7

RESISTANCE IN INDICATOR BACTERIA

7. Resistance in indicator bacteria

Highlights: In 2018, the most prominent changes in resistance in indicator *E. coli* from food-producing animals were a reduced occurrence of multidrug-resistance in isolates from broilers and pigs and an increase in fully susceptible isolates from pigs compared to previous years.

Resistance patterns and levels in indicator *E. coli* from poultry, pigs and cattle were overall similar to previous years. No resistance to cefotaxime and ceftazidime was detected when using the non-selective isolation method. Such phenotypes were detected using selective isolation methods in samples from broilers, thus indicating that they are present in a relatively small proportion of commensal *E. coli* of broilers. Furthermore, no colistin, meropenem and tigecycline resistance was detected. Currently, the zoonotic risk linked to transfer of resistance to critically important antimicrobials from animals to humans appears to be very limited in Denmark.

Imported chicken meat was more likely to contain *E. coli* producing ESBL/AmpC than Danish broilers and broiler meat. The levels were comparable to those of 2016. The most common ESBL/AmpC enzymes identified across the broiler sources were again the AmpC enzyme, CMY-2 and the ESBL enzyme, CTX-M-1. As previously, all samples examined for carbapenemase-producing *E. coli* (including OXA-48) were found negative.

7.1 Introduction

Escherichia coli are included in the DANMAP programme to monitor occurrence of antimicrobial resistance in different reservoirs through the food chain for the following reasons: i) they are present as commensals in the gut microbiota of healthy animals and humans, ii) they can acquire antimicrobial resistance both via mutations in chromosomal genes and horizontal transfer of antimicrobial resistance genes, and iii) they have the potential to cause infections in both animals and humans and to transfer antimicrobial resistance to pathogenic bacteria of the same or other species.

E. coli exhibiting resistance to third-generation cephalosporins via production of extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases (AmpCs) are one of the fastest spreading antimicrobial resistance problems in both humans and production animals worldwide. Several studies report similar ESBL/AmpC genes, plasmids and/or clones of *E. coli* isolates in animals, meat and human infections, which suggests a zoonotic link [Roer et al 2019. J Antimicrob Chemother. 74(3):557; Valcek et al 2019. J Antimicrob Chemother. 74(8):2171].

Carbapenemase-producing Enterobactericeae (CPE) are a great threat to human health, because carbapenems are last-line antimicrobial agents for treatment of infections caused by multidrug-resistant Gram-negative bacteria. Currently, CPE have been detected sporadically in production animals in EU but never in Denmark [EFSA/ECDC 2019. EFSA journal 17(2):5598].

Since 2014, isolation and antimicrobial susceptibility testing of indicator *E. coli* and ESBL/AmpC-producing *E. coli* have been performed according to the EU harmonised monitoring of antimicrobial resistance [Decision 2013/652/EU].

7.2 Indicator *Escherichia coli*

All isolates originated from caecal samples randomly collected from healthy pigs, broilers and cattle at slaughter. Only one isolate per farm was included. Susceptibility to the antimicrobials recommended by EFSA was measured by broth microdilution to determine minimal inhibitory concentrations (MIC). MIC distributions and occurrence of resistance among indicator *E. coli* are presented in the web annex (Table A7.1). These results

were obtained using the non-selective isolation procedure. Results obtained by using selective procedures for detection of cefotaxime-resistant *E. coli* are reported in section 7.3.

7.2.1 Indicator *E. coli* from broilers

From 184 representative pools of broiler caeca collected at Danish slaughterhouses, 174 *E. coli* isolates were obtained. A total of 166 of these were tested for antimicrobial resistance (Table 7.1). More than half (60%) of the isolates were susceptible to all antimicrobials tested (Figure 7.1). Moderate (13-20%) resistance to ampicillin, nalidixic acid, ciprofloxacin, sulfonamide, tetracycline and trimethoprim was observed. Low (2%) and very low (1%) chloramphenicol and gentamicin resistance was observed, respectively. No resistance to the remaining compounds tested, including antimicrobials that are critically important for human medicine (azithromycin, cefotaxime, ceftazidime, meropenem, colistin and tigecycline), was observed (Table 7.1).

Resistance to ampicillin, sulphonamide and trimethoprim was significantly higher in indicator *E. coli* from broilers than in those from cattle.

A total of 16 resistance profiles were detected among the 66 resistant isolates indicating relatively high diversity.

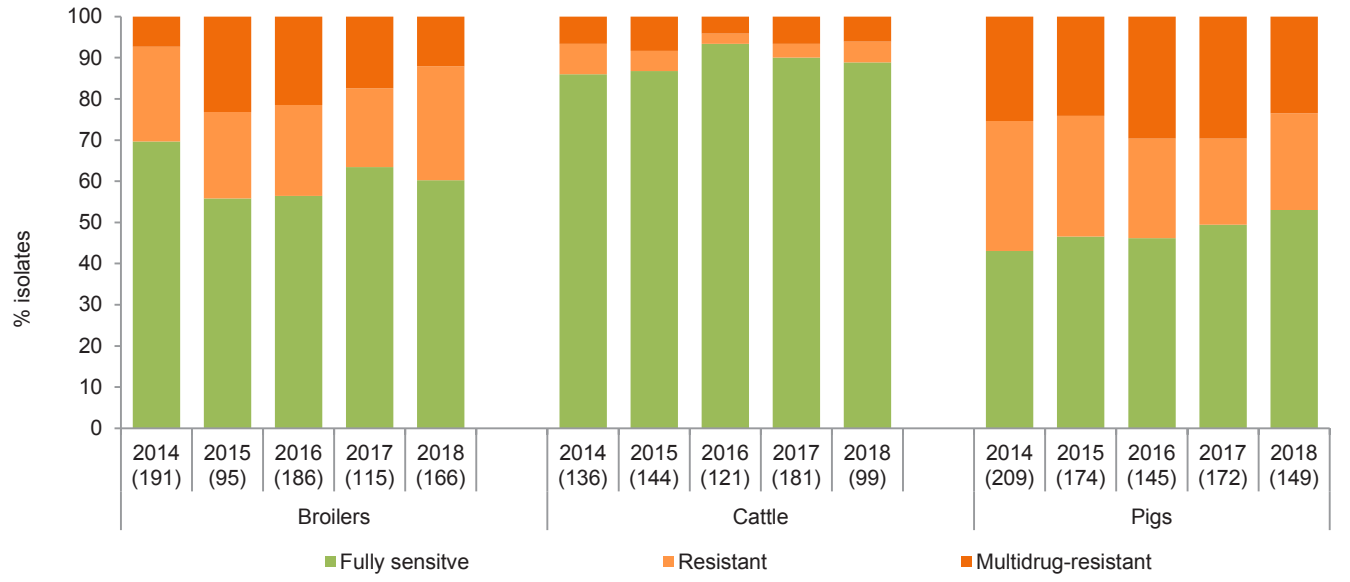
Table 7.1. Resistance (%) in *Escherichia coli* isolates from broilers, cattle and pigs, Denmark DANMAP 2018

	Broilers	Cattle	Pigs
Antimicrobial agent	Danish %	Danish %	Danish %
Ampicillin	17	7	27
Azithromycin	0	0	4
Cefotaxime	0	0	0
Ceftazidime	0	0	0
Chloramphenicol	2	5	7
Ciprofloxacin	13	0	1
Colistin	0	0	0
Gentamicin	1	0	<1
Meropenem	0	0	0
Nalidixic acid	13	0	1
Sulfonamide	20	7	30
Tetracycline	13	11	33
Tigecycline	0	0	0
Trimethoprim	13	1	23
Fully sensitive (%)	60	89	53
Number of isolates	166	99	149

Note: An isolate is considered fully sensitive if susceptible to all antimicrobial agents included in the test panel

Figure 7.1 Relative distributions (%) of fully sensitive, resistant and multidrug-resistant *Escherichia coli* isolates from broilers, cattle and pigs, Denmark

DANMAP 2018



Note: The number of isolates included each year is shown in parentheses. An isolate is considered fully sensitive if susceptible to all antimicrobial agents included in the test panel, and multidrug-resistant if resistant to 3 or more of the 12 antimicrobial classes included (Table 9.3)

The AMR profiles are listed in web annex Table A7.2. Most resistant isolates (53%) displayed resistance to one antimicrobial only or to two antimicrobials of the same class (see definition of classes in Table 9.5). Thus, 24%, 9%, 9%, 8% and 3% of resistant isolates showed resistance to quinolone (nalidixic acid and ciprofloxacin), sulfonamide, tetracycline, ampicillin and trimethoprim, respectively.

Co-resistance occurred in the remaining 31 (47%) resistant isolates. In some of the isolates (n = 11), resistance to compounds belonging to two antimicrobial classes was observed. Multidrug-resistance, i.e. resistance to 3 or more of the 12 antimicrobial classes in the *E. coli* test panel, was observed in 12% of all isolates (n = 20). Eighteen of the 20 isolates were resistant to ampicillin, sulfonamide, and trimethoprim, some isolates also in combinations with quinolones, tetracycline and/or chloramphenicol.

Compared to 2017, minor fluctuations in the occurrence of resistance to any antimicrobial were observed (Figure 7.2). However, over the last 10-year period, there has been a slow but statistically significant increase in resistance to chloramphenicol, nalidixic acid, ciprofloxacin, gentamicin, sulphonamide and trimethoprim.

The noticeable fluctuations observed in 2013-2015 have been associated to changes in antimicrobial usage patterns due to disease outbreaks in several flocks in 2015 [DANMAP 2015]. Thus, multidrug-resistant *E. coli* were significantly lower in 2014 compared to 2015 and 2016 (Figure 7.1). Afterwards, decreased antimicrobial use seemed to be accompanied by a decreasing trend in occurrence of multidrug-resistant isolates. However, the association between antimicrobial use and resistance is not straightforward, and in particular the occurrence of nalidixic acid and ciprofloxacin (quinolones) resistance at levels around 10% for at least the last 10 years has no clear explanation since there is virtually no fluoroquinolone use in the Danish poultry industry. Vertical transmission of *E. coli* resistant to fluoroquinolones and also other antimicrobials has been hypothesised [Bortolaia et al. 2010. Vet Microbiol. 142(3-4):379]. No statistically significant five-year trend was observed for the fully susceptible isolates.

7.2.2 Indicator *E. coli* from cattle

From 198 representative cattle caeca collected at Danish slaughterhouses, 187 *E. coli* isolates were obtained and 99 of these were tested for antimicrobial resistance (Table 7.1). The vast majority of isolates (89%) was susceptible to all tested antimicrobials. Very low (1%) occurrence of trimethoprim resistance and low (5-7%) occurrence of ampicillin, chloramphenicol and sulphonamide resistance were observed. Moderate occurrence of tetracycline resistance (11%) and no occurrence of resistance to the remaining antimicrobials tested were detected (Table 7.1).

Eight resistance profiles were detected in the 11 resistant isolates (web annex Table A7.2). Tetracycline resistance was detected in all resistant isolates either alone (one isolate) or in combination with resistance to additional compounds.

Compared to 2017, there were minor fluctuations in occurrence of resistances observed (Figure 7.2). When analysing the trends over a 10-year period a slow but statistically significant increase in ampicillin and chloramphenicol resistance was observed, whereas over the last five-year period a slow but significant decrease in occurrence of sulphonamide resistance occurred. There were no significant five-year trends in the occurrence of multidrug-resistant isolates and fully susceptible isolates (Figure 7.1).

7.2.3 Indicator *E. coli* from pigs

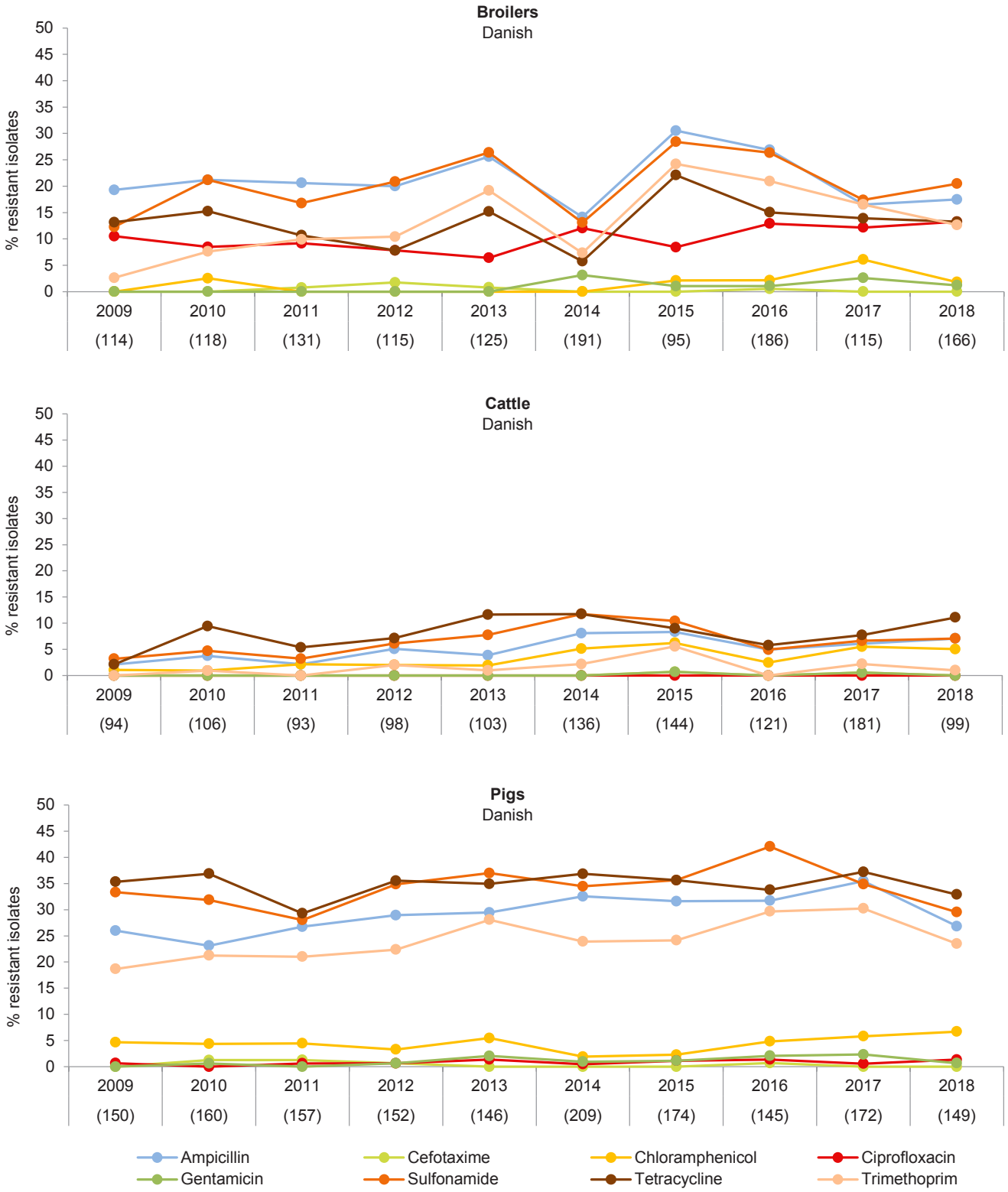
From 154 representative pig caeca collected at Danish slaughterhouses, 150 *E. coli* isolates were obtained and 149 of these were tested for antimicrobial resistance (Table 7.1). Approximately half (53%) of the isolates from pigs were susceptible to all antimicrobials tested. High (23-33%) occurrence of resistance to ampicillin, sulfonamide, tetracycline and trimethoprim and low (4-7%) occurrence of resistance to azithromycin and chloramphenicol were observed. Occurrence of resistance to the remaining antimicrobials tested was very low (≤ 1%, gentamicin, nalidixic acid and ciprofloxacin) or not detected (Table 7.1).

Noteworthy, the occurrence of azithromycin resistance increased from 1% in 2014-2017 to 4% in 2018, it is unknown whether increase in macrolide use since 2016 has induced this, but will be monitored carefully in the coming years.

Resistance to ampicillin, azithromycin, chloramphenicol, tetracycline and trimethoprim was significantly higher in indicator *E. coli* from pigs than in those from broilers. Furthermore, resistance to ampicillin, azithromycin, sulfonamide, tetracycline and trimethoprim was significantly higher in indicator *E. coli* from pigs than in those from cattle.

Figure 7.2 Resistance (%) among *Escherichia coli* isolates from broilers, cattle and pigs, Denmark

DANMAP 2018



Note: Number of isolates included each year is presented in the parenthesis

A total of 22 resistance profiles were detected among the 70 resistant isolates (see web annex Table A7.2). One third of the resistant isolates displayed resistance to one antimicrobial only. Thus, 20%, 6%, 4%, 3% and 1% of resistant isolates showed resistance to tetracycline, sulfamethoxazole, trimethoprim, ampicillin and chloramphenicol, respectively.

The remaining resistant isolates displayed varying resistance profiles, where 11 isolates exhibited resistance to antimicrobials of two classes (combinations of resistance to ampicillin, sulfonamide, tetracycline and trimethoprim). The remaining isolates (23% of all isolates) were classified as multidrug-resistant (n = 35) where co-resistance to ampicillin, sulfonamide, tetracycline and trimethoprim was common (ASuTTm resistance profile, n = 21). Each of the remaining resistance profiles was exhibited by few isolates indicating a wide diversity, and resistance to azithromycin (n = 6) were only observed in isolates resistant to three, four or five additional antimicrobial classes.

Compared to 2017, occurrence of resistance to several antimicrobials including ampicillin, gentamicin, sulfonamide, tetracycline and trimethoprim decreased (Figure 7.2). In addition, a minor increase in occurrence of chloramphenicol and ciprofloxacin resistance was observed. However, these changes in resistance levels were not statistically significant.

When analysing the trends over a 10-year period a slow but significant increase in ampicillin, gentamicin and trimethoprim resistance was observed whereas there was no statistically significant five-year trend in the occurrence of resistance to any antimicrobial. However, in the last five years, there has been a statistically significant increase in the percentage of fully susceptible isolates from 43% to 53% (Figure 7.1).

7.2.4 Perspectives

Antimicrobial resistance monitoring in commensal *E. coli* is considered a useful indicator of the selective pressure exerted by antimicrobial use on the intestinal microbiota of food-producing animals.

In 2018, there were relatively minor fluctuations in occurrence of antimicrobial resistance in indicator *E. coli* from broilers, cattle and pigs compared to previous years. However, the occurrence of multidrug-resistance was the lowest and the second-lowest detected in isolates from pigs and broilers, respectively, since 2014. In addition, the occurrence of fully susceptible *E. coli* increased significantly in pigs since 2014. This likely reflects the efforts undertaken in Denmark to control antimicrobial use in food-producing animals. Genomic characterisation would be relevant to understand the population structure of susceptible and resistant *E. coli* populations from food-producing animals, which may provide the basis to design interventions to boost the fully susceptible *E. coli* population.

The antimicrobial resistance phenotypes detected in animal-origin indicator *E. coli* mostly relevant to human health were ciprofloxacin resistance in *E. coli* from broilers and azithromycin resistance in *E. coli* from pigs, as in 2017. A slow but increasing trend in resistance to ciprofloxacin has occurred in the *E. coli* isolates from broilers over the last ten years (13% in 2018). Although the molecular bases of ciprofloxacin resistance have not been investigated, the phenotype indicated chromosomal mutations (in 88 of 91 ciprofloxacin resistant broiler isolates from 2014 to 2018), consequently linking the main risk to human health to the disease-causing potential of these strains. Resistance to azithromycin in isolates from pigs increased from 1% in 2017 to 4% in 2018. The potential human risk derived by infections with these strains and/or transfer of azithromycin resistance to pathogenic strains remains low - but will be monitored closely the following years.

Resistance to other antimicrobials relevant for human medicine such as colistin, cefotaxime, ceftazidime, meropenem and tigecycline was not detected, which indicates that the prevalence of these resistance phenotypes was not more than 2% in *E. coli* from pigs and cattle and below 3% in *E. coli* from broilers (see section 9.7). However, cefotaxime- and ceftazidime-resistant *E. coli* were detected, when using selective enrichment, which is more sensitive.

From a European perspective based on the last published data from 2016 and 2017, indicator *E. coli* from Danish broilers and calves < 1 year show noticeably low occurrence of resistance to any antimicrobial compared to the indicator *E. coli* from other countries apart from the Nordic countries [EFSA/ECDC 2018. EFSA journal 16(2):5182; EFSA/ECDC 2019. EFSA journal 12(2):5598]. Denmark is among the countries reporting the lowest occurrence of chloramphenicol and, more importantly, ciprofloxacin resistance in indicator *E. coli* from pigs, whereas the reported occurrence of ampicillin, azithromycin, sulfonamide, trimethoprim and tetracycline resistance was comparable to the average reported in the EU Member States.

7.3 ESBL/AmpC- and carbapenemase-producing *E. coli*

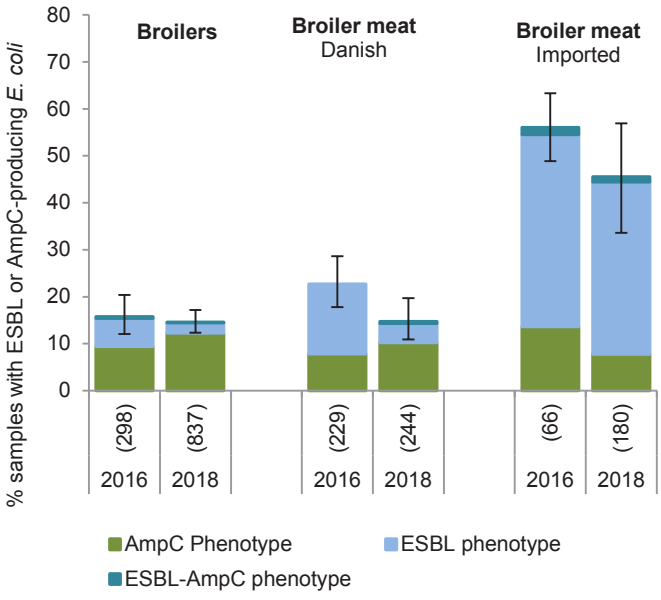
DANMAP 2018 includes ESBL/AmpC- and carbapenemase-producing *E. coli* from caeca of domestic broilers at slaughter and from Danish and imported broiler meat at retail in concordance with the EU regulation on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. Samples were collected randomly and cultured directly in a selective enrichment for detection of cefotaxime-resistant *E. coli* and carbapenemase-producing *E. coli* (including oxacillinase producing OXA-48-like enzymes). Subsequently, obtained *E. coli* isolates were phenotypically antimicrobial susceptibility tested by MIC determination against the panel of antimicrobials recommended by EFSA. In parallel, for most isolates whole genome sequencing (WGS) and in silico bioinformatics were applied to detect the ESBL/AmpC/CPE-encoding genes. MIC distributions and occurrence of resistance among ESBL/AmpC-producing *E. coli* isolates are presented in the web annex (Table A7.3 and A7.4).

7.3.1 ESBL/AmpC- and carbapenemase-producing *E. coli* from Danish broilers and broiler meat

A total of 837 samples from broilers and 244 samples from domestically produced broiler meat resulted in 124 (15%) and 36 (15%) ESBL/AmpC-producing *E. coli* isolates, respectively (Figure 7.3). In 2018, the number of investigated caeca samples of broilers at slaughter increased two-fold compared to 2016 (N = 298).

Figure 7.3 Occurrence (%) of samples with phenotypic ESBL- or AmpC-producing *E. coli* from broilers and broiler meat recovered by selective enrichment, Denmark

DANMAP 2018



Note: Number of samples tested per year is presented in the partenthesis. Confidence intervals for total proportion of samples positive for phenotypic ESBL and/or AmpC producing *E. coli* - calculated as 95% binomial proportions presenting Wilson intervals. Classification of CPE, ESB and AmpC phenotypes according to the scheme provided by EFSA (see section 9.7)

Table 7.2. Resistance (%) and beta-lactam resistance phenotype distributions (%) in ESBL/AmpC-producing *Escherichia coli* from broilers and broiler meat recovered by selective enrichment, Denmark

DANMAP 2018

	Broilers	Broiler meat	
Antimicrobial agent	Danish %	Danish %	Import %
Ampicillin	100	100	100
Azithromycin	0	0	4
Cefepime	81	83	93
Cefotaxime	100	100	100
Cefotaxime/clavulansyre	85	69	17
Cefoxitin	85	72	20
Ceftazidime	100	100	95
Ceftazidime/clavulansyre	82	69	17
Chloramphenicol	<1	6	23
Ciprofloxacin	23	22	72
Colistin	0	0	4
Ertapenem	0	11	1
Gentamicin	0	0	11
Imipenem	0	0	0
Meropenem	0	0	0
Nalidixic acid	23	22	70
Sulfonamide	16	22	70
Temocillin	0	0	0
Tetracycline	18	17	56
Tigecycline	0	0	0
Trimethoprim	9	14	50
CPE phenotypes	0	0	0
AmpC phenotypes	83	69	17
ESBL phenotypes	15	28	80
ESBL-AmpC phenotypes	1	3	2
Other phenotypes	2	0	0
Number of isolates	124	36	82

Note: Classification of CPE, ESBL and AmpC phenotyes according to the scheme provided by EFSA (see section 9.7)

For broilers, the prevalence of samples positive for ESBL/AmpC-producing *E. coli* was similar to what was observed in 2016 (15% vs. 16%), whereas the prevalence in broiler meat was lower in 2018 compared to 2016 (15% vs. 23%, respectively), though not statistically significant. The reduction was mainly due to a statistically significant reduction in ESBL phenotypes (4% in 2018 vs. 15% in 2016). The prevalence of ESBL phenotypes among broilers also declined significantly between 2016 and 2018 from 6% to 2% (Figure 7.3).

No CPE isolates were recovered, suggesting that we can be 95% certain that CPE isolates are only present in 0.4% or less of domestic broilers at slaughter (see section 9.7). Low level phenotypic resistance to ertapenem was, however, observed in four isolates from broiler meat suggesting these to be

Table 7.3 Number of ESBL and AmpC enzymes detected in *E. coli* isolates from broilers and broiler meat recovered by selective enrichment, Denmark DANMAP 2018

	Broilers		Broiler meat			
	Danish		Danish		Import	
Enzymes	2016	2018	2016	2018	2016	2018
CMY-2	12	52	16	23	8	10
CMY-98	0	0	0	1	0	0
CTX-M-1	7	11	22	9	22	23
CTX-M-14	0	0	0	1	0	1
CTX-M-14b	0	0	0	0	1	2
CTX-M-32	0	0	0	0	0	1
SHV-12	1	2	4	1	3	19
SHV-2	0	0	0	0	0	1
TEM-52B	3	0	7	0	2	11
TEM-52C	1	0	0	0	0	5
Chromosomal AmpC	10	15	3	1	1	2
Unknown	0	0	0	0	0	1
Not available	14	44	0	0	0	6
Number (%) positive samples	48 (16%)	124 (15%)	52 (23%)	36 (15%)	37 (56%)	82 (46%)
Number of tested samples	298	837	229	244	66	180

Note: ESBL/AmpC enzymes and MLSTs are determind by WGS. For 2018 data, all MLST and ESBL/AmpC enzyme combinations are listed in web annex table A7.6

CPEs. WGS typing, however, revealed neither known carbapenemase nor oxacillinase encoding genes present in these *E. coli* isolates. In contrast, all four isolates harboured the CMY-2 encoding gene (Table 7.3) causing the phenotypic expression of the weak carbapenemase activity caused by porin-deficiency as described by Manneri et al. 2018 [FEMS Microbiol. Lett. 282:238].

Overall, the 124 ESBL/AmpC-producing *E. coli* isolates from broilers exhibited 100%, 100%, 85%, and 81% resistance to cefotaxime, ceftazidime (3rd generation cephalosporins), cefoxitin (2rd generation cephalosporin), and cefepime (4th generation cephalosporin), respectively (Table 7.2). Similar levels of resistance to cephalosporins were observed among the 36 ESBL/AmpC -producing *E. coli* isolates from domestically produced broiler meat that conferred resistance to cefotaxime (100%), ceftazidime (100%), cefoxitin (72%), and cefepime (83%), respectively (AMR profiles listed in web annex Table A7.5).

A total of 80 of the 124 ESBL/AmpC -producing *E. coli* isolates from broilers were whole genome sequenced (WGS). The MLST and ESBL/AmpC Enzymes combinations are listed in web annex Table A7.6. Among the AmpC phenotypes, 52 harboured the CMY-2 encoding gene. Of those, 43 (83%) belonged to the same clonal lineage of multi-locus sequence type (MLST) ST2040, indicating a clonal expansion from a point source. ST2040 harbouring an IncX plasmid replicon has been described as associated with the production of poultry in Finland [Oikarainen et al. 2019. Vet. Microbiology 231:100]. CMY-2 encoded ST2040 *E. coli* isolates appear to be newly introduced in Danish broilers from an unknown origin. Only one *E. coli* isolate of this genotype was observed in 2016. Furthermore, singletons or a few isolates of CMY-2 encoded *E. coli* isolates were also attributed to other MLSTs such as ST48, ST580, ST752, ST1101, ST295 and ST1286; where the two latter types also harbouring CMY-2 were observed in 2016. Similarly to previous years, a subset of 15 (29%) AmpC-producing *E. coli* isolates conferred resistance due to the presence of an upregulated AmpC promotor (Table 7.3).

In broilers, 11 ESBL-producing *E. coli* isolates were also detected. They all harboured the CTX-M-1 encoding gene and belonged to a variety of different MLSTs including ST155, ST212, ST1818, ST88, ST1640 and ST752, of which the latter two types also harbouring CTX-M-1 were observed in 2016 (see web annex Table A7.6).

All of the 36 ESBL/AmpC -producing *E. coli* isolates from domestically produced broiler meat were typed by WGS. The vast majority, 23 (64%) AmpC phenotypes harboured the CMY-2 encoding gene, of which 16 belonged to ST2040 as observed in the Danish broilers. Similarly, the most common ESBL gene from the broiler meat was also CTX-M-1 and was found in nine isolates, attributed to ST162, ST1640, ST2309, ST3232, ST1640, ST752, and ST1818. The later three MLSTs harbouring CTX-M-1 were also observed among isolates originating from Danish broilers.

The ESBL/AmpC -producing *E. coli* isolates from Danish broilers and broiler meat exhibited varying levels of resistance to

other antimicrobials. No resistance to tigecycline, temocillin, gentamicin, azithromycin and colistin was detected. (Table 7.2). Resistance to quinolones was moderate in both sources, and lower than in 2016 in broilers (23% vs. 40%) but not in broiler meat (22% v. 15%). In 2018, a 100% concordance between resistance to ciprofloxacin and nalidixic acid was observed for both sources, suggesting chromosomal mutations in the topoisomerases genes gyrA and parC.

Different combinations of multidrug-resistance were observed among the ESBL/AmpC-producing *E. coli* isolates (web annex Table A7.5). The multidrug-resistance was primarily including ampicillin, chloramphenicol, tetracycline, sulphonamides, and trimethoprim in addition to the cephalosporins. Thus, co-resistance, likely attributable to the presence of class 1 integrons, was observed: i) to tetracycline in 18% and 17% of the isolates, ii) to sulfonamides in 16% and 22% of the isolates and iii) to trimethoprim in 9% and 14% of the isolates from Danish broilers and broiler meat, respectively (Table 7.2).

7.4.2 ESBL/AmpC- and carbapenemase-producing *E. coli* from imported broiler meat

A total of 180 samples from imported broiler meat resulted in 82 (46%) ESBL/AmpC-producing *E. coli* isolates (Table 7.2), which was significantly higher than the 36 (15%) in the domestically produced broiler meat. No CPE isolates were recovered in any of the imported broiler meat samples.

The prevalence of ESBL/AmpC-producing *E. coli* positive samples was similar to 2016 (46% vs. 56%), and no significant change in prevalence from 2016 to 2018 for neither ESBL nor AmpC phenotypes was observed (Figure 7.3). In contrast to the isolates from Danish broiler meat, the ESBL phenotype (80%) was more common than the AmpC phenotype (17%) in imported broiler meat. Two isolates exhibited both ESBL and AmpC phenotype.

The ESBL/AmpC-producing *E. coli* isolates exhibited 100%, 95%, 20%, and 93% resistance to cefotaxime, ceftazidime, cefoxitin, and cefepime, respectively (Table 7.2).

All of the 82 ESBL/AmpC-producing *E. coli* isolates from imported broiler meat were available for WGS. Among the 80% displaying an ESBL phenotype, the CTX-M-1-encoding gene was the most common found in 23 isolates belonging to 18 different MLSTs (see web annex Table A7.6). Moreover, 19 and 11 ESBL-producing *E. coli* isolates harboured the SHV-12- and TEM-52-encoding genes, respectively. Among the 19 *E. coli* isolates encoding SHV-12, the majority belonged to two MLSTs, ST1011 (n = 6) and ST117 (n = 6). For the *E. coli* isolates encoding TEM-52B, ST115 (n = 5) was dominant. The CMY-2-encoding gene was the most common among the 17% of *E. coli* isolates exhibiting the AmpC phenotype. All the AmpC-producing *E. coli* isolates were singleton MLSTs except for two isolates belonging to ST2040, which was the most prevalent MLST in Danish broilers and broiler meat .

The 82 ESBL/AmpC-producing *E. coli* isolates from imported broiler meat generally exhibited higher levels of resistance and co-resistance compared with those from Danish broiler meat. See AMR profiles listed in web annex Table A7.5. No resistance to tigecycline and temocillin was observed and only low levels of resistance to gentamicin (11%), azithromycin (4%) and colistin (4%) were detected (Table 7.3).

Resistance to quinolones was very high with 72% (n = 59) and 70% (n = 57) resistance to ciprofloxacin and nalidixic acid, respectively. This suggests that chromosomal mutations in the topoisomerases genes *gyrA* and *parC* have occurred and is similar to observations in isolates from Danish sources.

7.4.5 Perspectives

The number of samples collected for the monitoring of ESBL/AmpC-producing *E. coli* isolates from Danish broilers and imported broiler meat increased in 2018 compared to previous years, strengthening the monitoring system and the results hereof. In 2018, we observed a similar scenario as in previous years with a consistent lower level of ESBL/AmpC-producing E. coli in Danish broilers and broiler meat compared to imported broiler meat.

The most common ESBL/AmpC enzymes identified across the broiler sources were again the AmpC enzyme, CMY-2, and the ESBL enzyme: CTX-M-1. As in previous years we observed that all the ESBL/AmpC enzymes identified, were associated with large number of MLSTs. Interestingly, the majority of the AmpC enzyme, CMY-2, from Danish broilers and broiler meat were ST2040, a relative new introduction of an unknown origin in 2018. The pattern of MLSTs seems to fluctuate across years and in 2016 different MLSTs were most prevalent such as ST429 harbouring CMY-2.

Each year, the ESBL/AmpC enzymes and the MLSTs are compared by whole genome sequencing and phylogenetic SNP analysis to isolates from human bloodstream infections to elucidate potential zoonotic transmission. The vast majority of the food and veterinary ESBL/AmpC-producing *E. coli* isolates are rarely congruent to those of human bloodstream infections. This is supported by similar observations in other European countries. Only in very few cases, have commensal non-pathogenic MLSTs with similar ESBL/AmpC enzymes been identified in both sectors. A higher number of similar cases might be identified expanding the monitoring to also include ESBL/AmpC -producing *E. coli* isolates from human diarrheal and urine tract infections.

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Textbox 7.1

Antimicrobial resistance in prawns and pangasius fillets imported from Asia

Background: Global aquaculture production is expanding as the worldwide demand for protein increases [1]. Asia and other low and middle-income countries (LMIC) produce more than 90% of the global aquaculture both for domestic use and for export. In Denmark and other Northern European countries, pangasius fillets (*Pangasianodon hypophthalmus*) and especially, the Vannemei Prawns (*Litopenaeus vannamei*) are popular imports from Asia. Low to middle income countries generally struggle with sanitation, infrastructure and separation of clean and used water [2]. LMIC with a lower level of governance and less regulation including use of antibiotics are more likely to develop more antimicrobial resistance [3].

Despite strict requirements to products imported into the EU, it may be difficult to avoid microbial contamination in products from third countries and all bacteria can carry resistance genes independent of their ability to cause disease. This also includes resistance to antimicrobials critically important to humans (CI) and resistance types not present in the importing country. WHO note that among the CI antimicrobial classes; quinolones, 3rd and higher generation cephalosporins, macrolides, glycopeptides and polymyxins (Table 1) are drugs classified as highest priority (CIHP) [4].

To assess the risk of importing fish and seafood products contaminated with bacteria resistant to critically important antibiotics, we carried out a survey of antimicrobial resistance in indicator bacteria found in Vannemei prawns and pangasius fillets in Danish supermarkets.

Data and data sources: A total of 67 *Escherichia coli,* 204 *Enterococcus faecalis* and 65 *Enterococcus faecium* strains were isolated from 97 pangasius fillet and 203 Vannemei prawn samples collected in Danish supermarkets [5]. The samples were selected representatively during the period of May 2017 to May 2018. All *E. coli* and a total of 140 *E. faecalis* and 65 *E. faecium* isolates were MIC tested. Furthermore, all samples were screened for ESBL-, AmpC-, carbepenem- and OXA-48-producing *E. coli* using selective enrichment. Isolation, MIC testing and whole genome sequencing was performed as described in section 9.4-9.7 with a few moderations.

Results and discussion: Among the 67 ***Escherichia coli*** isolates, 60% were resistant to at least one antimicrobial in the test panel. Interestingly, the levels of resistance to the antimicrobials, such as ampicillin (9%), tetracycline (22%), sulphonamide (9%) and chloramphenicol (6%) were lower than in isolates originating from Danish pigs and broilers and comparable to isolates from Danish cattle (Figure 7.1). In contrast, resistance to CIHP antimicrobials were observed in 54% (n = 36) of the *E. coli* isolates from imported fish and prawns, and of these, all were resistant to the flouroquinolones (ciprofloxacin). One of the 36 isolates

Table 1 Classification of antimicrobials used to assess antimicrobial resistance in bacteria from prawns and fish from Asia

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Critically Important and of highest priority:
3rd, 4th and 5th gen. cephalosporins (cefepime, cefotaxime, ceftazidime); glycopeptides (teicoplanin, vancomycin, azithromycin); macrolides (azithromycin, erythromycin); polymyxins (colistin); quinolones and fluoroquinolones (ciprofloxacin, nalidixic acid)
Critically Important:
Aminoglycosides (gentamicin); aminopenecillins(ampicillin, temocillin); carbapenems and other penems (ertapenem, imipenem, meropenem); glycylcyclines (tigecycline); Lipopeptides (daptomycin) and oxazolidinones (Linezolid)
Highly important:
Amphenicols (chloramphenicol; 1st and 2nd gen. cephalosporins and cephamycins (cefoxitin); streptogramins (quinupristin, dalfoprisitn); sulfonamides, inhibitors and combinations (sulphamethoxazole) and tetracyclines (trimethoprim, tetracycline)

Note: Based on the 6th revision of the WHO report on critically important antimicrobials for human medicine, WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2019

was resistant to 3rd. generation cephalosporins (cefepime and ceftazidime) and one to polymyxins (colistin). None of the isolates was resistant to macrolides (azithromycin), carbapenems (meropenem) or glycylcyclines (tigecycline).

Only 7 of the 36 ciprofloxacin resistant isolates were also resistant to nalidixic acid, suggesting that the ciprofloxacin resistance may be due to plasmid-mediated resistance rather than the chromosomal mutation driven resistance (Figure 1). A total of 49% of the *E. coli* isolates from the imported fish and prawns carried the phenotypic profile suggesting plasmid-mediated resistance. Ciprofloxacin resistance levels are quite low in Denmark and plasmid-mediated quinolone resistance is considered very rare in Danish foods and animals [6]. A study examined the DANMAP indicator *E. coli* isolates from 2014-18 from Danish broilers, cattle, pigs and meat. Here only 122 of the 2,863 Danish *E. coli* isolates were resistant to ciprofloxacin (4.3%) and only 6 of these (0.2%) isolates were susceptible to nalidixic acid.

Four ESBL-producing *E. coli* isolates from prawns were detected on the selective media. The WGS analysis showed that two strains harboured $bla_{CTX\text{-}M\text{-}15}$ and *qnrS1,* as the only identified resistance genes. Both of these isolates exhibited phenotypic resistance to ciprofloxacin, but not nalidixic acid. The other two strains carried $bla_{CTX\text{-}M\text{-}55}$ and were multidrug-resistant, of which one also had the *qnrS1.* In the fourth isolate, 15 resistance genes were detected in addition to the ESBL-gene (Table 2), encoding for all the investigated classes of the CIHP antimicrobials as well as plasmid-mediated quinolone and colistin resistance.

No carbapenemase- or OXA-48 producing *E.coli* was detected by the selective enrichment screening.

Among the 104 ***Enterococcus faecalis*** isolates, 34% were resistant to at least one of the eleven compounds in the test panel. *E. faecalis* is intrinsically (i.e. naturally) resistant to streptogramin A and B (quinupristin-dalfopristin), and interpretation of the MIC testing for this drug was therefore not evaluated. Tetracycline was the most common resistance found in 32% of the isolates, and 4% were resistant to chloramphenicol. One isolate was resistant to gentamycin (CI) and 7% of the isolates were resistant to erythromycin (CIHP). All multidrug-resistant strains (n = 3) exhibited resistance to chloramphenicol, tetracycline, and erythromycin.

Almost all of the 65 ***Enterococcus faecium*** isolates had MIC values higher than the microbiological cut-off of MIC<1 µg/ml for streptogramins (97%), however only four isolates exceeded the clinical breakpoint of MIC>4 µg/ml. Most isolates were also resistant to tetracycline (64%). Resistance to erythromycin (CIHP) was detected in 22% of the isolates. Resistance to chloramphenicol and ciprofloxacin were each detected in one strain only. Multidrug-resistance was found in 20% of the strains.

Table 2 Phenotypic and genotypic traits of the four ESBL-producing *E. coli* isolates from Asian prawns recovered by selective enrichment

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Isolate number	Phenotypic resistance profile	Genotypic resistance profile
1	AMP CIP FEP FOT TAZ	$bla_{CTX\text{-}M\text{-}15}$, *qnrS1*
2	AMP CIP FEP FOT TAZ	$bla_{CTX\text{-}M\text{-}15}$, *qnrS1*
3	AMP CHL CIP FEP FOT TAZ TET TMP	$bla_{CTX\text{-}M\text{-}55}$, *aadA5,* $bla_{TEM\text{-}1B}$, *dfrA17, floR, qnrS1, tet(A)*
4	AMP AZI CHL CIP COL FEP FOT FOX GEN NAL SMX TAZ TET TMP	$bla_{CTX\text{-}M\text{-}55}$, *aac(3)-Iid, aadA22, aadA5, aph(3')-Ia, aph(6)-Id, dfrA17, mcr-1, mph(A), Inu(F), FloR, GyrA S83L, qnrS1, Sul2, sul3, tet(A)*

Note: AMP=Ampicillin; AZI=Azithromycin; CIP=Ciprofloxacin; CHL=Chloramphenicol; COL=Colistin; FEP=Cefepime; FOT=Cefotaxime; FOX=Cefoxitin; GEN=Gentamicin; NAL=Nalidixic acid; SMX=Sulphamethoxazole; TAZ=Ceftazidime; TET=Tetracycline; TMP=Trimethoprim

continued ... Textbox 7.1

No resistance to the last-line CI drugs; linezolid or vancomycin was detected in any of the enterococci isolates by the methods applied. It is possible that more sensitive methods such as WGS or culture on selective media may have detected resistance to some of the last-line drugs.

Conclusion: Most of the observed phenotypic resistances have previously been observed in Danish food or production animals. However, we cannot exclude that these products may pose a risk to consumers by introducing AMR genes that are very rare in domestic food sources. No carbapenem, linezolid or daptomycin resistance was found, all of which are antimicrobials prioritised for treatment of human infections with multidrug-resistant enterococci and staphylococci. Plasmid-mediated quinolone resistance is quite rare in Danish foods, but was quite abundant in the imported prawns and fish products. One isolate carried resistance genes to ESBL, macrolide and plasmid-mediated colistin and flouroquinolone resistance alongside genes coding resistance to several of the critically important and highly important antimicrobial groups.

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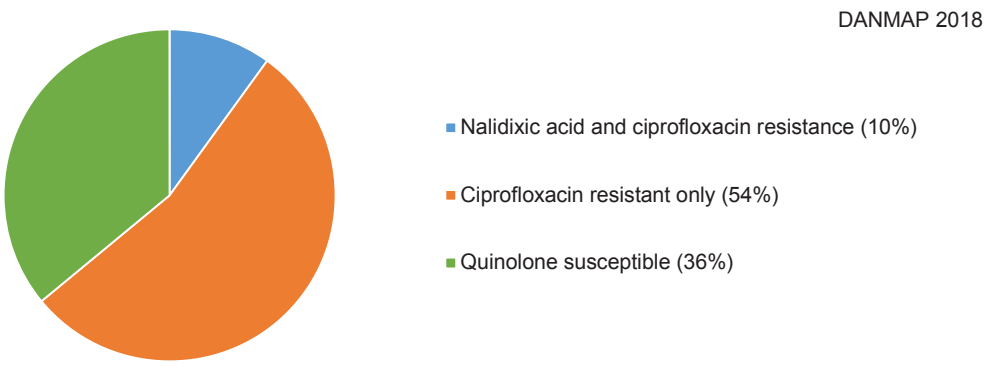
Figure 1 Quinolone resistance distribution in 67 *E. coli* isolated from prawns and fish from Asia



Textbox 7.2

ESBL- and pAmpC-producing *Escherichia coli* – comparison of isolates of animal origin and isolates obtained from human bloodstream infections

Background: ESBL- and pAmpC-producing bacteria are widespread in both humans and animals worldwide. Several studies have suggested that the presence of *E. coli* with identical Multilocus Sequence Types (STs) and ESBL-/pAmpC-genes in animals, meat products and human infections could be caused by zoonotic transmission. In this study, we investigated possible zoonotic links between isolates originating from different origins based on MIC determination and whole genome sequencing combined with bioinformatic analysis.

Materials and methods: ESBL- and pAmpC-producing *E. coli* isolates from production animals and meat obtained in 2017 and 2018 (Table 7.3 and DANMAP 2017) were compared with *E. coli* 2018-isolates from human bloodstream infections (BSI) having similar phenotypic characteristics (Textbox 8.2). Possible clonal relationships between isolates sharing the same combination of ST and ESBL-/pAmpC-genes, were identified by whole-genome-based single-nucleotide polymorphism (SNP) analysis. Genomes were further characterized and phylogenetic analysis initiated if < 100 SNPs were observed between isolates of both domains. Horizontal gene transfer by plasmids encoding ESBL/pAmpC enzymes was not investigated.

Results: During 2017-2018, a total of 209 isolates from production animals and meat products were carrying ESBL/pAmpC-encoding genes, including: 80 isolates from broilers, 112 isolates from broiler meat (Danish and imported), 10 isolates from beef and seven isolates from pork. From human BSI, a total of 352 ESBL/pAmpC positive isolates were collected in 2018.

When comparing the *E. coli* isolates from human BSI and *E. coli* isolates of animal origin, the same combinations of STs and ESBL/pAmpC were detected 11 times; ST10, ST23, ST69, ST88, ST162, ST362, ST74, and ST1434 in combination with CTX-M-1, ST10 in combination with CTX-M-32, ST69 in combination with CMY-2, and ST69 in combination with SHV-12. For each of the 11 combinations, a SNP-based comparison was performed. Only for ST69 with CTX-M-1 less than 100 SNPs between isolates of human and animal origin were observed.

The ST69 CTX-M-1-producing *E. coli* encompassed one isolate from imported beef obtained in 2017 and four isolates from patients with BSI in 2018. Twenty-one, 126, 154, and 227 SNPs were observed between the isolate from beef and the four isolates from human infections indicating a close clonal relatedness.

Discussion and conclusion: ST69 *E. coli* is a frequent cause of urinary tract infections and BSI in humans but also found in poultry, pork/pigs and beef/cattle [Amee *et al.* 2012, CID 55: 712-719]. The number of SNP (>10) observed between the isolate from beef and the human BSI do not indicate an outbreak or a direct transmission, but the difference of 21 SNPs indicates high clonal relationship and a possible zoonotic link of *E. coli* ST69 with CTX-M-1. A more comprehensive analysis of the zoonotic transmission in Denmark could in the future also include isolates from other human sources than BSI.

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Textbox 7.3

Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries

Background: Traditional AMR monitoring, as performed in DANMAP and similar national programmes, is limited to a handful of bacterial species with pathogenic or indicator potential. The majority of AMR genes are however located in species that are not being monitored. Since many bacterial species have unknown requirements and have therefore never been cultured, it would be impractical or even impossible to meet their growth conditions. In order to elucidate the livestock-associated AMR gene reservoir, also called the resistome, we use shotgun metagenomics.

Materials and method: As part of the European Union-funded EFFORT project (www.effort-against-amr.eu) we collected 25 animal faecal samples from each of 181 pig and 178 poultry herds located in nine European countries and pooled each set for a herd-level sample. From each pool, DNA was extracted, sheared and paired-end sequenced on an Illumina HiSeq instrument. On average, more than 50 million pieces of DNA were sequenced per sample and were analysed *in silico* against the ResFinder database. For details, please see the publication [Munk et al. 2018, Nat. Microbio. 3: 898].

The numbers of assigned sequences were adjusted to both the sample-specific number of bacterial sequences and gene-specific lengths, such that the "Fragments Per Kilobase reference gene per Million bacterial fragments" (FPKM) was obtained.

Results: The total AMR load varied drastically between samples, both as a function of host animal species and country of origin (Figure 1). For both pig and poultry, the Dutch and Danish farms had lower combined AMR loads than other countries. Over half the Danish poultry farms were below 500 FPKM AMR, whereas the Italian pig farms had the highest loads, frequently in excess of 10,000 FPKM.

In addition to the abundance, we calculated AMR gene diversity and richness. Across all samples, more than 400 different AMR genes were detected. Interestingly, high AMR gene richness in one livestock species was associated with high richness in the other species in the same country. Despite the lower combined load, poultry had more unique AMR genes. Danish pig herds had lower expected gene richness (66 unique genes) compared to any other herds.

Several important AMR genes, including linezolid-resistance gene *optrA* and colistin-resistance gene *mcr-1*, were detected and found to differ significantly in abundance between countries. National veterinary drug use level was significantly associated with the combined AMR load measured by metagenomics.

Conclusion: The detailed metagenomic snapshot of European livestock showed a staggering diversity of AMR that is not normally observed in traditional monitoring. This baseline study is being followed by additional monitoring in more livestock species including turkey, fish and veal calves. The shotgun sequence data are being used to answer completely new questions and highlights the significant value of metagenomic monitoring going forward.

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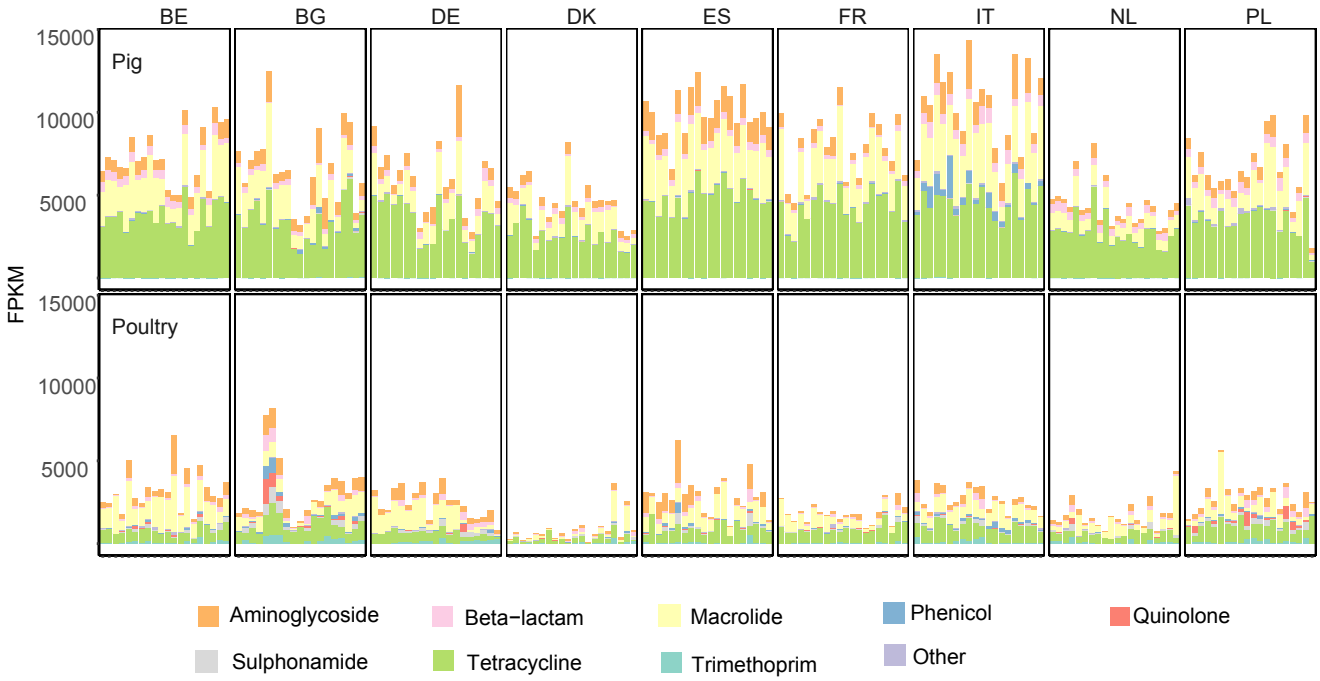
Figure 1 Distribution of antimicrobial resistance genes across livestock species and countries. Each stacked bar represents a single herd in which AMR was quantified using a metagenomic approach



Note: The overall height of the bar represents the combined load, whereas the individual colors denote the antimicrobial drug classes corresponding to the individual 400+ AMR genes that were quantified. Top and bottom panels are pig and poultry respectively. Source: EFFORT projec published in Munk et al. 2018, Nat. Microbio. 3: 898

BE=Belgium; BG=Bulgaria; DE=Germany; DK=Denmark; ES=Spain; FR=France; IT=Italy; NL=Netherlands; PL=Poland.
FPKM=Fragments Per Kilobase reference gene per Million bacterial fragments