Exposure assessment of extended-spectrum beta-lactamases/AmpC beta-lactamases-producing in meat in Denmark

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Abstract
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-producing E. coli (ESBL/AmpC-PEC) 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 genotypes in each type of meat.

Broiler meat represented the largest part of the estimated ESBL/AmpC contaminated pool of meat (83.8%) compared to pork (12.5%) and beef (3.7%). CMY-2 was the genotype with the highest relative importance for human exposure (58.3%). However, it 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 28.8% for human exposure through meat. 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. Nonetheless, more detailed surveillance data are required to determine the contribution of meat compared to other sources, such as pets and hospitals.

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. 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, such as CMY-2, is increasing (EFSA, 2011).

Economically and socially, the burden of antimicrobial resistance is quite significant (De Kraker et al., 2011). 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 (WHO, 2011b). Recently, a possible link has been made 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-producing bacteria (ESBL/AmpC-PB) infections.
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.

In 2009, the first Danish surveillance study in meat and live animals 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).

The aim of this project was to determine the relative importance of the various ESBL/AmpC-producing $E. coli$ (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.

**Material and Methods**

Data about the fresh and frozen meat available for consumption in 2009, 2010 and 2011 were collected and divided into six categories: imported and domestically produced broiler meat, pork and beef (DTU, 2011). Furthermore, the prevalence of the different genotypes found in each category of meat was obtained (DANMAP, 2009, 2010, 2011). In the DANMAP 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 resulting in positive or negative outcome.

To calculate the relative importance of each type of meat and of each genotype for human exposure, the prevalence of ESBL/AmpC genotypes was weighted by the amount of meat available for consumption: Weighted prevalence (WP) = Prevalence*Meat available for consumption. All the WPs were added to have an ESBL/AmpC Total Pool. Finally, the WP of each genotype was divided by the ESBL/AmpC Total Pool: Relative importance (RI) = WP/ (ESBL/AmpC Total Pool).

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. However, the described procedure was also applied to each year separately. 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.

Using the software @Risk 4.5 Palisade Corporation®, beta distributions were created to assess the 95% credibility interval (CI) for each the genotype and hereby estimate how high the true prevalence could be given the available data. The beta distributions were made through a simulation using 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-PEC in each type of meat within the same 3-year period.

**Results**

During the period 2009 to 2011, broiler meat contributed the most to the total human exposure to ESBL/AmpC-PEC representing 83.8% of the total exposure. Danish broiler meat contributed with 37.0% and imported broiler meat with 46.8%. Danish and imported pork constituted 6.2% and 6.3% of the ESBL/AmpC positive meat available for consumption, respectively. Beef had minor relevance representing 1.2% in beef of Danish origin and 2.5% of imported origin.

The relevance of each genotype varied between the 3 years (Figure 1). Most important was the increase in the
role CMY-2 in Danish broiler meat from 4.6% in 2009 to 11.8% in 2010, increasing to 53.5% of the total exposure in 2011. Moreover, CMY-2 constituted the genotype that meat consumers were most exposed to (58.3% across the 3-year period). CTX-M-1 was the most frequently isolated genotype in pork and beef at 8.2% and 2.5% of the total exposure, respectively. Including broiler meat, CTX-M-1 represented 28.8% of the total ESBL/AmpC positive meat in the Danish market from 2009 to 2011. Overall, Danish meat presented a lower relative importance and imported meat contributed to 55.7% of the human exposure.

Figure 1 – 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, red and orange, and broiler meat is in green, brown and yellow colours.

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. However, it has not been detected in meat samples so far. This ESBL genotype was estimated to be present in Danish pork and beef below prevalences of 0.7% and 1.0%, respectively, given the available data. The prevalence of CTX-M-1 was 2.0% in imported pork (DANMAP, 2009, 2010, 2011), but this estimate is uncertain due to the low number of samples. The estimated upper limit of prevalence for this genotype was 3.8% in imported pork.

Discussion
A definitive cause-effect association cannot be established through our approach. The limited genotype overlap between the meat and human reservoirs indicates that meat might play a minor role for human cases of ESBL/AmpC-PEC.

Considering that cephalosporins have not been used in poultry in Denmark for more than 10 years it is possible that the high prevalences detected in Danish broiler meat might be caused by practices upstream in the production pyramid. This hypothesis has also arisen in Sweden (Börjesson et al., 2013; SV ARM, 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 where cephalosporins were used as a prophylactic measure until recently. Cross-contamination through the environment, humans or animals, as well as off-label use of cephalosporins, should also be considered and investigated. Moreover, it should be studied whether the animal feed is an important source of ESBL/AmpC-PEC. However, the fact that CMY-2 and CTX-M-1 were also the genotypes detected in Swedish broiler meat (SV ARM, 2010) and both of them being the only ESBL/AmpC genes persistently detected in Danish broiler meat from 2009 to 2011, supports 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).

The discontinuation of the use of cephalosporins in pigs is the most likely explanation for the reduction on the
ESBL/AmpC-PEC prevalence seen in these animals in 2011 (Agersø & Aarestrup, 2013). Other factors could also have contributed to this diminution, i.e. the “Yellow card” initiative, adopted almost at the same time and that initially led to a 25% decrease in use of antimicrobials in livestock (Aarestrup, 2012; Alban et al., 2013).

In humans, the so-called “pandemic CTX-M-15” genotype dominated both in blood and urine ESBL-PEC isolates, with 68.0% and 59.3%, respectively (DANMAP, 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). Consequently, 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. As CTX-M-1, CTX-M-14 was detected both in human infections and meat.

ESBL/AmpC genes found in humans and meat overlapped to a limited extent. 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.

This was one of the main motives to assess the uncertainty. This investigation is especially interesting to apply to genotypes that were found in live animals and not in meat. The upper 95% CI expected prevalence for ESBL/AmpC genes not detected in meat (that is the case of CTX-M-15), considering the DANMAP sampling within the 3-year 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 0.7% for imported broiler meat. Such low prevalences points against a role of meat for the CTX-M-15 PEC infections in humans. Nonetheless, very little is known about the duration of carriage, dominance in the human gut microflora and different ESBL/AmpC-PEC’s ability to survive in the human gut environments.

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). Considering the possible emergence of CMY-2 E. coli and its relevance for human exposure through meat, it is important that its prevalence is calculated in the upcoming years.

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, so a precautionary approach should probably be taken.

In Denmark, there are limited additional options to control ESBL/AmpC occurrence through the reduction in use of cephalosporins 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. Nonetheless, the role of other sources should be assessed, including other foodborne sources, pets and hospitals, so further studies are needed. ESBL/AmpC-PB is a very complex subject and their emergence, ecology and dynamics is nowadays poorly understood. 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 urgently be further investigated. Finally, we suggest that data collection, in human, food and animal reservoirs, become harmonized across the EU. This would not only allow comparison of data between countries, but also facilitate studying risk factors and suggest optimal control strategies.

**Conclusion**

The genotype overlap between the two reservoirs – humans and meat from poultry, pigs and cattle – is low,
suggesting that meat might not have such a relevant role for ESBL/AmpC-PEC human infections in Denmark in 2009-2011 as previously thought. Poultry meat was most relevant for human ESBL/AmpC-PEC exposure followed by pork and beef, and Danish meat had a lower relative importance than imported meat. CTX-M-1 and CTX-M-14 producing \textit{E. coli} were found in both humans and animals, but the occurrence of these two genotypes in humans was not very frequent. "Pandemic" CTX-M-15 was not found in meat samples, but it should not be ruled out that it can occur in meat, because it has previously been detected in live animals although at a low prevalence. Future studies aiming to obtain a better understanding of the issue of ESBL/AmpC-PEC is required, unveiling the factors that lead to ESBL/AmpC emergence and explaining the interface between several sources and humans to clarify the increasing occurrence of human cases.

Acknowledgements

We thank the Technical University of Denmark and the Statens Serum Institute of Denmark for their collaboration. Moreover, we acknowledge the European Union for funding ERASMUS internship.

References

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