


Figure 1 – Proportion of 3rd generation cephalosporin resistant isolates from *E. coli* bloodstream infections across Europe reported to EARS-Net from 2009 to 2011. Source: ECDC 2013

ESBL/AmpC-producing bacterial infections are on the increase worldwide (Pitout & Laupland, 2008). The European Antimicrobial Resistance Surveillance Network (EARS-Net) stated that no country from the 28 countries reporting to this programme demonstrated decreasing trends over the last few years (Figure 1) (ECDC, 2012). It should be noted that in 2011 a high proportion of 3rd generation cephalosporin resistant *E. coli* isolates were ESBL producers. In line with this, for 2011, more than half of the countries reported proportions between 85% and 100% of 3rd generation resistant *E. coli* that were also ESBL producers(ECDC, 2012). The same occurred in previous years (ECDC, 2010, 2011). In Denmark, a steady increase has been observed from 2005 (1.1%) and through 2011 (8.5%) (Figure 2) (ECDC, 2013).

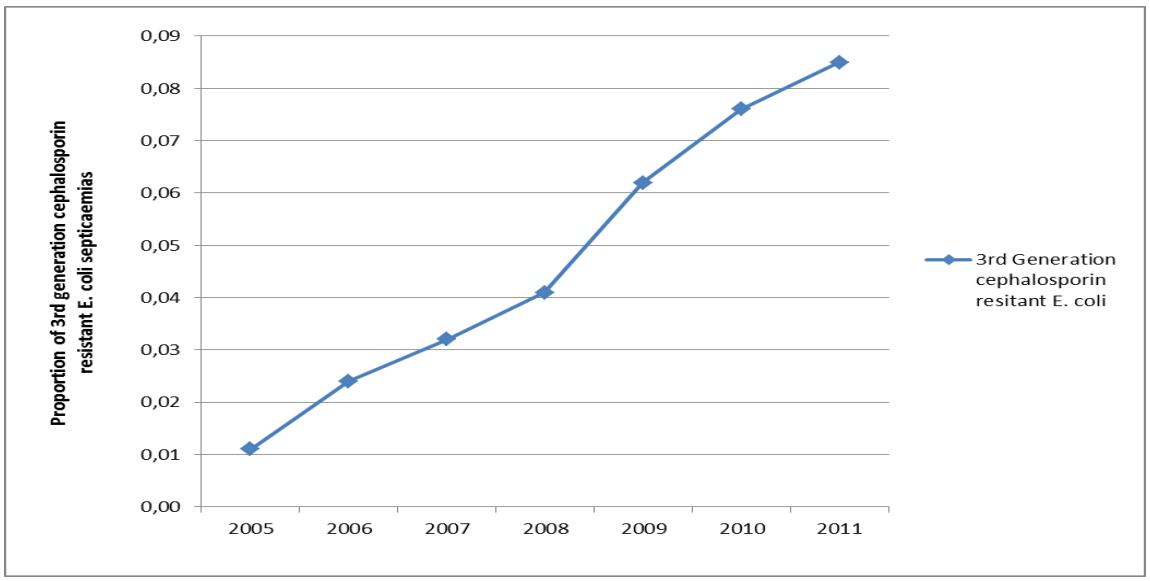


Figure 2 – Proportion of 3rd generation cephalosporins resistant *E.coli* from *E. coli* bloodstream infections in Denmark reported to EARS-Net from 2005 to 2011. Source: ECDC 2013

The importance of the increase of ESBL/AmpC proportion among *E. coli* septicaemias can also be reflected in the augmented case fatality risk. Melzer & Petersen (2007) found a mortality risk of 23.7% in non-ESBL-producing *E. coli* septicaemias compared with 60.8% in ESBL-producing *E. coli* (ESBL-PEC) bloodstream infections. It should be taken into account that these results might be overestimating the general ESBL mortality risk, because bacteraemia are frequently associated with critical underlying disease, delay in appropriate treatment and older age (Colodner *et al.*, 2004; Nasa *et al.*, 2012; Rottier *et al.*, 2012; Trecarichi *et al.*, 2012). A meta-analysis of 32 studies about the different outcome when comparing septicaemias by ESBL-producing and non-producing bacteria, found 2.32 higher odds of dying when infected with an ESBL producer. Even after adjusting for inadequate empirical treatment and sepsis severity, there was a statistically significant association (Rottier *et al.*, 2012).

In general, the burden of antimicrobial resistance is quite significant. De Kraker *et al.* (2011) estimated for the year of 2007 in the 31 participating countries of the European Antimicrobial Resistance Surveillance System (EARSS) that 2,712 early deaths and 120,065 extra hospital days could be related to 3rd generation cephalosporins resistant *E. coli* bloodstream infections. Moreover, the same study calculated an economic burden of €18.1 million for the total costs related to the excess hospital stays (De Kraker *et al.*, 2011).

Another worrying fact is that ESBL/AmpC resistance leads to an increased use of carbapenems, since these compounds are widely regarded as the first choice for treatment of severe ESBL/AmpC infections (Paterson, 2000; Pitout & Laupland, 2008; Rupp & Fey, 2003). The use of this class of drugs is intrinsically related to the emergence of carbapenemases (Wilcox, 2009), which have the ability to hydrolyse all the beta-lactams and are extremely drug-resistant, and therefore being a matter of great concern (EFSA, 2011). Additionally, it has been suggested that previous use of carbapenems may be a risk factor for occurrence of ESBL-PEC in patients with bloodstream infection (Martínez *et al.*, 2006).

1.2. Possible ESBL/AmpC-producing bacteria sources

There are several potential ESBL/AmpC-PB sources that can be summarized in five major groups: foodborne, direct animals-to-human transmission, human-to-human transmission, environment and infections obtained abroad during travelling. Travelling is considered a source by itself since surveillance programmes are normally set at a national level. Moreover, it is more difficult to unveil the specific source when infection is acquired abroad. To our knowledge, the true importance of each of these possible sources for ESBL/AmpC dissemination and their relative relevance for human cases is unknown.

Recently, a possible link has been highlighted to food and food-producing animals (EFSA, 2011; SAGAM, 2009), stressing the importance of scrutinizing the role of food animal products to human ESBL/AmpC-PB infections.

1.3. The Danish situation

Denmark, as the other Nordic countries, has always been considered having a low animal prevalence of antimicrobial resistance, due to the conscious use and restrictive measures related to antibiotics. The “Yellow card” initiative (Alban *et al.*, 2013) and the cephalosporins ban for use in pigs (Agersø & Aarestrup, 2013) are examples of the precautionary Danish policy related to antimicrobial use in animal production.

From 2000 to 2008, DANMAP reports consistently showed very low prevalence of 3rd generation cephalosporin resistant bacteria both in live animals and in Danish meat (below 2%). ESBL have been reported in diagnostic submissions and have shown an increasing tendency over the years (DANMAP, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009). In

2009, the first surveillance study with enriched culture method was performed, nowadays described as the preferred method for ESBL/AmpC producers isolation (EFSA, 2011), followed by genetic background investigation (Agersø *et al.*, 2012). Since then, this protocol has been repeated yearly, revealing an increasing prevalence in Danish broiler meat from 3% in 2009 (data not published) to 44% in 2011 (DANMAP, 2011). In the same period, imported broiler meat had prevalences between 36% and 50% (DANMAP, 2009, 2010, 2011).

1.4. Purpose of the study

The aim of this project was to determine the relative importance of the various ESBL/AmpC-PEC genotypes in different meat types with respect to exposure of Danish meat consumers. Moreover, the genotype overlap between isolates found in meat and human cases was evaluated to assess the relative importance of meat as a source of ESBL/AmpC-PEC infections in humans.

2. Materials and methods

To assess the relative importance of each genotype and each type of meat for human exposure it was necessary to collect data about the meat available for consumption. This was divided into six categories: imported and domestically produced broiler meat, pork and beef. Furthermore, the prevalence of the different genotypes found in each category of meat were obtained. For this purpose, data from the surveillance programme DANMAP was used (DANMAP, 2009, 2010, 2011).

2.1. Meat consumption patterns

According to surveillance data from DANMAP 2011, the frozen and chilled fresh meat available for consumption in Denmark were 128,200 tonnes of broiler meat, 198,800 tonnes of beef and 272,000 tonnes of pork (Figure 3) (DANMAP, 2011).

Consumption was calculated as: Domestically produced – Exported + Imported (DTU, 2011).

In 2011, 59.2% of all the meat available for consumption in Denmark was of domestic origin (DANMAP, 2011). A closer look at these data revealed that 64.7% of broiler meat, 50.5% of beef and 64.7% of pork were of Danish origin (DANMAP, 2011).

There was no significant difference between the meat available for consumption in 2011 and the 2 previous years (*DTU*, 2011).

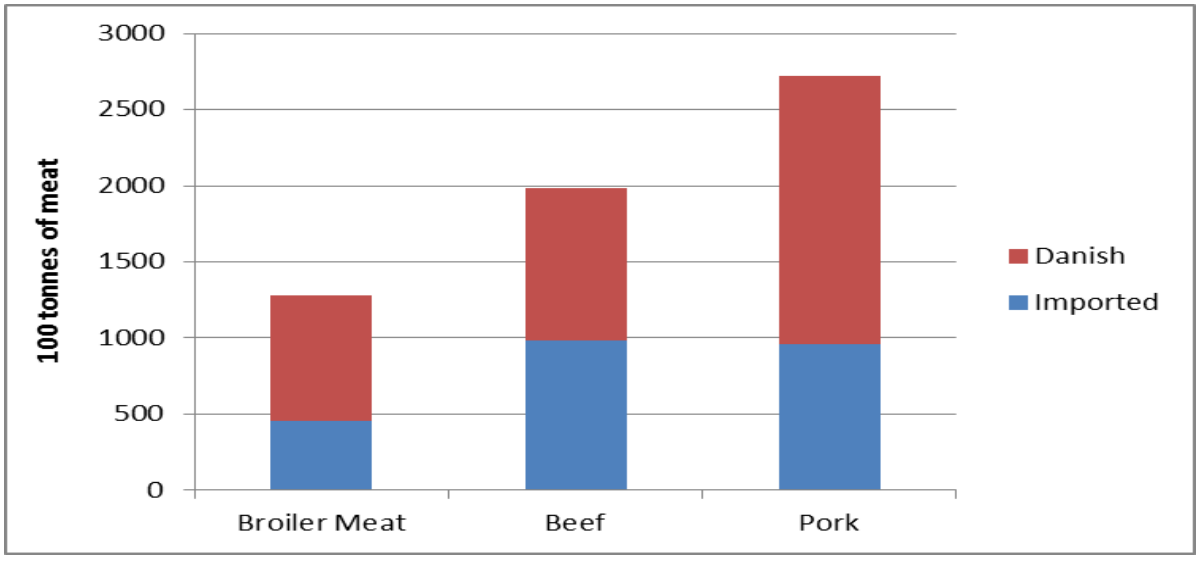


Figure 3 – Fresh and frozen meat available for consumption in Denmark in 2011. Source: DANMAP 2011

2.2. ESBL/AmpC-producing E. coli occurrence in meat

The data describing the ESBL/AmpC prevalence was collected from the Danish surveillance programme for antibiotic use and antibiotic resistance (DANMAP, 2009, 2010, 2011). In this programme detection of ESBL/AmpC-PEC is conducted using a selective culture method with

antimicrobials. This method has a very high sensitivity and is qualitative, hence only resulting in positive or negative outcome.

2.3. Relative importance of each type of meat for human exposure

The relative importance of each type of meat and of each genotype was obtained by weighting the prevalence of ESBL/AmpC genotypes by the amount of meat available for consumption. DANMAP data were used for the prevalence values. Due to the low frequency of positive samples for pork and beef and the low frequency of testing in general, a pool of the 3 years' sampling was used.

The genotype group "Others" included TEM-52 and unknown (i.e. where it was impossible to determine a specific genotype) in beef; unknown genotypes in pork; and TEM-20, TEM-52, up-regulated AmpC and unknown genotypes in broiler meat (Appendix I).

2.4. ESBL/AmpC-producing E. coli distribution

Using the software @Risk 4.5 Palisade Corporation®, beta distributions were performed to assess the 95% credibility interval (CI) for each the genotype based on the sample data for each source. The main objective of this procedure was to conduct an uncertainty analysis and hereby estimate how high the true prevalence could be.

The beta distributions were made through a simulation based on 1,000,000 iterations. The distributions were defined by (s+1, n-s+1), where s represents the number of positive samples for each genotype in each type of meat from 2009 to 2011, and n represents the total number of samples tested for ESBL/AmpC in each type of meat within the same 3-year period.

The beta distribution output was the probability of occurrence of a number of positive ESBL/AmpC specific genotype in a determined type of meat. The number of positive samples represents a discrete outcome.

Beta distributions were performed to take into account the uncertainty related to the low number of samples in the surveillance programme. This procedure was of particular relevance considering that some genotypes may have not been found through the Danish sampling surveillance and yet still be present in meat and play a role for ESBL/AmpC-PEC human cases. Their distribution was set as (0+1, n-0+1). Actually, some genotypes were not found in meat during the surveillance programme, but were found in live animals. The beta distributions are shown in Appendix II.

The lower the number of positive samples found, the lower the maximum prevalence that could be hiding in the meat.

3. Results

3.1. Relative importance of each type of meat for human exposure

As illustrated in Figure 4, during the period 2009 to 2011, broiler meat contributed the most to the total human exposure to ESBL/AmpC representing 84.8% of the total exposure. Danish broiler meat contributed with 36.8% and imported broiler meat with 48.0%. Danish pork constituted 5.4% of the ESBL/AmpC positive meat available for consumption, while imported pork represented 6.1%. Finally, beef seemed to have a minor relevance representing around 3.6% (1.2% of Danish origin and 2.4% imported origin) of the total human exposure to ESBL/AmpC through meat. The relative importance of Danish broiler meat increased markedly from 2009 to 2011, while the relative importance of pork and imported broiler meat decreased between 2009 and 2011.

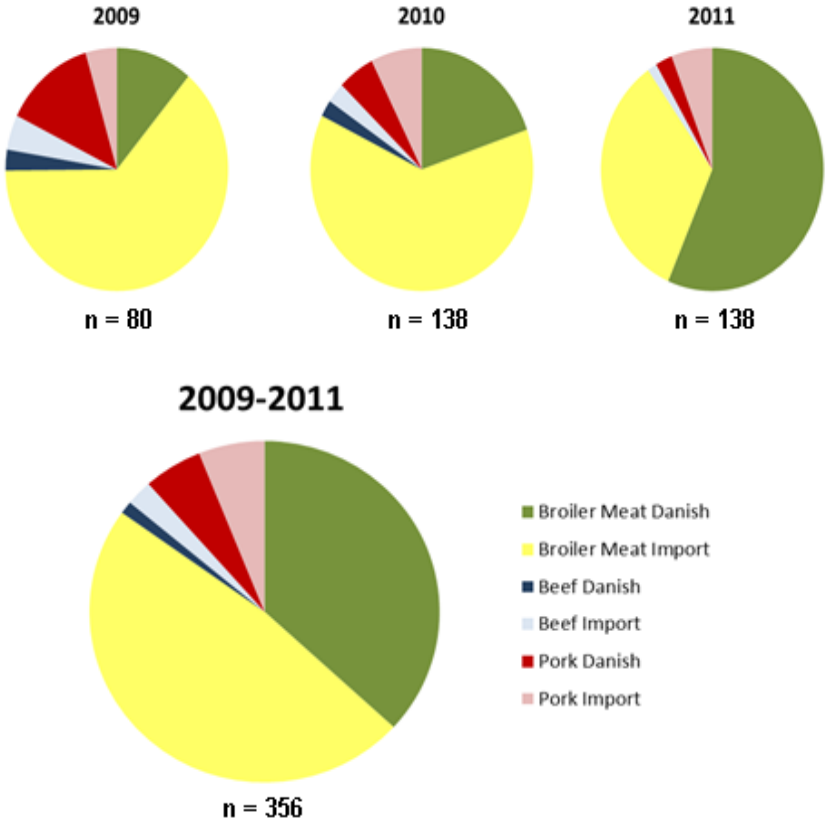


Figure 4 – Relative importance of each type of meat and their respective origin to human ESBL/AmpC-PEC exposure, based on the data from 2009 to 2011.

The relevance of each genotype varied between the 3 years, as shown in Figure 5. Most important, was the increase of the role CMY-2 in Danish broiler meat from 5.6% in 2009 to 16.0% in 2010, increasing to 53.5% of the total exposure in 2011. Moreover, CMY-2 constituted the ESBL/AmpC genotype that meat consumers were most exposed to (58.0% across the 3-

year period). Broiler meat was the source that contributed the most to this specific exposure (56.6% from broiler meat, 0.8% from pork and 0.6% from beef).

CTX-M-1 was the most frequently isolated genotype in pork and beef: 7.7% and 2.4% of the total exposure, respectively. Including broiler meat, CTX-M-1 represented 28.9% of the total ESBL/AmpC positive meat in the Danish market from 2009 to 2011.

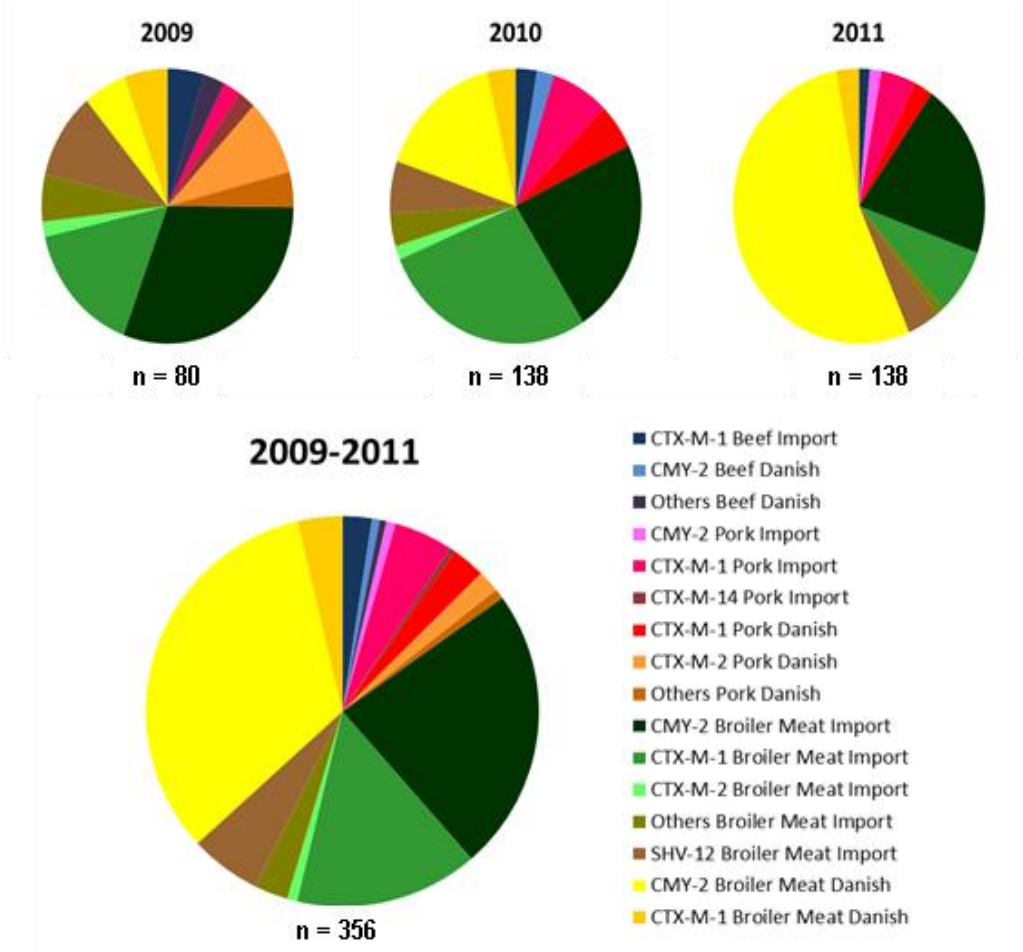


Figure 5 – The relative importance of each type of meat for human exposure considering the genotypes found in DANMAP surveillance from 2009 to 2011. The beef genotypes are represented in blue colours, while pork is in pink and red, and broiler meat is in green, brown and yellow colours.

Considering the origin of meat positive for ESBL/AmpC, a major shift occurred from 2010 to 2011. Both in 2009 and 2010 Danish meat represented around 27% of the positive ESBL/AmpC meat contributing to human exposure, against 73% of imported origin. In 2011, the relative importance for human exposure of the Danish meat rose to 59% and the imported meat was 41%. However, across the 3-year period, imported meat still contributed the most to the human exposure (57%).

3.2. Prevalence estimations for each genotype

CTX-M-15 was the genotype most commonly found in human cases. It was found on rare occasions in Danish pigs and cattle at slaughter: two positive samples in 2009 (n=786) and one in 2011 (n=777) in pigs, and one positive sample in 2010 (n=192) and another in 2011 (n=186) in cattle. However, it has not been detected in meat samples so far. The same was the case with CTX-M-14. Through our calculations, it can be shown that these ESBL genes might be present in Danish pork and beef in an upper limit of the estimated prevalence of 0.7% and 1.0%, respectively.

The prevalence of CTX-M-1 in imported pork found by DANMAP was 0.2%, but there was a high degree of uncertainty due to the small number of positive samples. The estimated upper limit of prevalence for this genotype was 3.8% in imported pork. Both CTX-M-1 and CTX-M-14 were found in human cases in Denmark.

Imported broiler meat was the type of meat where the highest diversity of ESBL/AmpC genes was found through in the DANMAP programme. CMY-2 and CTX-M-1 had an upper limit of estimated prevalence of 24.5% and 17.9%, respectively.

4. Discussion

This study evaluated the relative importance of various types of meat for the consumers' exposure to ESBL/AmpC-PEC and their potential importance for human cases. We concluded that broiler meat was the type of meat that contributed the most for human exposure (84.8%), while CMY-2 was the genotype with the highest relative importance (58.0% of the total exposure through meat). However, this genotype has only been found in rare occasions in Danish human infections.

A definitive cause-effect association cannot be established through our approach. A genotype overlay is not a proof that can be used for a proper source attribution, but the absence of a genotype overlap provides evidence against a possible link. Therefore, some inferences can be made. With this in mind, the limited genotype overlap between the two reservoirs indicate that meat might play a minor role for human cases of ESBL/AmpC-PEC.

4.1. Data and methods

A critical analysis should be done to the exposure assessment due to some possible bias that was not possible to avert. The meat consumption patterns were measured through the fresh and frozen meat available for consumption. This is the amount of meat which is in the market during a year, since it is calculated as: domestically produced – export + import (DTU, 2011). The values used do not represent the amount of meat that was actually consumed. However, it was assumed that possible waste of meat occurred in the same proportion for all the types of meat.

Moreover, the bacterial capacity to survive in frozen and fresh meat is different (Black *et al.*, 2010) and it was not taken into account. Once again, if the proportion between fresh and frozen meat is identical and considering the *E. coli* ability to survive in different types of meat similar, the final results are not affected.

It was not possible to extend the study to other types of meat, as turkey or horse, due to lack of data on ESBL/AmpC-PEC prevalences. Nonetheless, this exposure assessment covers the three types of meat that represent the vast majority of meat consumed in Denmark.

DANMAP reported high prevalences in imported broiler meat from 2009 to 2011; varying from 36% to 50% (DANMAP, 2009, 2010, 2011). Regarding Danish broiler meat ESBL/AmpC prevalence suffered a sudden increase in 2011 from 8.6% to 44% (DANMAP, 2011). Considering cephalosporins are not used in poultry in Denmark for more than 10 years it is possible that the problem is upstream in the production pyramid. This hypothesis has also arisen in Sweden (Börjesson *et al.*, 2013; SVARM, 2010). Danish and Swedish broiler parents come from the same Swedish breeding stock, which in turn is supplied by a Scottish grand-parent breeding company. It is possible that cephalosporins were used at this point of the

production chain as a prophylactic measure. Cross-contamination through the environment, humans or animals, as well as off-label use of cephalosporins, should also be considered and investigated. However, the fact that CMY-2 and CTX-M-1 were also the genotypes detected in Swedish broiler meat (SVARM, 2010) and both of them being the only ESBL/AmpC genes persistently detected in Danish broiler meat from 2009 to 2011, strengths the hypothesis of a common source upstream in the production pyramid. Co-resistance patterns may have facilitated the spread and maintenance of ESBL/AmpC-PEC (Börjesson *et al.*, 2013). Transmission between flocks is also possible, although might represent a low contribution due to the *all in/ all out* policy and strict biosecurity implemented in Denmark. Nevertheless, the use of biocides for farm disinfection has been considered has a possible contributor for antimicrobial resistance (Schets *et al.*, 2012). This problem should be investigated not only in broiler farms but also in cattle and pig farms.

Imported broiler meat presented a great variety of genotypes (DANMAP, 2011), possibly due to the different importing countries and various causes of ESBL/AmpC emergence.

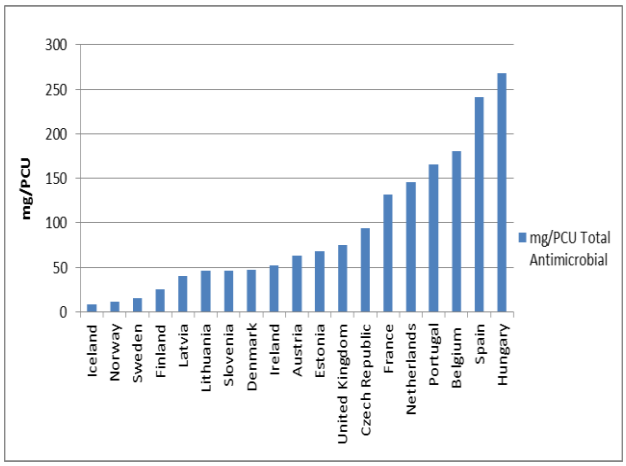


Figure 6 – Antimicrobials sold in 2010 for veterinary purposes in several European countries adjusted with a population correction unit. Source: ESVAC 2010.

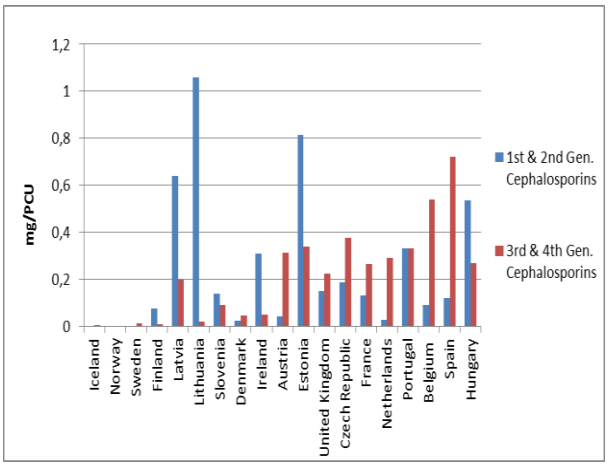


Figure 7 – 1st & 2nd generation and 3rd & 4th generation cephalosporins sold in 2010 for veterinary use in several European countries. Measure adjusted with a population correction use. Source: ESVAC 2010.

Denmark has one of the lowest antimicrobial uses in livestock of the EU and demonstrates a very prudent use of cephalosporins in animals (Figures 6 and 7) (ESVAC, 2010). Although the use of cephalosporins in pig production was already low, a voluntary ban on this antimicrobials use was made in 2010 for this livestock sector (Figure 8). The discontinuation of its use is the most likely explanation for the reduction on the ESBL/AmpC-PEC prevalence seen in pigs in 2011 (Agersø & Aarestrup, 2012). Other factors could also have contributed to this diminution. As the "Yellow card" initiative, adopted by the Danish Veterinary and Food Administration to

reduce the overall antimicrobial consumption, that took place almost at the same time and initially led to a 25% decrease in use of antimicrobials (Aarestrup, 2012; Alban *et al.*, 2013).

A decrease of the prevalence of ESBL/AmpC-PEC in pork from 1.9% in 2009 to 0.9% in 2011 was also observed, although not statistical significant (Agersø & Aarestrup, 2012).

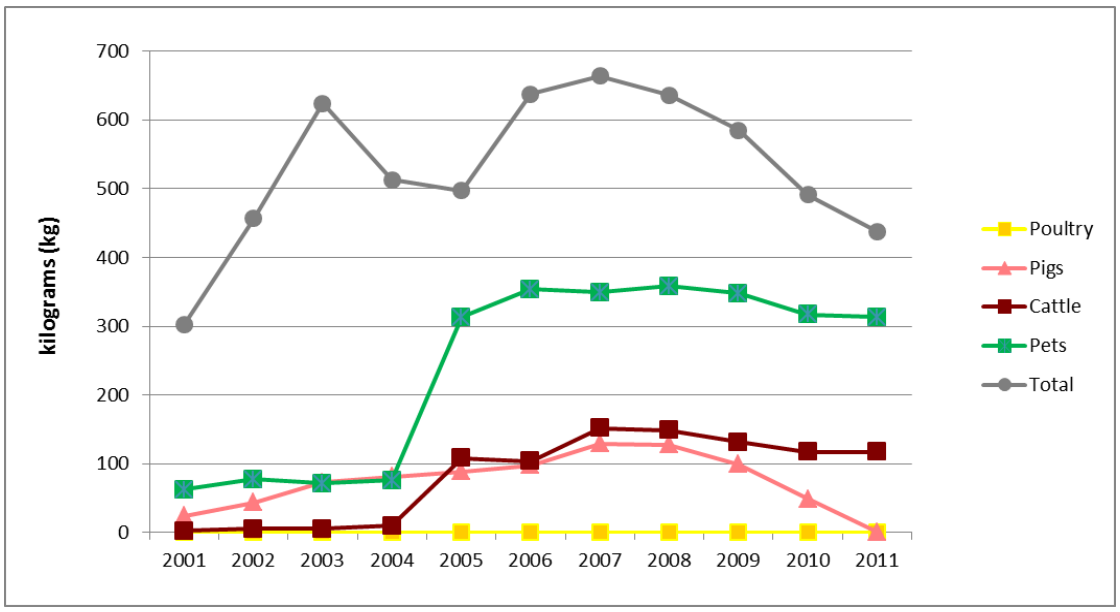


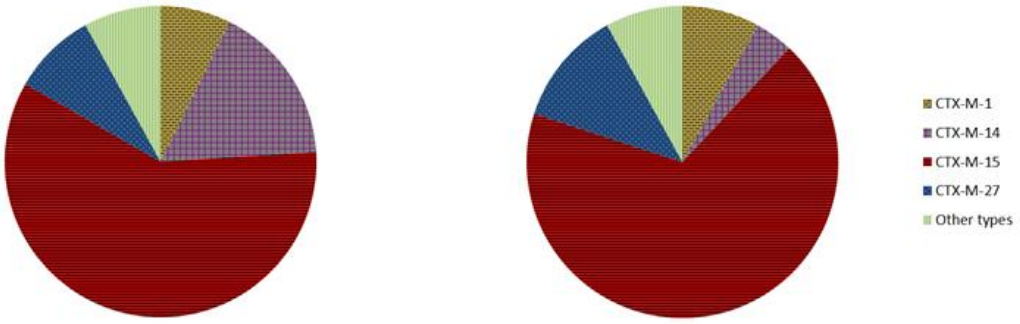
Figure 8 – Kg of cephalosporins sold in Denmark for veterinary use. The increase seen in pets and cattle, and consequently in the total, from 2004 to 2005 is an artefact due to changes in the calculation method. Human data is not displayed. Since 2008, human consumption has been above 2500 kg. Source: DANMAP 2001-2011.

4.2. The relative importance of meat and the overlapping with genotypes detected in humans

The relative importance of each genotype found in meat for human exposure was calculated considering a 3-year pool. This may be considered a long period for prevalence estimation due to the explosive emergence and quick evolution of ESBL/AmpC, but resulted in a wider perspective of the situation. Furthermore, the procedure was also repeated for the 3 years separately.

Some changes were observed in the relative importance of each genotype from 2009 to 2011. Mainly minor shifts happened due to sporadic detection of some ESBL/AmpC genes and were due to the limited sample sizes. However, the most evident change occurred with the emergence of CMY-2 in Danish broiler meat from 5.6% in 2009 to 53.5% in 2011, having also repercussions on the relative importance of Danish broiler meat and Danish meat in general. This was due to the increasing prevalence of this genotype in Danish broiler meat. Especially, CMY-2 and CTX-M-2 in imported broiler meat and CTX-M-1 both in broiler imported meat and imported pork were consistently represented over the 3 years.

In humans, the so-called “pandemic CTX-M-15” genotype dominate both in blood and urine ESBL-PEC isolates, with 68.0% and 59.3%, respectively (Figure 9) (DANMAP, 2011). Results from 2007 are not as descriptive as the ones from 2011, but CTX-M-15 was approximately detected in 70% of the cases, while CTX-M-1 was found in roughly 5% of them (DANMAP, 2009). In 2011, CTX-M-1 was detected in 7.3% of urine and 8.0% of blood isolates (DANMAP, 2011). However, it should be noted that, particularly for human ESBL-PEC septicaemia, the number of cases on which these results are based is quite small (n=25). Subsequently, it is possible that the true ESBL genotype distribution is slightly diverse from what was found and some genotypes causing infections may have not been detected.



(A) n = 275 (B) n = 25

Figure 9 – (A) – Proportion of each genotype detected in ESBL-PEC urinary tract infections in humans in Denmark in 2011. (B) - Proportion of each genotype detected in ESBL-PEC bloodstream infections in humans in Denmark in 2011. Source: DANMAP

ESBL/AmpC genes found in humans and meat were, as previously described, only congruent to a limited extent. Just considering the detection of the same genotypes in both reservoirs (CTX-M-1 and CTX-M-14), only 24.0% of the urinary tract infections cases and 12.0% of the sepsis ones, in the worst case scenario, could be ascribed to meat.

It should be highlighted, however, that although some genotypes were not found in meat they were found in live animals, indicating their possible presence in meat. This is the case of CTX-M-15, which was detected in very low prevalences in Danish pigs and in Danish cattle. The very low prevalence probably explains the reason for why it was not detected in meat.

This was one of the main motives which propelled us to perform an uncertainty analysis through the use of beta distributions. As expected, the mode prevalence was equal or very similar to the prevalence found in DANMAP and the uncertainty was higher with smaller sample sizes. This investigation is especially interesting to apply to genotypes that were found in live animals and not in meat. The maximum expected prevalence for ESBL/AmpC genes not detected in meat, considering the DANMAP sampling within the 3-year aforementioned period,

was 0.7% for Danish pork, 0.9% for imported pork, 1.0% for Danish beef, 1.4% for imported beef, 0.9% for Danish broiler meat and, finally, 0.7% for imported broiler meat. Through our calculations, these are the upper 95% credibility limit for the expected prevalences for CTX-M-15 in each type of meat. Such low prevalence makes us doubt about a possible relevant role of meat for the CTX-M-15 producing *E. coli* infections in humans. Nonetheless, very little is known about the duration of carriage, dominance in the human gut microflora and different ESBL/AmpC's ability to survive in human gut, to reach a definitive conclusion.

The homogeneity of the ESBL/AmpC genes found in *E. coli* from imported beef is mostly likely a result of the very low number of positive samples: one positive sample per year. In Danish beef just two positive samples were found from 2009 to 2011, but from different genotypes (data not published). Due to this low prevalence, beef is the type of meat in which the uncertainty about the true ESBL/AmpC genotype distribution is the highest. It is advisable to increase the sampling process to achieve a better understanding of the genotype distribution in beef.

It is of great relevance to highlight that CMY-2 was detected in human infections, but it was not possible to calculate its prevalence in humans. Nevertheless, the positive number of samples was low (data not published). CMY-2 is frequently seen in humans in the USA (ECDC, 2012). Considering the possible emergence of CMY-2 *E. coli* and its high relevance for human exposure through meat, it is important that its prevalence is calculated in the upcoming years.

Depoorter *et al.* (2012) performed a quantitative exposure assessment to 3rd generation cephalosporin resistant *E. coli* through the consumption of broiler meat in Belgium, based to some extent on previous risk assessment models for *Salmonella* and *Campylobacter.* Although this study's approach is different, Depoorter *et al.* (2012) also highlighted the lack of some important data to evaluate the impact of animal foodborne sources to human exposure.

4.3. Control options

Meat might not have such a high impact for human ESBL/AmpC-PEC infections as firstly thought. However, this does not exclude the importance of animal production, and even meat, for ESBL/AmpC dissemination. Therefore, and attending to the relevance of this subject, a precautionary approach should be taken. In Europe, the use of cephalosporins in poultry is prohibited and in Denmark a voluntary ban was implemented by the pig industry to their use in pigs in 2010. Cephalosporins are still used in Danish cattle, especially for intra-mammary injections, but Danish beef do not seem to be a relevant source of ESBL/AmpC exposure for humans. Regarding the high ESBL/AmpC-PEC prevalences in Danish broiler meat, measures have recently been taken to assure the non-use of cephalosporins on the top of the production pyramid (Jan Dahl, DAFC, personal communication, 2013).

In Denmark, there are limited new options to control ESBL/AmpC occurrence through the reduction of cephalosporins use in livestock. It is known that other antimicrobials can co-select ESBL/AmpC genes. Therefore, measures to promote the reduction of antimicrobial use in general, such as the "Yellow card" scheme, may have some influence.

However, downstream the production chain, other options can be explored. In first place, some studies have documented the existence of some risk factors for ESBL/AmpC growing in animals' gut (Persoons *et al.*, 2010). The impact of probiotics in the control of this type of bacteria should also be studied.

Secondly, cross contamination during slaughter should be inspected and, if relevant, hygiene should be improved. Other option to consider is to implement decontamination after slaughter.

Animal trade has been considered a promoter of ESBL/AmpC dissemination. DANMAP has consistently reported higher prevalences of ESBL/AmpC-PEC in imported meat, suggesting that non-Danish produced meat involves a higher risk of being exposed to ESBL/AmpC-PEC. European common measures should preferably be implemented to fight this hazard, rather than individual, national policies.

Table 1 – Suggested measures to control ESBL/AmpC-PB.

Possible source	**Measures**
Human-to-human and nosocomial infections	• Improve hospital hygiene • Stricter guidelines, both for hospital and community use of cephalosporins
Animal-to-human transmission	• Stricter guidelines for the use of cephalosporins in pets
Travelling	• International measures to control ESBL/AmpC-PB • Advice to travellers about hygiene measures to prevent becoming infected with ESBL/AmpC-PB
Environment	• Reduce the use of cephalosporins and antimicrobials in general; this should decrease ESBL/AmpC-PB dissemination
Foodborne	• Reduce the use of cephalosporins and other antimicrobials in general in livestock • Production measures to reduce ESBL/AmpC-PB dissemination • Improve slaughter hygiene • Decontamination after slaughter

Nonetheless, a judicious use of resources must be done. In that sense, the role of other sources should be assessed, inclusively for other foodborne sources. This would allow us to point the reservoir where most effort should be applied to control ESBL/AmpC-PEC. Table 1 present some general measures to control ESBL/AmpC-PEC, and ESBL/AmpC-PB in general.

It is possible that meat is the source for at least a small part of the human cases, but to obtain more precise conclusions, further studies need to be performed. ESBL/AmpC-PB is a very complex subject and their emergence, ecology and dynamics is nowadays poorly understood.

As previously mentioned, a wider study about the ESBL/AmpC genotypes in human cases in Denmark is necessary. An extensive characterization of human patients must be done. Moreover, CMY-2, and other AmpC, prevalence need to be calculated.

Just one study has been performed about the duration of carriage and prevalence in healthy individuals in Denmark. Hammerum *et al.* (2011) performed a study in healthy army recruits. From 84 participants, 7% were carriers of ESBL/AmpC-PB, suggesting the existence of a human reservoir. Additionally, studying the risk factors in healthy humans could be a relevant step for source attribution and important to prioritize control measures. The relevance of ESBL/AmpC carriage for the occurrence of community acquired and nosocomial infections should also be assessed.

Furthermore, very little is known about the impact of control strategies, which hampers prioritisation of measures to prevent the emergence and dissemination of ESBL/AmpC-PB. This is a critical point that must be further investigated urgently.

Finally, we suggest that data collection, in human, food and animal reservoirs, become harmonized across the EU. This would not only allow us to actually compare data between countries, but also facilitates to study risk factors and suggest optimal control strategies.

5. Conclusions

Lack of data disabled us from performing a full risk assessment for ESBL/AmpC-PB with source attribution. However, we were able to undertake an exposure assessment to evaluate the relative role of meat for ESBL/AmpC human cases.

Our study concluded that poultry meat is the most relevant for human ESBL/AmpC-PEC exposure, followed by pork and beef. In general, Danish meat has a lower relative importance compared with imported meat. Nevertheless, the prevalence of ESBL/AmpC-PEC in Danish broiler meat significantly increased in 2011, leading to a considerable growth in its relative importance. High prevalences in broiler meat are probably related to cephalosporins use in the top of the production pyramid and measures are being taken to control the problem.

CMY-2 was the ESBL/AmpC gene seen in meat with the highest relevance for food safety. It was detected in low proportions in human infections, but its prevalence in humans was impossible to calculate. We suggest that this situation should be carefully followed and CMY-2 prevalence in urinary and bloodstream human infections should be calculated.

CTX-M-1 and CTX-M-14 producing *E. coli* were found in both human and animal reservoirs, but the relevance of both genotypes for human infections is not very significant.

“Pandemic” CTX-M-15 was not found in meat samples. Nonetheless, it should not be ruled out the possibility of its existence in meat, especially because it has previously been detected in live animals, although at a low prevalence.

The complexity of this subject has not been transposed. A better understanding of the entire problematic is required, unveiling the factors that lead to ESBL/AmpC emergence and explaining the interface between several sources and humans. This may turn it possible to clarify the increasing occurrence of human cases. With that purpose, future studies are urgently needed, especially to complement the data on the human side.

In conclusion, the genotype overlap between the two reservoirs – human and animal - is small, suggesting that meat might not have such a relevant role for ESBL/AmpC-PEC human infections in Denmark as it was firstly thought.

Review on ESBL/AmpC-producing bacteria potential sources

This chapter intends to provide an overview on the current knowledge about the several possible sources of ESBL/AmpC-PB. These potential sources can be gathered into five major groups: foodborne, human-to-human transmission, animal-to-human transmission, environment and acquired abroad during travelling.

1. Foodborne

1.1. Meat

Meat has been extensively discussed as a potential source of ESBL/AmpC-PB, taking the Danish situation as an example. Nevertheless, some Dutch studies assign a more important role of retail chicken meat and poultry meat as vectors for ESBL/AmpC-PB transmission to humans (Kluytmans *et al.*, 2013; Leverstein-van Hall *et al.*, 2011; Overdevest *et al.*, 2011). In fact, the predominant ESBL genes found in chicken meat were identical to the ones found in human rectal specimens (Overdevest *et al.*, 2011). These differences between the Dutch and Danish situations may be due to the higher livestock's antimicrobial use in the Netherlands compared to Denmark. However, it should be noted that these studies do not reached a definitive conclusion or any cause-effect association between meat consumption and the occurrence of ESBL/AmpC-PB related infections.

ESBL/AmpC-PB were detected in other animals, such as ducks, rabbits, turkeys or ostriches (Blanc *et al.*, 2006; Carneiro *et al.*, 2010; Ma *et al.*, 2012; Randall *et al.*, 2011), thus, all these types of food-producing animals are potential sources for human gut colonization by ESBL/AmpC-PB. Furthermore, game animals have also been identified as possible reservoirs (Poeta *et al.*, 2009). However, the role of these types of potential meat for ESBL/AmpC-PB acquisition by humans is not fully understood, but is likely to have less impact than the meat of domesticated animals.

1.2. Fish

1.2.1. Wild fish

Regarding wild fish, it is well known that the antimicrobial pressure they suffer is much lower than the antibiotic stress experienced by aquaculture fish. Still, wild fish may be exposed to ESBL/AmpC-PB due to water contamination with faecal content of other animals and humans.

Sousa *et al.* (2011) assessed the carriage of ESBL-PEC in the faecal samples of *Sparus aurata* captured in the West coast of Portugal. From 118 analysed samples, five were ESBL

positive (4.2%), being TEM-52 and SHV-12 the genotypes detected. Thus, this study suggests that wild fish can be a reservoir and a potential vehicle to ESBL-PEC human infections.

1.2.2. Aquaculture fish

Aquaculture fish are exposed to various stressors, ranging from physical to chemical, caused by human intervention (due to processes to enhance production, such as regular stocking, feeding, protection from predators) and several other environmental constrains (Barton, 2002; Cabello, 2006; Naylor & Burke, 2005; Sørum, 2006). This generally has negative repercussions in the fish immune system, increasing the possibility of infections and their rapid spread. Moreover, the widespread and unrestricted use of prophylactic antibiotics in the industry of aquaculture, commonly used to prevent bacterial infections resulting from sanitary shortcomings in fish rearing**,** normally remain in the aquatic environment, exerting their selective pressure for long periods of time and, thus, leading to the emergence of antibiotic-resistant bacteria in these aquaculture environments (Cabello, 2006). Although the use of cephalosporins in aquaculture is not authorized (Serrano, 2005) (except in properly justified situations), the extend use of other antimicrobials may lead to the emergence of ESBL/AmpC-PB due to co-resistance patterns. Additionally, sometimes water contamination with antibiotics may be originated from sewage, agriculture or industrial wastewater (Cabello, 2006).

In Asia, the Integrated Multi-Trophic Aquaculture (IMTA), in which the by-products (wastes) from one species (fish or terrestrial animals) are recycled to become food for another, is a common practice (Cabello, 2006; Dang, *et al.*, 2011; Petersen & Dalsgaard, 2003). This facilitates the transmission of ESBL/AmpC-PB from terrestrial animals to aquaculture fish.

To our knowledge there are no studies performed in Europe to evaluate the existence of ESBL/AmpC-PB in aquaculture fish. Jiang *et al.* (2012) found an ESBL-PEC prevalence of 1.5% in farmed fish in China, while Ishida *et al.* (2010) found 5.1% prevalence in Egypt. However, it should be highlighted that both studies used non-selective culture methods.

Alderman & Hastings (1998) suggested that the risk for the public health resulting from the usage of antimicrobial agents in aquaculture was very low. Presently, this still probably be true in Europe and North America where decreasing trends in the use of antibiotics in aquaculture have been observed, mostly due to more restricted legislation apply to this industry (Heuer *et al.*, 2009; Petersen & Dalsgaard, 2003). However, the same is not applicable to the Asian continent. Asia, leaded by China, have the largest world's aquaculture environment (Heuer *et al.*, 2009; Jiang *et al.*, 2012), supplying European countries with great amounts of aquaculture fish, which is imported frozen and despite reducing the microbial load, the risk of bacterial contamination should also be assessed.

To date there are no studies reporting the presence of ESBL/AmpC-PB in fish meat, hindering the implementation of an exposure assessment. Sørum (2006) stated that the transfer

of antimicrobial resistant determinants between human and fish bacteria occurred much faster than the same process with terrestrial animal husbandry. This highlights the need to study and control ESBL/AmpC-PB emergence in fish.

1.3. Milk and milk products

ESBL/AmpC-PB have been found in milk and milk products (Amador *et al.*, 2009; Gundogan & Yakar, 2006). That is not surprising once cows are often exposed to antibiotics. Moreover, cephalosporins are commonly used to treat mastitis and for the dry-cow therapy (Merck, 2012). Actually, in Denmark, the majority of the cephalosporins used in cattle are intra-mammary injections (DANMAP, 2011). The biggest issue is that cephalosporins are excreted in milk.

Ultra pasteurized milk is not likely to carry any bacteria. However, some products made with raw or pasteurized milk may constitute a route for ESBL/AmpC-PB transmission to humans (Amador *et al.*, 2009; Gundogan & Yakar, 2006). The same is also true in the case of milk from other animal species, such as ewe or goat (Amador *et al.*, 2009).

Amador *et al.* (2009) found that 80.9% of the *Enterobacteriaceae* isolates from 6 different types of Portuguese cheese harbored genes from the TEM ESBL family. No CTX-M or SHV ESBL family genes were detected.

Gundogan & Yakar (2006) assessed the presence of ESBL-producing *Klebsiella* species in Turkey in raw and pasteurized milk, white cheese and ice cream. ESBL-producing *Klebsiella* were detected in the four types of samples, with an average prevalence of 35% (n=43).

These studies reflect the need to control the presence of these bacteria in the milk, but also to improve the good hygienic practices during milking and production of cheese.

1.4. Eggs

The importance of the egg as an ESBL/AmpC-PB source is not well documented (Egea *et al.*, 2011). Moreover, there is limited data on the prevalence of ESBL/AmpC-PB in the chicken egg production.

It is relevant not only to assess the importance of the egg as an ESBL/AmpC-PB vehicle aliment, but also the possibility of cross contamination in the kitchen through the eggshell.

Bortolaia *et al.* (2010) investigated the occurrence of ESBL-PEC in flocks of chicken egg layers reared in Danish organic systems. From de 60 cloacal swabs collected from live animals, 3% were positive for CTX-M-producing *E. coli*. The beta-lactamase genes found were from de groups CTX-M-1, -2, -9 and TEM-1. It must be noted that this study was conducted in organic farms where the antimicrobial use is generally lower. Actually, there was no history of antimicrobial or biocide use on the farms where the samples were collected.

Egea *et al.* (2011) assessed the possibility of eggshell contamination with ESBL-PEC, by analysing 72 eggshells from several supermarkets and no positive samples were found.

The sanitation procedures for chicken eggshells may also explain the absence of ESBL/AmpC-PB on this product. In the EU, washing egg procedures are not allowed, but other processes (gaseous ozone, chloride ultraviolet radiation, dirty eggs exclusion) are applied in order to reduce the coliform load, what may contribute to the nonappearance of ESBL/AmpC-PB in the above mentioned study.

The available data on this subject is scarce and more studies should be conducted to accurately assess the importance of eggs in the transmission of ESBL/AmpC-PB.

1.5. Fruits and vegetables

The existing microflora on raw fruits and vegetables is usually harmless for the human health, although pathogenic microorganisms are occasionally found. Several human infection outbreaks resulting from vegetables or fruits contamination have been reported (Beuchat, 2002). A recent example was the presence of *E. coli* O104:H4 in sprouts that caused an outbreak in Germany (Buchholz *et al.*, 2011).

Furthermore, fruits and vegetables may be contaminated with resistant bacteria at any point from production to consumption (Hassan *et al.*, 2011; Österblad *et al.*, 1999). The use of antibiotics during cultivation, use of contaminated water or contaminated fertilizers may enhance the colonization of fruits and vegetables by resistant bacteria (Österblad *et al.*, 1999).

Hassan *et al.* (2011) performed a study to assess the bacterial load and the occurrence of some disease-causing enteric bacteria on raw vegetables sold in Saudi markets and found that 2.3% of the isolates were ESBL producers and 14.8% were resistant to extended-spectrum beta-lactam antibiotics. Other studies did not found ESBL/AmpC-PB, but found resistance to first and second generation cephalosporins in isolates from vegetables, fruits and fruit juice (Hamilton-Miller & Shah, 2001; Lateef *et al.*, 2004; Österblad *et al.*, 1999)

Antibiotic resistant bacteria detection in fruits and vegetables is particularly worrying since these products are often eaten raw. Moreover, they may constitute a risk for kitchen cross-contamination. Future studies need to be performed to cover the little data available on the subject and to assess the role of these products in ESBL/AmpC-PB human infections.

2. Human-to-human transmission

Humans are also an ESBL/AmpC-PB reservoir. The increase number of carriers leads to an increased risk of other individuals become carriers as well (Levin, 2001; Valverde *et al.*, 2004).

Besides the study on army recruits in Denmark (Hammerum *et al.*, 2011), few studies were performed to assess ESBL/AmpC-PB carriage in healthy individuals. Geser *et al.* (2012) found that 5.8% of the faecal samples collected from 586 humans in Switzerland yielded ESBL producers, confirming the existence of a human reservoir.

Duration of carriage has not been extensively studied and the results available show great variability. Alsterlund *et al.* (2012) found that carriage could last more than 58 months, after following patients from an ESBL outbreak. Apisarnthanarak *et al.* (2008) detected an average ESBL-producing bacteria (ESBL-PB) carriage of 98 days among outpatients, during a 6 month follow-up. It is of great relevance to study the factors that influence these variations in the time of carriage of ESBL-PB by unhealthy patients, as well as to determine the duration of carriage in healthy individuals.

The increase of carriers in the community, and consequently of the resistome, may jeopardize the decrease of the ESBL/AmpC-PB in nosocomial settings (Lipsitch & Samore, 2002; Valverde *et al.*, 2004).

Indeed, nosocomial ESBL/AmpC-PB infections may have a very important role, especially when considering severe infections. Before 2000, the majority of the ESBL cases were nosocomial, often caused by *Klebsiella spp.* (Livermore *et al.*, 2007). It is of great relevance to determine the proportion of ESBL/AmpC-PB infections that are nosocomial. A great number of these infections may lead us to focus more on the hospital practices and hygiene.

3. Animal-to-human transmission

3.1. Pet animals

Regarding companion animals, ESBL/AmpC-PB transmission should be observed as a bidirectional phenomenon, driven by the close contact of the two reservoirs; it is well known that pets are commonly let free inside the house and close face-to-fur contact is very frequent (Wieler *et al.*, 2011). Additionally, considering meat a possible vehicle of ESBL/AmpC-PB, pets often eat the same food of their owners, which can select the same gut microflora in both reservoirs. Considering these factors, it is not surprising that several studies found similar ESBL/AmpC genotypes in humans and pets, including the CTX-M-15 genotype (Costa *et al.*, 2008; Damborg *et al.*, 2011; Ewers *et al.*, 2010; Huber *et al.*, 2013; Lefebvre *et al.*, 2006; Tamang *et al.*, 2012; Wedley *et al.*, 2011). Meyer *et al.* (2012) suggested that having pet animals could be a risk factor for human ESBL-PB acquisition. Nonetheless, this study is based on a very limited number of positive samples (n=8).

The status of family member that pets gained during the last decades also changed the perception of welfare and the expected medical care services. This led to no differences between the antimicrobials used for human and pet treatments and also to an increase in antimicrobial usage in companion animals. The use of cephalosporins in pet animals has been on the rise (Peter Damborg *et al.*, 2011) and strong evidences have shown that their use contributed for ESBL/AmpC-PEC appearance in dogs and horses (Damborg *et al.*, 2009; Damborg *et al.*, 2012)

Compared to food-producing animals, little attention has been given to companion animals regarding the possibility of ESBL/AmpC-PB transmission to humans. With rare exceptions, surveillance programmes do not include pet animals (Guardabassi *et al.*, 2004). However, considering the actual knowledge, the role of companion animals should not be neglected.

3.2. Contact with farm animals

Considering that farm animals are ESBL/AmpC-PB reservoirs, humans may be exposed not only through meat, but also by direct contact.

Moodley & Guardabassi (2009) investigated the transmission of plasmids carrying ESBL genes between pigs and farm workers. The results indicated that there was, indeed, transmission of these mobile elements between the reservoirs. If farm workers are at a higher risk of carriage of ESBL/AmpC-PB, their importance as carriers should be investigated.

More recently, Huijbers *et al.* (2013) performed a study in the Netherlands to assess if living in areas of high broiler densities could implicate a higher risk of carrying ESBL-producing *Enterobacteriaceae*. However, this hypothesis was not confirmed. It should be noted that several factors may have influenced the results.

The importance of contact with farm animals may be extended to other species. Kozak *et al.* (2009) found that small rodents living nearby pig farms carried antimicrobial resistant *E. coli* more frequently than the ones living in natural areas. Furthermore, it seemed to occur an association between the type of resistance found in pigs and small rodents. However, the sampling was too small to prove it statistically. This suggests that animal production may also have impact on the bacterial resistance found in wild animals.

3.3. Wild animals

ESBL/AmpC-producing bacteria have also been reported in wild animals (Bonnedahl *et al.*, 2009; Costa *et al.*, 2006, Costa *et al.*, 2008; Guenther *et al.*, 2010; Literak *et al.*, 2010; Pinto *et al.*, 2010; Poeta *et al.*, 2009; Silva *et al.*, 2011; Simões *et al.*, 2010; Simões *et al.*, 2012) and some of them harbour the same genes detected in human cases. Considering that no apparent exposure had occur in these animals, resistance bacteria should not be confined within the reservoir where resistance emerged (da Costa *et al.*, 2013).

Wild animals may contribute to the maintenance and dissemination of ESBL/AmpC-PB. This has especial interest for public health when these animals' habitat is in urban areas and, thus, the contact with humans is closer. That is the case of seagulls. Simões *et al.* (2010) found that 32% of *E. coli* isolated from seagull faeces in beaches of Porto region, in Portugal, were ESBL positive. Moreover, CTX-M-15 was the most detected genotype, as well as it was in another study involving hospitalized patients in Porto (Machado *et al.*, 2006). This may raise the possibility of a link between the two reservoirs, seagulls and humans.

4. Environment

As da Costa *et al.* (2013) stated: “The environment is the melting pot of antimicrobial resistance”. Environmental niches may play a central role for ESBL/AmpC dispersion and maintenance and their relevance have been neglected. The actual number of genotypes within the environmental resistome is not known and the acquisition of these genes by human pathogens is also not well studied (Allen *et al.*, 2010; da Costa *et al.*, 2013).

Through the dispersion of antimicrobial residues, these molecules may still exert selective pressure far away from the place where they firstly were used (Allen *et al.*, 2010; Tello *et al.*, 2012). The sewage released from hospitals and pharmaceutical industries (Guardabassi *et al.*, 1998) plays an important role in the dissemination of resistant genes in the environment. Additionally, horizontal gene transfer may occur between commensal and pathogenic bacteria.

Wastewater treatment plants are potential hotspots for antimicrobial resistance acquisition through horizontal gene transfer (Guardabassi *et al.*, 2002). Moreover, effluent treatments do not guarantee that antimicrobial resistant bacteria are not released in the receiving water and so, dispersed in the environment (Reinthaler *et al.*, 2003).

Currently, no sewage treatment is able to eliminate selective antibiotic concentrations (da Costa *et al.*, 2013). This is an important topic for future studies that could help us breaking the antimicrobial resistant bacteria cycle.

Zarfel *et al.* (2013) found, in Austrian sewage sludge, ESBL-PEC genetically identical to the bacteria detected in human urinary tract infections, suggesting the possibility of recirculation of these genes. Thermal treatment and lime stabilization followed by dehydration was suggested as the preferable method to reduce pathogen concentration in sewage sludge (Reinthaler *et al.*, 2010). However, it is not possible to guarantee that no ESBL/AmpC-PB are in the sludge and, therefore, its use as fertilizer can contaminate the environment (da Costa *et al.*, 2013)

The spread of ESBL/AmpC-PB in the environment and their transmission to new hosts is one of the most complex topics related with this subject. This is due to the numerous possible pathways and influencing factors on the spread of these antimicrobial resistance genes.

5. Travelling

ESBL/AmpC-PB acquisition while travelling abroad is considered a source *per se* since the ESBL/AmpC reality varies between countries and the risk to acquire these bacteria is different considering diverse parts of the world (Meyer *et al.*, 2012; Tängdén *et al.*, 2010). Furthermore, surveillance programmes are normally performed at a national level and so, is more adequate to consider travel abroad separately. Additionally, the true source is commonly more difficult to determine in abroad acquired infections.

Few studies were performed to evaluate the role of travelling in the acquisition of ESBL/AmpC-PB and some of them have important limitations. Meyer *et al.* (2012) identified travel to Greece and to Africa as potential risk factors for colonization with ESBL-PEC. However, it should be noted that these conclusions are based on a very small number of positive ESBL samples (n=8) and therefore can be considered speculative. Tham *et al.* (2010) assessed the prevalence of ESBL-PEC in stool samples of Swedish patients with travellers' diarrhoea. ESBL-PEC was found in 24% of the patients analysed (n=242). Nonetheless, stool samples were not collected before travelling abroad, so it is not possible to know if the patient was already carrying the resistant bacteria before travelling or not.

In a study performed by Tängdén *et al.* (2010), faecal swabs were collected from 100 volunteer individuals before and after travelling. No one had ESBL-producing *Enterobacteriacea* before travelling. After their return, 24 were already ESBL-PB carriers.

In developing countries, the prevalence of antimicrobial resistant bacteria is commonly higher due to poor hygiene, lack of antimicrobial usage control, overcrowding and absence of good sewage disposal systems (Memish *et al.*, 2003; van der Bij & Pitout, 2012). Currently, a considerable number of people from developed countries is travelling to developing countries in order to do surgical procedures there, to avoid longer waiting times and benefit from lower treatment costs (Van der Bij & Pitout, 2012). It is estimated that this "medical tourism" to India, for instance, will increase around 30% each year until 2015 (Pitout, 2010). This is particularly worrying considering that hospitalization has been suggested as a risk factor for ESBL-PB colonization (Colodner *et al.*, 2004).

Additionally, Freeman *et al.* (2008) stated a higher risk of urinary tract infections caused by ESBL-PEC among New Zealand travellers to India. Moreover, in this study, 3 out of 10 patients had a relapse after treatment with carbapenems.

Globalization and the easy access to travel are facilitating the spread of ESBL/AmpC-PB. Travelling to countries with high prevalence of ESBL/AmpC-PB can be considered a risk factor for colonization and may be a relevant vehicle for ESBL/AmpC-PB dissemination within the population. Joint efforts are mandatory to fight adequately this problem.

Final comments

ESBL/AmpC-PB is a very complex issue, with a very intricate network of connections and influencing factors. These bacteria prevalence and spreading is not well understood, neither controlled.

The great mobility of ESBL/AmpC genetic material helps to explain their dissemination through several ecological niches and adds an extra layer of complexity to control this public health problem.

The absence of knowledge on the effectiveness of measures to control ESBL/AmpC-PB, hampering the prioritization of procedures, is the biggest concern we are facing nowadays.

Furthermore, since new antimicrobials classes have not been discovered since the 70's (Aminov, 2010), the balance between the pros and cons of the way antibiotics are used needs to be rethought.

During the last years, the scientific community turned its attention to the possibility of foodborne sources, especially meat, being vastly implicated in the human ESBL/AmpC-PB infection cases, neglecting the importance of other potential sources. Furthermore, the use of cephalosporins as a driving force for the occurrence of this resistance is becoming not highlighted has it should. As Ewers *et al.* (2012) stated: "The opinion that animal ESBL-producing *E. coli* is a major source of human infections is oversimplified, and neglects a highly complex scenario".

Thus, it is necessary to scrutinize the contribution of other possible sources; principally considering that meat seems not to be as relevant as firstly was predicted, at least in some country cases.

Control measures to face this situation are urgently needed and should be set on a global scale.

For a better understanding of the theme, several areas of scientific knowledge (Human and Veterinary Medicine, Microbiology, Pharmacology, Genetics, among others) should collaborate in order to improve the knowledge on ESBL/AmpC-PB. A broad perspective is required to achieve solutions for this public health problem.

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APPENDIX

APPENDIX I – *Prevalence Estimations*

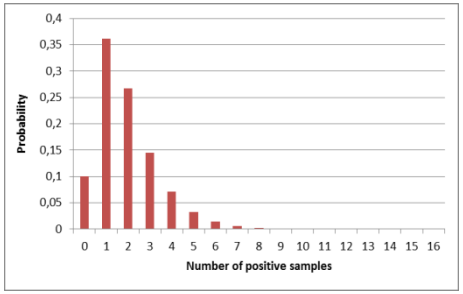
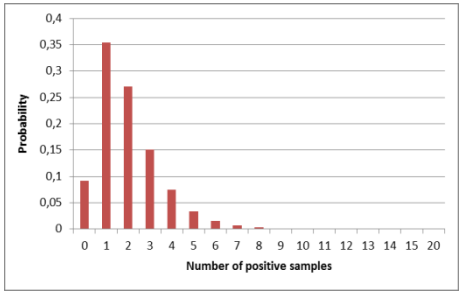
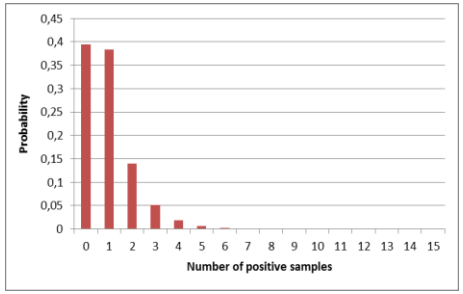
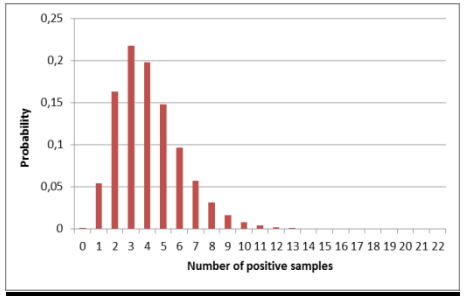
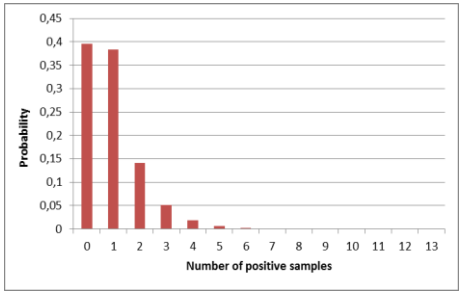
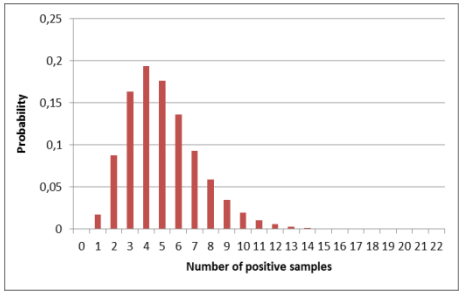
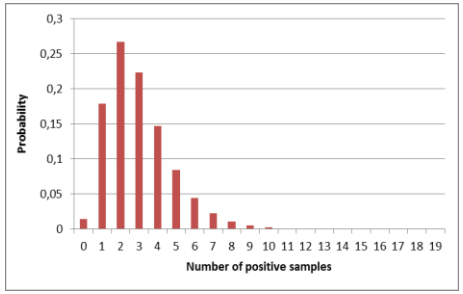
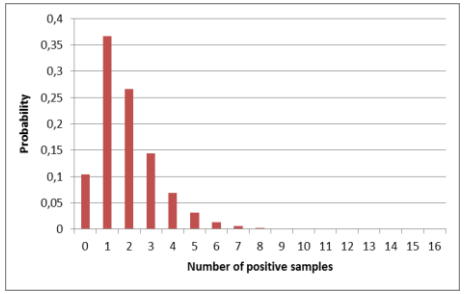
Table 2 – Lower limit, mode and upper limit of prevalence calculated for each genotype in each type of meat through beta distribution with a 95% CI. The total number of samples for each type of meat performed from 2009 to 2011 for DANMAP is represented by n, while s is the number of positive samples for each genotype in each type of meat. For a better understanding and comparison with the uncertainty analysis the genotype prevalence within each type of meat is shown.

Meat type	Origin	Total number of samples for each type of meat n	Number of positive samples for each type of meat	Prevalence 2009-2011	Genotype	Number of positive samples for each genotype s (prevalence)	Beta Distribution			Detected in humans (DANMAP 2011)
							Lower Limit of Prevalence	Mode Prevalence	Upper Limit of Prevalence	
PORK	Danish	562	7	1.2%	CTX-M-1	4 (0.7%)	0.4%	0.7%	1.8%	Yes
					CTX-M-2	2 (0.4%)	0.2%	0.4%	1.2%	No
					Unknown	1 (0.2%)	0.0%	0.2%	0.9%	N/A [1]
					Others	0 (0.0%)	0.0%	0.0%	0.7%	N/A [2]
	Imported	448	11	2.4%	CTX-M-1	9 (2.0%)	1.1%	2.0%	3.8%	Yes
					CTX-M-14	1 (0.2%)	0.0%	0.2%	1.3%	Yes
					CMY-2	1 (0.2%)	0.0%	0.2%	1.3%	Yes [3]
					Others	0 (0.0%)	0.0%	0.0%	0.9%	N/A
BEEF	Danish	382	2	0.5%	TEM-52	1 (0.3%)	0.0%	0.3%	1.3%	No
					CMY-2	1 (0.2%)	0.0%	0.3%	1.3%	N/A
					Others	0 (0.0%)	0.0%	0.0%	1.0%	N/A
	Imported	298	3	1.1%	CTX-M-1	3 (1.1%)	0.3%	1.0%	3.0%	Yes
					Others	0 (0.0%)	0.0%	0.0%	1.4%	N/A

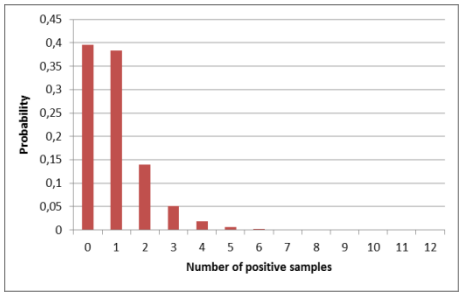
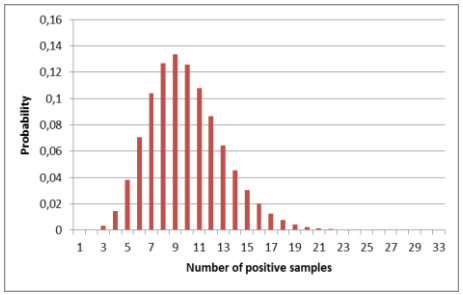
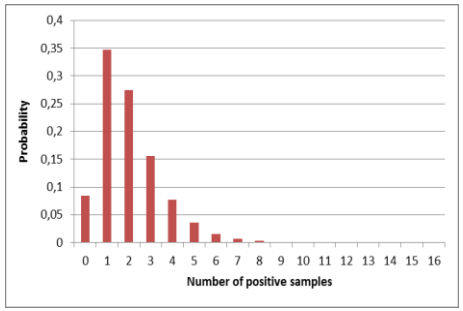
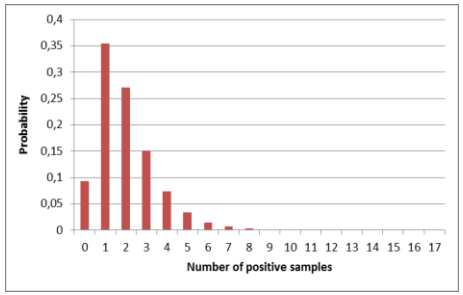
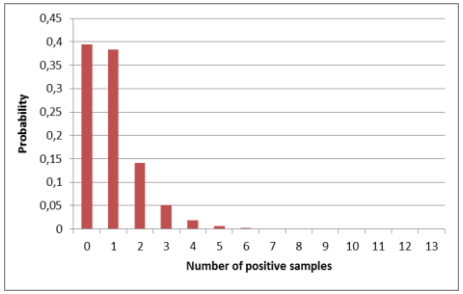
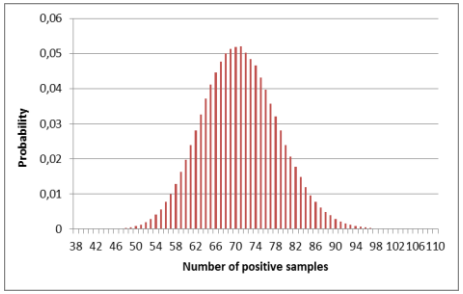
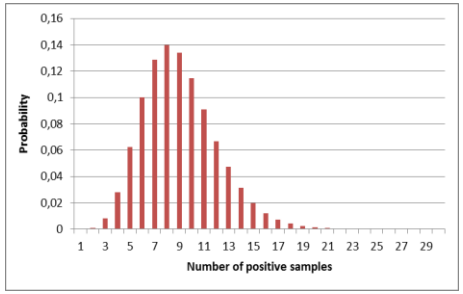
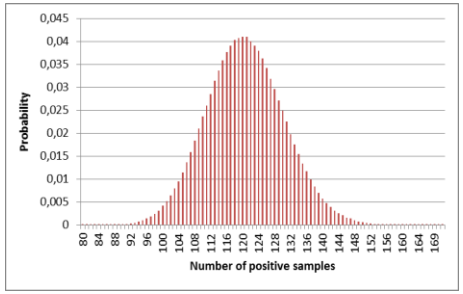
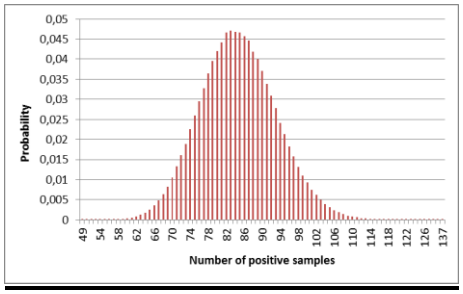
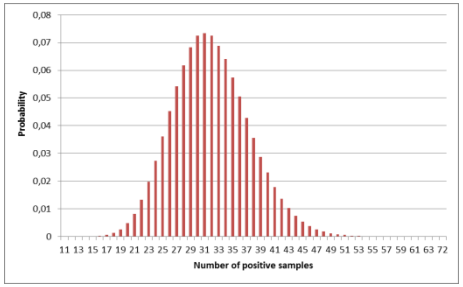
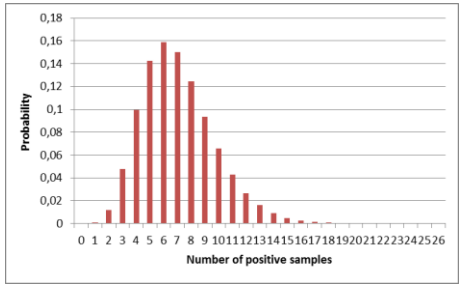
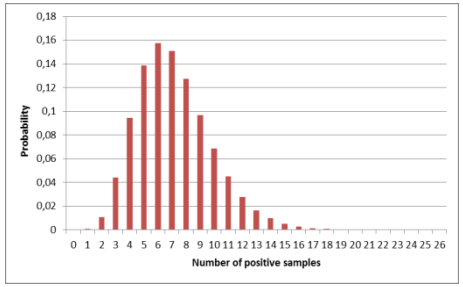
BROILER MEAT	**Danish**	**440**	**78**	**17.8%**	**CMY-2**	**70 (16.0%)**	**12.7%**	**16.1%**	**19.8%**	**Yes**
					CTX-M-1	**8 (1.8%)**	**0.9%**	**1.8%**	**3.6%**	**Yes**
					Others	**0 (0.0%)**	**0.0%**	**0.0%**	**0.9%**	**N/A**
	Imported	**571**	**255**	**44.7%**	**CMY-2**	**120 (21.0%)**	**17.9%**	**21.0%**	**24.5%**	**Yes**
					CTX-M-1	**84 (14.7%)**	**12.1%**	**14.5%**	**17.9%**	**Yes**
					SHV-12	**31 (5.4%)**	**3.9%**	**5.4%**	**7.5%**	**No**
					TEM-52	**6 (1.1%)**	**0.5%**	**1.1%**	**2.3%**	**No**
					Unknown	**6 (1.1%)**	**0.5%**	**1.1%**	**2.3%**	**N/A**
					CTX-M-2	**5 (0.9%)**	**0.4%**	**0.9%**	**1.9%**	**No**
					TEM-20	**1 (0.2%)**	**0.0%**	**0.2%**	**0.9%**	**No**
					Up-regulated AmpC	**1 (0.2%)**	**0.0%**	**0.2%**	**0.9%**	**No**
					SHV-2a	**1 (0.2%)**	**0.0%**	**0.2%**	**0.9%**	**No**
					Others	**0 (0.0%)**	**0.0%**	**0.0%**	**0.7%**	**N/A**

(1) N/A is applied to “Unknown” genotypes found in meat in DANMAP. It was not possible to determine which was the genotype detected.
(2) N/A is applied to “Others”, since it may include genotypes that were present or not in human samples.
(3) CMY-2 was detected in humans but its prevalence was not possible to calculate. However, it is known that few positive CMY-2 samples were detected.

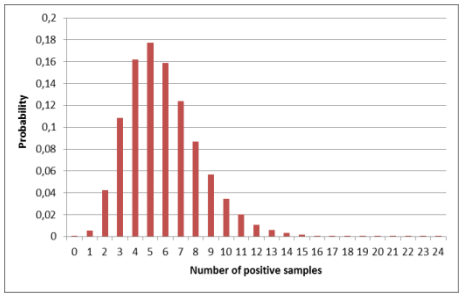
APPENDIX II - *Beta Distributions*

Danish Beef	Danish Beef	Danish Beef	Imported Beef
			
CMY-2	TEM-52	Others	CTX-M-1
Imported Beef	Danish Pork	Danish Pork	Danish Pork
			
Others	CTX-M-1	CTX-M-2	Unknown

The x and y axis vary between the displayed graphs.

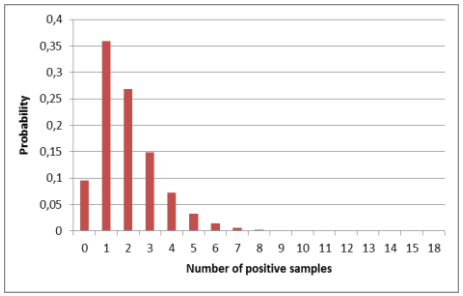
Danish Pork	Imported Pork	Imported Pork	Imported Pork
			
Others	CTX-M-1	CTX-M-14	CMY-2
Imported Pork	Danish Broiler Meat	Danish Broiler Meat	Imported Broiler Meat
			
Others	CMY-2	CTX-M-1	CMY-2
Imported Broiler Meat	Imported Broiler Meat	Imported Broiler Meat	Imported Broiler Meat
			
CTX-M-1	SHV-12	TEM-52	Unknown

Imported Broiler Meat



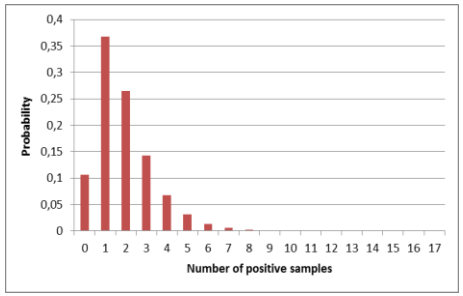
CTX-M-2

Imported Broiler Meat



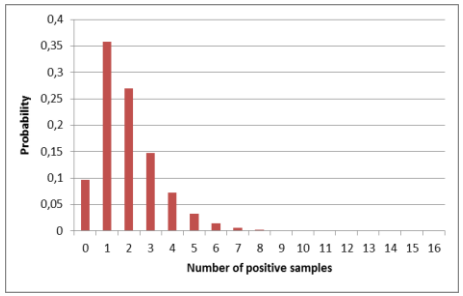
TEM-20

Imported Broiler Meat



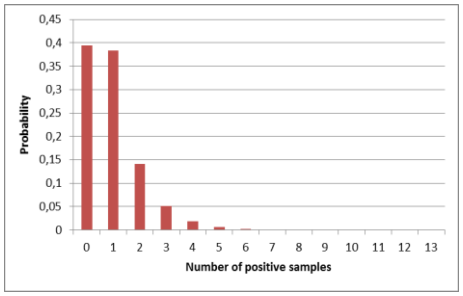
SHV-2a

Imported Broiler Meat



AmpC

Imported Broiler Meat



Others

APPENDIX III - *Other activities enrolled during the internship*

NOSOVE 2013 Meeting and Course

Copenhagen, 14th to 17th January 2013

The Nordic Society for Veterinary Epidemiology (NOSOVE) annual meeting focused on "Introduction to R" and "Advanced prevalence estimation using R". The course work load was equivalent to 1 ECTS.

Seminar about the Danish policy on antimicrobial use in food animals

Copenhagen, Danish Agriculture & Food Council, 8th February 2013

Joint meeting between the Danish Veterinary & Food Administration, the Danish Agriculture & Food Council and the US delegation to discuss the Danish policy on antimicrobial use in food animals, the several initiatives that took place in Denmark in the last few years about this subject and their consequences. The American strategy was also revised.

Speaker at the University of Copenhagen

Copenhagen, University of Copenhagen, 18th February 2013

Presentation of "Risk Assessment of ESBL in Meat" at the Faculty of Health and Medical Sciences of the University of Copenhagen. This lecture was intended for the 4th year Master's Degree on Veterinary Medicine students belonging to the Elite Module and focused on the project structure and on the several risk assessment steps according to the OIE approach.

Burden of Foodborne Diseases Seminar

Copenhagen, Technical University of Denmark, 19th February 2013

A seminar organized by the National Food Institute and the Technical University of Denmark about the state-of-art and the progress made in the last few years on the estimation of the burden of disease in several fields of the food science.

ESBL Seminar

Copenhagen, University of Copenhagen, 1st March 2013

Speaker and participant on the ESBL seminar at the Population Biology group of the University of Copenhagen. This meeting gathered antimicrobial resistance and ESBL experts from several scientific areas to present and discuss their knowledge on ESBL. I presented the project "Risk Assessment of ESBL in Meat".

SVEPM Congress 2013

Madrid, 20th to 22nd March 2013

The annual congress of the Society of Veterinary Epidemiology and Preventive Medicine (SVEPM) took place in Madrid. The lectures covered various topics related to Epidemiology and Preventive Medicine. I was enrolled in the "Basic Epidemiological Concepts" workshop by Peter Cripps and Kenton Morgan from the University of Liverpool and I also presented the poster "Exposure assessment of ESBL in meat" in a poster tour. The poster was awarded one of the three Poster Prizes.

Danish Agriculture & Food Council Statistics Group Meeting

Copenhagen, Axelborg, 11th April 2013

I was one of the speakers, presenting "ESBL – Exposure assessment used to assess the role of meat".

Workshop on Prezi™ Presentations

Copenhagen, IT Learning Center, 15th April 2013

Workshop "Learn to make a Prezi presentation in only two hours" by Loet Rammelsberg and Søren Larsen. The practical workshop focused on the basic tools to create a Prezi and present it.

Workshop on SimHerd™

Åarhus, Research Centre Foulum, Åarhus University, 23rd and 24th April 2013

Workshop with Jan Tind Sørensen, Søren Østergaard, Jehan Ettema and Anne Kudahl on SimHerd™. The programme had a very strong practical component, focusing on the creation of scenarios, interpretation of results and possible uses of the model.

Presentation of the project

Copenhagen, Danish Agriculture and Food Council, 29th April 2013

Presentation of the project to 4th year veterinary medicine students from the food track in the veterinary curriculum at the University of Copenhagen. The draft report was used for a student work project within the subject Risk Assessment.

Presentation of the project

Copenhagen, University of Copenhagen, 30th April 2013

Presentation of the project to the Population Biology group at Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen.

APPENDIX IV - *Poster Prize Winner SVEPM Madrid 2013*

FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

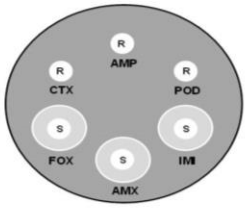
Exposure assessment of ESBL in meat

Luís Pedro Carmo[1, 2], Liza Rosenbaum Nielsen[1], Paulo Martins da Costa[2], Lis Alban[3]

[1] Department of Large Animal Sciences, University of Copenhagen, Denmark, lpgcarmo@gmail.com
[2] Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Portugal
[3] Danish Agriculture & Food Council, Denmark

What are ESBL?

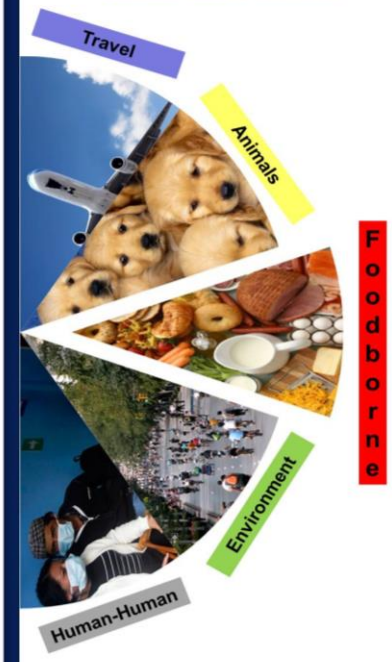
- Plasmid-encoded enzymes found in *Enterobacteriaceae*
- Confer resistance to β-lactam antibiotics
  i.e. penicillins, 2nd, 3rd and 4th generation cephalosporins and monobactams
- ESBL often associated with treatment failure leading to life-threatening human infections
- The increasing prevalence worldwide is worrying



Project objective

To evaluate the relative importance of different types of meat for human ESBL exposure and assess their relevance for human cases

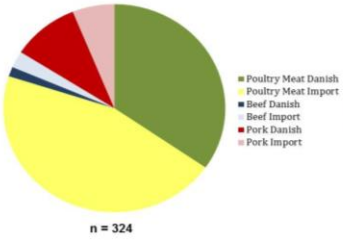
Potential ESBL sources



Relative importance of each type of meat

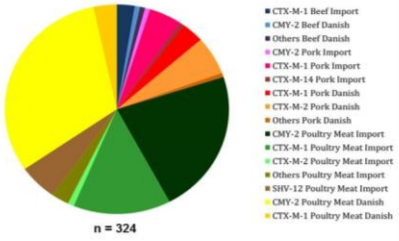
Exposure assessment performed using data from surveillance programme DANMAP (2009-2011). The contribution of each meat type to the human exposure was based on the prevalence of the identified genes in the ESBL positive samples weighted by the meat consumption patterns in Denmark

ESBL in meat sources 2009-2011



n = 324

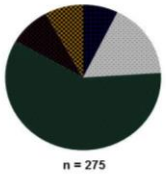
ESBL-genes found in meat 2009-2011



n = 324

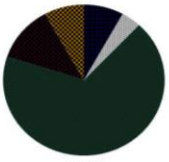
ESBL-genes found in humans 2011

In *E. coli*-isolates from urine



n = 275

In *E. coli*-isolates from blood



n = 25

Discussion and Conclusion

- In Danish human isolates of *E. coli* the ESBL gene CTX-M-15 is the most frequently detected and it has not been found in meat samples from 2009 to 2011
- The genotype overlap between human ESBL cases and those found in meat is small. This suggests that meat might play an inferior role for ESBL dissemination in Denmark than previously thought
- A source attribution model is needed to reveal the relative importance of several sources of ESBL to humans, such as pets, environment, travel and nosocomial infections, and this study is relevant to achieve that purpose

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