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Impact of selective and non-selective media on prevalence and genetic makeup of ESBL/pAmpC-producing *Escherichia coli* in the broiler production pyramid

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ABSTRACT

Presence of extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (pAmpC)-producing *Escherichia coli* (ESBL/pAmpC-EC) in humans and animals is alarming due to the associated risks of antibiotic therapy failure. ESBL/pAmpC-EC transmission between the human and animal compartments remains controversial. Using cefotaxime-supplemented (selective) media, we recently showed high sample prevalence of ESBL/pAmpC-EC in an integrated broiler chain [i.e. Parent Stock (PS), offspring broilers and their carcasses]. Here, we used a different approach. In parallel with the selective isolation, samples were processed on non-selective media. *E. coli* isolates were tested for ESBL/pAmpC-production and those found positive were genotyped. For carcasses, total *E. coli* were enumerated. This approach enabled us to estimate prevalence at the isolate level, which mirrors ESBL/pAmpC-EC colonisation levels. We showed that although present in many animals, ESBL/pAmpC-EC were overall subdominant to intestinal *E. coli*, indicating that high sample prevalence is not associated with high levels of resistance in individual hosts. This is a relevant aspect for risk assessments, especially regarding the immediate exposure of farm personnel. An exception was a particularly dominant B2/bla_{CMY-2} lineage in the gut of imported PS chicks. This predominance obscured presence of latent genotypes, however bias towards particular ESBL/pAmpC-EC genotypes from the selective method or underestimation by the non-selective approach did not occur. At the slaughterhouse, we showed a link between total *E. coli* and ESBL/pAmpC-EC on carcasses. Mitigation strategies for reducing consumers' exposure should aim at suppressing ESBL/pAmpC-EC in the broiler gut as well as controlling critical points in the processing line.

1. Introduction

The increased incidence of extended-spectrum β-lactamase (ESBL)- and plasmid-mediated AmpC β-lactamase (pAmpC)-producing *Enterobacteriaceae* (ESBL/pAmpC-E) in livestock and in the food chain is cause for concern due to the vital role of third-generation cephalosporins (3GCs) in infection control (OIE, 2015; WHO, 2017). The simultaneous rise of difficult to treat nosocomial and community-acquired infections by ESBL/pAmpC-E has supported the hypothesis that food systems with high occurrence of resistant bacteria, such as the broiler production system (Blanc et al., 2006; Dierikx et al., 2013; Laube et al., 2013), could act as reservoirs of resistant bacteria that contribute to the public health burden (Dorado-García et al., 2018). The contribution of these reservoirs to the human burden has been a point of debate (Lazarus et al., 2015; Evers et al., 2017; Dorado-García et al., 2018). Nevertheless, the zoonotic transmission potential prompted the conduct of numerous investigations and the global onset of monitoring programs over the last twenty years (NORM-VET, 2013; DANMAP, 2016; Dame-Korevaar et al., 2019). Early monitoring studies assessed antimicrobial resistance (AMR) at the isolate level by determining the percentage of resistant strains within a given collection (Moreno et al., 2007). The isolate level approach overlooked the role of the host and was deemed inadequate to detect emerging or latent AMR types, shifting the focus of investigations at the sample (host) level (i.e. percentage of samples colonised with resistant bacteria) with the use of antibiotic supplemented (hereafter selective) media (Davison et al., 2000; Moreno et al., 2007). However, surveillance programs, such as EFSA's annual AMR monitoring (EFSA/ECDC, 2018), combine both

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methods since prevalence at the isolate level provides valuable insights on AMR epidemiology and acts as a proxy for colonisation level in the studied population, especially in large-scale studies in which enumeration of resistant isolates can be labour-intensive.

We recently showed a high sample prevalence of ESBL/pAmpC-producing *E. coli* (ESBL/pAmpC-EC) in the broiler production system, with substantial transfer between subsequent production levels (Apostolakos et al., 2019). Here, we determined prevalence at both the sample and isolate levels using both selective and non-selective media. Data from the two methodologies were then compared with one another to assess potential differences in the obtained prevalence and genotype diversity of ESBL/pAmpC-EC in broiler production.

2. Materials and methods

2.1. Sample collection

Sampling was performed as previously described (Apostolakos et al., 2019). Briefly, three production chains of an integrated broiler production company in Northern Italy were monitored. In 40 sampling visits, faecal samples from 20 randomly selected healthy birds were taken by cloacal swabs in the farms, and 20 carcasses were collected at the slaughterhouse (see Supplementary Fig. 1 in Apostolakos et al., 2019). In total, 820 samples were collected. We sampled Parent Stock chickens at the age of one-day (PS chicks) and at the production farms (PS breeders). PS chicks samples for one chain were not collected due to time constraints. Sampled breeders were not combined with other flocks. The offsprings of the sampled PS breeders were sampled in four fattening farms per production chain at the start (one-day-old broiler chicks) and the end (~30-days-old broilers) of the production cycle. Finally, 20 carcasses from the previously sampled broilers were collected at the slaughterhouse after the chilling process. All flocks except for three received antibiotic treatments for therapeutic reasons unrelated to this study (see Supplementary Fig. 1 in Apostolakos et al., 2019).

2.2. Isolation and detection

In addition to the selective isolation with cefotaxime (Apostolakos et al., 2019), samples were simultaneously processed on non-selective media, following the same procedure. Briefly, cloacal swabs were directly streaked on Eosin Methylene Blue agar (EMB) and incubated at 37 ± 0.5 °C for 20 ± 2 h. Carcasses were rinsed with 400 mL of Buffer Peptone Water (BPW), rinsates were incubated (37 ± 0.5 °C for 20 ± 2 h) and streaked on EMB (0.1 mL). Rinsates and three serial dilutions (10^{-1} to 10^{-3}) were plated on EMB (0.1 mL) for subsequent enumeration. The limit of quantification (LOQ) was 1 Log CFU/mL rinsing water. At least two (average three) morphologically typical *E. coli* colonies on EMB (metallic green sheen) were isolated from each sample (cloacal swabs and carcass rinsates) and screened for ESBL/pAmpC production by combination disk diffusion test using cefotaxime (30 μg) and ceftazidime (30 μg) discs with and without clavulanic acid (10 μg) and according to CLSI guidelines (CLSI, 2018). Additionally, a cefoxitin disc (30 μg) was used to detect potential AmpC-producers. Overall, 1309 isolates from cloacal swabs of birds and 555 from carcasses (total 1864) were screened for ESBL/pAmpC production.

2.3. Molecular characterisation

ESBL/pAmpC gene groups (Dierikx et al., 2012) and *E. coli* phylogroups (Clermont et al., 2013) were detected by multiplex PCRs for all phenotypically resistant isolates. For a selection of 33 isolates from EMB (and 119 isolates from CTX-EMB, Apostolakos et al., 2019), ESBL/AmpC genes were sequenced (Macrogen, Spain) after amplification (Dierikx et al., 2012) to identify gene variants (Supplementary Table 1). This selection was done considering the variability of ESBL/pAmpC and *E. coli* groups per sampling (at least one isolate per phylogroup-*bla* gene combination per sampling). Moreover, isolates with an AmpC phenotype, but negative for pAmpC genes by multiplex PCR, were analysed for chromosomal mutations in the *ampC* promoter/attenuator (cAmpC) according to Haldorsen et al. (Haldorsen et al., 2008).

2.4. Data analysis

2.4.1. Prevalence

Prevalence of ESBL/pAmpC-EC at the sample level was calculated for each production stage based upon faecal samples or carcasses being positive to ESBL/pAmpC-EC (at least one isolate). Prevalence at the isolate level was calculated as the proportion of ESBL/pAmpC-EC isolates to the total number of isolates tested per production stage. Differences in prevalence between CTX-EMB and EMB media were tested using logistic regression analysis, with the positive/negative (for ESBL/pAmpC-EC) samples or isolates being the dependent variable and the medium in question (CTX-EMB vs. EMB) being the predictor. All analyses were adjusted for clustering of observations at the chain and farm levels using cluster-robust standard errors. Statistical analysis was performed using STATA (StataCorp, College Station, USA).

2.4.2. Differences in genotypes

The Simpson's index (SI) of diversity (Simpson, 1949) was used to measure the diversity of ESBL/pAmpC-EC strains in terms of gene groups, gene variants and phylogroups (of resistant strains) per sampling stage and isolation medium as the probability that two strains randomly selected from a given sampling stage and medium would belong to different gene groups, gene variants, and phylogroups, respectively. Differences in SI values between media were tested for significance using a two proportion z-test. The proportional similarity index (PSI) (Garrett et al., 2007) was used to measure the overlap between media in terms of gene groups, gene variants and phylogroups. PSI values range from 0 % (no similarities) to 100.0 % (total overlap). All analyses accounted for clustering of data at the farm-chain level (Apostolakos et al., 2019).

2.4.3. Contamination of carcasses

To study the correlation between the loads of total *E. coli* on EMB and putatively ESBL/AmpC growing on CTX-EMB, we followed the methodology of Reich et al. (Reich et al., 2016) and analysed the data with two methods: first, only samples with countable loads on CTX-EMB were included. In a second attempt, *E. coli* loads of samples negative for the quantitative but positive for the qualitative method on CTX-EMB, were arbitrarily set to 0.9 Log CFU/mL (i.e. just below LOQ) whereas for samples negative for both methods, loads were set to 0.0 Log CFU/mL. Data were not normally distributed, so the Spearman's rank correlation coefficient was used to assess correlation between total *E. coli* and ESBL/AmpC-EC. Further, to test the correlation between log-transformed concentrations and prevalence (sample level) of ESBL/AmpC-EC in carcasses, a linear regression analysis adjusted for clustering at the chain-farm level was performed.

3. Results

3.1. Prevalence

3.1.1. Isolate level

Overall, 146 out of 1864 (7.8 %, 95 % Confidence Interval [95 %CI] 4.3–13.9 %) *E. coli* isolates were phenotypically resistant to ESCs (Supplementary Table 1). The highest (71.2 %, 95 %CI 59.6–80.6 %) and lowest (0.53 %, 95 %CI 0.07–3.7 %) prevalence was respectively found for PS chicks and PS breeders (Fig. 1). In fattening broilers, the proportion of resistant isolates was almost double in the beginning of the cycle compared to the end, but remained low throughout the production cycle (Fig. 1). At the end of the production pyramid, 27 out of

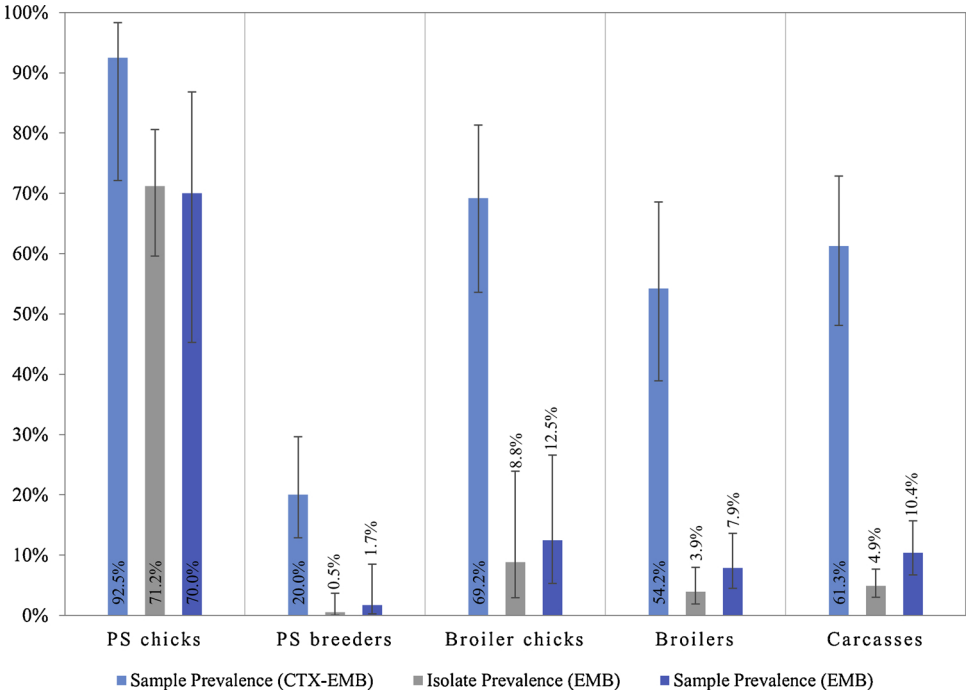

Fig. 1. Prevalence of ESBL/pAmpC-EC in the broiler production pyramid. Error bars represent corresponding 95 % confidence intervals. Data labels (percentages) are placed inside and outside of their respective columns. Abbreviations: PS, Parent Stock; EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1 mg/L cefotaxime.

555 (4.9 %, 95 %CI 3.0–7.0 %) tested isolates from carcasses were phenotypically resistant to 3GCs. In contrast, CTX-EMB showed an overall high sensitivity (prevalence 95.5 %, 95 %CI 91.8–97.6 %) in detecting ESBL/pAmpC-EC as only 24 isolates out of 537 growing on selective media were not positive for ESBL/pAmpC production. Differences in prevalence between the two media were all statistically significant, both overall and at each production level (all p-values = 0.000, except PS breeders with p = 0.001).

3.1.2. Sample level

There was an overall significant difference (p = 0.000) in the estimation of sample prevalence when using selective and non-selective media. Out of 820 samples, only 12.6 % (95 %CI 7.9–19.3 %) was found positive (at least one ESBL/pAmpC-EC recovered) with EMB whereas the prevalence for CTX-EMB was 60.3 % (95 %CI 51.3–68.1 %) (Apostolakos et al., 2019). On average, the estimated prevalence was 6.3 times lower with EMB compared to CTX-EMB when estimating prevalence at the production level (Fig. 1). Differences in prevalence between the two media were statistically significant at each production level (all p-values = 0.000, except PS chicks with p = 0.016 and PS breeders with p = 0.025).

There were 303/820 samples negative with CTX-EMB. For 5 of those samples (4 cloacal and 1 carcass, 1.7 %) ESBL/pAmpC-EC were recovered in the non-selective medium. Additionally, in 14 out of 40 samplings no ESBL/pAmpC-EC positive samples could be detected on EMB (prevalence 0 %) while in all samplings at least one positive sample was found on CTX-EMB (data not shown).

3.2. Differences in genes and phylogroups

All comparisons of resistant isolates between EMB and CTX-EMB showed no significant differences in genotype diversity (SI) and (high) similarity (PSI ≥ 75 %) in their distribution frequencies (Table 1, Supplementary Table 1). At the production level, significant difference was found only for the richness of phylogroups in PS chicks, although all resistant isolates from both media were carrying bla_{CMY-2} and thus showed no differences in terms of resistance genes (Figs. 2 and 3). In PS breeders, where prevalence of ESBL/pAmpC-EC was the lowest, only one resistant isolate of phylogroup A with cAmpC was recovered from EMB while five types of genes and phylogroups were recovered from CTX-EMB, indicating an underestimation of genotype diversity when the non-selective medium was used (Table 1, Fig. 2). In broilers and carcasses, the abundance of genotypes was not significantly different (Table 1). For these production levels, PSI values ranged from 73 to 83%, 55–76 %, and 73–89 % for EBSL/AmpC gene groups, EBSL/AmpC gene variants and phylogroups, respectively, showing that ESBL/pAmpC-EC gene and phylogroup distributions were generally similar between the two isolation media (Table 1).

3.3. Contamination of carcasses

The median load of putative *E. coli* counted with EMB was 3.1 Log CFU/mL (min 1 Log CFU/mL, max 5.2 Log CFU/mL) with all samples having countable *E. coli* loads. The ratio of median loads on CTX-EMB (1.66 Log CFU/mL) (Apostolakos et al., 2019) and EMB was 3.6 % indicating that ESBL/pAmpC-*E. coli* load was 1.5 log cycles lower, thus representing a minor proportion of the total *E. coli* population.

Correlation between total *E. coli* on EMB and putatively ESBL/pAmpC-*E. coli* on CTX-EMB was not significant when all carcasses (n = 240) were taken into account and values were adjusted for non-countable loads on CTX-EMB. Conversely, there was a slight but significant correlation ($p < 0.05$) when only samples with countable loads on CTX-EMB were included (n = 146). In addition, there was a significantly positive correlation (β-coefficient 0.80, 95 %CI 0.30–1.2, p = 0.004) between prevalence and concentrations on the selective medium, indicating that batches of carcasses with a higher sample prevalence also had higher concentrations of ESBL/pAmpC-EC.

4. Discussion

We screened a large collection of isolates from three broiler production chains with the simultaneous use of selective and non-selective media to assess the epidemiology and genetic makeup of ESBL/pAmpC-EC and gain insights on the differences of the two methodologies.

The non-selective medium has significantly underestimated occurrence of ESBL/pAmpC-EC, as sample prevalence was on average six times lower compared with the selective medium and several farms were negative for presence of ESBL/pAmpC-EC. Previous studies have

Table 1
Comparison of genotype abundance and distributions between ESBL/pAmpC-EC from EMB and CTX-EMB.

Production Stage	Index	Gene groups		Gene variants		Phylogroups	
		EMB	CTX-EMB	EMB	CTX-EMB	EMB	CTX-EMB
PS chicks	SI	0.0 %	0.0 %	0.0 %	NC	**33.7 %**	**10.5 %**
	PSI	1.0 %		1.0 %		86 %	
PS breeders	SI	NC	72.7 %	NC	80.0 %	NC	84.8 %
	PSI	NC		NC		25 %	
Broiler chicks	SI	71.4 %	71.0 %	80.6 %	68.3 %	68.4 %	72.2 %
	PSI	74 %		55 %		73 %	
Broilers	SI	62.5 %	57.0 %	82.2 %	76.9 %	78.4 %	77.1 %
	PSI	73 %		76 %		68 %	
Carcasses	SI	59.0 %	51.3 %	63.9 %	77.5 %	80.9 %	78.2 %
	PSI	83 %		74 %		89 %	
Overall	SI	70.4 %	69.5 %	81.1 %	78.5 %	81.0 %	78.8 %
	PSI	75 %		87 %		77 %	

Values in bold differ significantly ($p < 0.05$). Abbreviations: EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1 mg/L cefotaxime; PS, parent stock; PSI, proportional similarity index; SI, Simpson index; NC, not calculated (only one isolate from EMB).

shown that addition of cefotaxime in isolation media is crucial to accurately estimate ESBL/pAmpC-EC prevalence, especially when their sample levels are low (Cavaco et al., 2016; EFSA/ECDC, 2018). Based on these findings EFSA's "specific 3GC-resistance monitoring" was implemented in 2014 to supplement the non-selective "standard monitoring", which determines 3GC resistance rates of randomly selected isolates (EFSA/ECDC, 2018).

In contrast to the sample prevalence approach that detects even very low numbers in individual samples, isolate prevalence provides an estimate of the levels of resistance in the studied population (Mo et al., 2014; EFSA/ECDC, 2016, 2018). Further, this method is relevant for risk assessment, since ESBL/pAmpC-EC are thought to follow random pathways in their transfer along the food chain (EFSA/ECDC, 2018). In our study, the overall levels of resistance were low (7.8 %) and in line with previous field (Horton et al., 2011) and experimental (Bouder et al., 2010) studies. High proportion of resistant isolates was observed in PS chicks, with all isolates belonging to phylogroup B2 and carrying bla_{CMY-2} (Figs. 2 and 3). Therefore, it is not only possible that the B2/bla_{CMY-2} lineage has been introduced in the broiler pyramid through the import of PS chicks, as previously discussed (Apostolakos et al., 2019), but also that it was predominant in the developing *E. coli* population of young hatchlings and thus more likely to disseminate in successive production levels (Fig. 2). We subsequently observed a significant decrease of resistance rates for PS breeders with only one ESBL/pAmpC-producing isolate out of 188 tested. Unfavourable conditions for bla_{CMY-2}-carrying plasmids could justify this trend (Dame-Korevaar et al.,

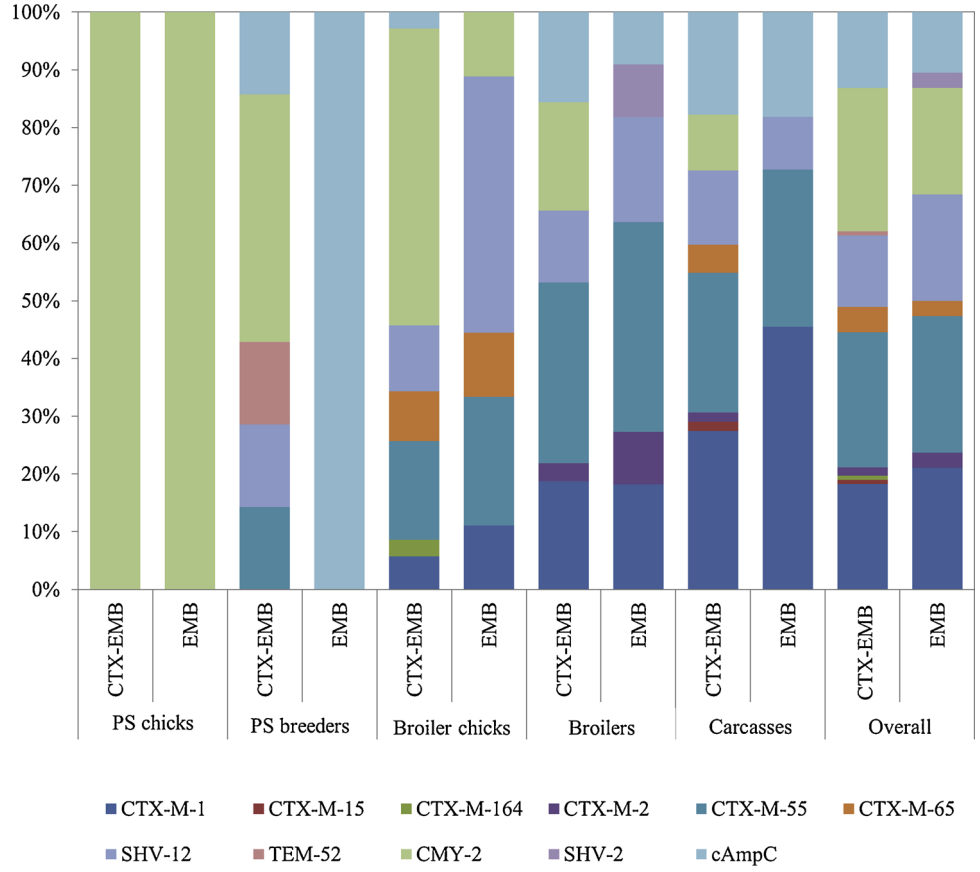

Fig. 2. Distribution of ESBL/pAmpC genes in the broiler production pyramid. Abbreviations: cAmpC, isolates with chromosomal mutations in the *ampC* promoter/attenuator; EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1 mg/L cefotaxime.

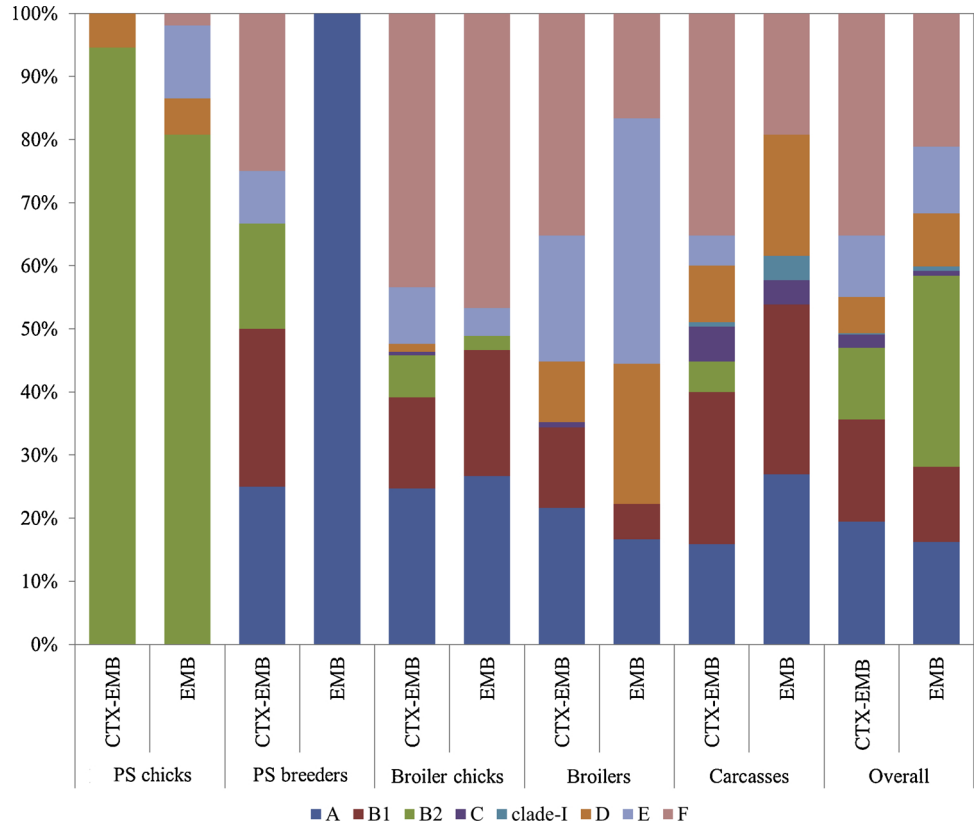

Fig. 3. Distribution of *E. coli* phylogroups in the broiler production pyramid. Abbreviations: EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1 mg/L cefotaxime.

2017). Furthermore, opposed to the high host prevalence observed with the selective approach, the levels of resistance were low for broilers and carcasses, with less than 10 % of isolates being ESBL/pAmpC-producing. Nonetheless, the higher shedding densities of ESBL/pAmpC-EC in chickens compared to other animals (Horton et al., 2011; Reich et al., 2013) and the promiscuity of ESBL/pAmpC-carrying plasmids (de Been et al., 2014), justify the spread of ESBL/pAmpC-EC in individual animals and thus the high sample prevalence observed in our study. However, ESBL/pAmpC-EC seems to be a subdominant part of the *E. coli* population present in the broiler gut flora (EFSA/ECDC, 2016).

To explore whether CTX-EMB induces a selection bias over particular ESBL/pAmpC-EC genotypes or, conversely, if EMB underestimates presence of certain genotypes, we compared the two datasets. Overall, PSI and SI values showed no significant differences in the genetic makeup of ESBL/pAmpC-EC strains from the two media. In PS chicks, the predominant B2/bla_{CMY-2} seems to have obscured presence of two additional, low occurring phylogroups that were discovered carrying bla_{CMY-2} with EMB (Fig. 3). In PS breeders, where prevalence was the lowest, only one cAmpC isolate was recovered with EMB whereas four additional ESBL/pAmpC genes were found with CTX-EMB (Fig. 2). In broilers and carcasses, no significant differences were found between the two approaches in terms of genotype abundance and distribution (Table 1). Therefore, bias towards particular ESBL/pAmpC-EC genotypes from the selective method or underestimation by the non-selective approach did not occur.

At the slaughterhouse level, contamination of carcasses with ESBL/pAmpC-EC defines exposure rates of chicken meat consumers (Depoorter et al., 2012). We showed that ESBL/pAmpC-EC were subdominant to total *E. coli*. Furthermore, the proportions found were respectively one order of magnitude lower and higher than those reported by Reich et al. (Reich et al., 2016) and von Tippelskirch et al. (von Tippelskirch et al., 2018) while total *E. coli* loads were comparable. In contrast to these studies, a significantly positive correlation between total *E. coli* and putative ESBL/pAmpC-EC was found, meaning that carcasses with higher total *E. coli* loads are expected to have higher levels of ESBL/pAmpC-EC. However, a methodological difference with the aforementioned studies is that we sampled the whole carcass area by rinsing and not smaller areas such the neck skin or breast. Further, our results corroborate the findings of Pacholewicz et al. (Pacholewicz et al., 2015), who followed the same sampling methodology, and proved that total *E. coli* can be used as a proxy of the status and fate of ESBL/pAmpC-EC in carcasses during processing. Moreover, batches of carcasses with high sample prevalence correlated with higher concentrations of ESBL/pAmpC-EC. Hence, measures to reduce the numbers of ESBL/pAmpC-EC at the slaughterhouse could have an effect on the number of contaminated carcasses (or its parts) reaching retail level. Interventions should first aim at primary farming by reducing the numbers of ESBL/pAmpC-EC in the broiler gut before slaughter (Ceccarelli et al., 2017), to decrease the unavoidable effect of contamination by faecal leakage during evisceration. Processing parameters, such as temperature and duration of scalding, can form additional control points (Pacholewicz et al., 2015). Finally, microbiological criteria for ESBL/pAmpC-EC could help monitor the progress of such interventions (Depoorter et al., 2012; Reich et al., 2016) and carcasses from farms with high prevalence could be directed to abattoirs with better hygiene records (EFSA, 2011).

5. Conclusion

By estimating prevalence on the isolate level, we showed that although carried by many animals, ESBL/pAmpC-EC were overall subdominant to intestinal *E. coli*. An exception to this situation was a particularly dominant B2/bla_{CMY-2} lineage in the gut of PS chicks, which obscured the presence of latent genotypes. However, major differences in acquired genotypes between the selective and non-selective isolation methods were not observed. At the slaughterhouse,

improvement of hygiene and establishment of microbiological criteria can help reduce ESBL/pAmpC-EC numbers on carcasses.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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References

Apostolakos, I., Mughini-Gras, L., Fasolato, L., Piccirillo, A., 2019. Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing *Escherichia coli* across the broiler production pyramid. PLoS One 14, e0217174. https://doi.org/10.1371/journal.pone.0217174.

Blanc, V., Mesa, R., Saco, M., Lavilla, S., Prats, G., Miró, E., Navarro, F., Cortés, P., Llagostera, M., 2006. ESBL- and plasmidic class C β-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. Vet. Microbiol. 118, 299–304. https://doi.org/10.1016/j.vetmic.2006.08.002.

Bouder, A., Kempf, I., Hellard, G., Dheilly, A., Le Devendec, L., 2010. Persistence and spread of qnr, extended-spectrum beta-lactamase, and ampC resistance genes in the digestive tract of chickens. Microb. Drug Resist. 17, 129–134. https://doi.org/10.1089/mdr.2010.0096.

Cavaco, L., Hendriksen, R., Agersø, Y., Aaby Svendsen, C., Nielsen, H., Guerra, B., Peran, R., Hasman, H., 2016. Selective Enrichment of ESBL, AmpC and Carbapenemase Producing *E. Coli* in Meat and Cecal Samples - Additional Validation for Poultry Samples. 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2016), Amsterdam, the Netherlands.

Ceccarelli, D., van Essen-Zandbergen, A., Smid, B., Veldman, K.T., Boender, G.J., Fischer, E.A.J., Mevius, D.J., van der Goot, J.A., 2017. Competitive exclusion reduces transmission and excretion of extended-spectrum-β-lactamase-producing *Escherichia coli* in broilers. Appl. Environ. Microbiol. 83, 1–13. https://doi.org/10.1128/AEM.03439-16.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58–65. https://doi.org/10.1111/1758-2229.12019.

CLSI, 2018. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. CLSI Supplement M100, 27th ed. Clinical and Laboratory Standards Institute, Wayne, PA.

Dame-Korevaar, A., Fischer, E.A.J., Stegeman, A., Mevius, D., van Essen-Zandbergen, A., Velkers, F., van der Goot, J., 2017. Dynamics of CMY-2 producing *E. coli* in a broiler parent flock. Vet. Microbiol. 203, 211–214. https://doi.org/10.1016/j.vetmic.2017.03.024.

Dame-Korevaar, A., Fischer, E.A.J., van der Goot, J., Stegeman, A., Mevius, D., 2019. Transmission routes of ESBL/pAmpC producing bacteria in the broiler production pyramid, a literature review. Prev. Vet. Med. 162, 136–150. https://doi.org/10.1016/j.prevetmed.2018.12.002.

DANMAP, 2016. The Danish integrated antimicrobial resistance monitoring and research programme. DANMAP 2016 - Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark. National Food Institute, Technical University of Denmark, Lyngby.

Davison, H.C., Woolhouse, M.E.J., Low, J.C., 2000. What is antibiotic resistance and how can we measure it? Trends Microbiol. 8, 554–559. https://doi.org/10.1016/S0966-842X(00)01873-4.

de Been, M., Lanza, V.F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N., Tooming-Klunderud, A., Heederik, D.J.J., Fluit, A.C., Bonten, M.J.M., Willems, R.J.L., de la Cruz, F., van Schaik, W., 2014. Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 10, e1004776. https://doi.org/10.1371/journal.pgen.1004776.

Depoorter, P., Persoons, D., Uyttendaele, M., Butaye, P., De Zutter, L., Dierick, K., Herman, L., Imberechts, H., Van Huffel, X., Dewulf, J., 2012. Assessment of human exposure to 3rd generation cephalosporin resistant *E. Coli* (CREC) through consumption of broiler meat in Belgium. Int. J. Food Microbiol. 159, 30–38. https://doi.org/10.1016/j.ijfoodmicro.2012.07.026.

Dierikx, C.M., van der Goot, J.A., Smith, H.E., Kant, A., Mevius, D.J., 2013. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. PLoS One 8, e79005. https://doi.org/10.1371/journal.pone.0079005.

Dierikx, C.M., van Duijkeren, E., Schoormans, A.H.W., van Essen-Zandbergen, A., Veldman, K., Kant, A., Huijsdens, X.W., van der Zwaluw, K., Wagenaar, J.A., Mevius, D.J., 2012. Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. J. Antimicrob. Chemother. 67, 1368–1374. https://doi.org/10.1093/jac/dks049.

Dorado-García, A., Smid, J.H., van Pelt, W., Bonten, M.J.M., Fluit, A.C., van den Bunt, G., Wagenaar, J.A., Hordijk, J., Dierikx, C.M., Veldman, K.T., de Koeijer, A., Dohmen, W., Schmitt, H., Liakopoulos, A., Pacholewicz, E., Lam, T.J.G.M., Velthuis, A.G., Heuvelink, A., Gonggrijp, M.A., van Duijkeren, E., van Hoek, A.H.A.M., de Roda Husman, A.M., Blaak, H., Havelaar, A.H., Mevius, D.J., Heederik, D.J.J., 2018. Molecular relatedness of ESBL/AmpC-producing *Escherichia coli* from humans, animals, food and the environment: a pooled analysis. J. Antimicrob. Chemother. 73, 339–347. https://doi.org/10.1093/jac/dkx397.

EFSA/ECDC, 2018. European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. EFSA J. 16, 5182. https://doi.org/10.2903/j.efsa.2017.4694.

EFSA/ECDC, 2016. European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. EFSA J. 14, 4380. https://doi.org/10.2903/j.efsa.2016.4380.

EFSA, 2011. European Food Safety Authority. Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. EFSA J. 9, 2322. https://doi.org/10.2903/j.efsa.2011.2322.

Evers, E.G., Pielaat, A., Smid, J.H., van Duijkeren, E., Vennemann, F.B.C., Wijnands, L.M., Chardon, J.E., 2017. Comparative exposure assessment of ESBL-producing *Escherichia coli* through meat consumption. PLoS One 12, e0169589. https://doi.org/10.1371/journal.pone.0169589.

Garrett, N., Devane, M.L., Hudson, J.A., Nicol, C., Ball, A., Klena, J.D., Scholes, P., Baker, M.G., Gilpin, B.J., Savill, M.G., 2007. Statistical comparison of *Campylobacter jejuni* subtypes from human cases and environmental sources. J. Appl. Microbiol. 103, 2113–2121. https://doi.org/10.1111/j.1365-2672.2007.03437.x.

Haldorsen, B., Aasnaes, B., Dahl, K.H., Hanssen, A.-M., Simonsen, G.S., Walsh, T.R., Sundsfjord, A., Lundblad, E.W., 2008. The AmpC phenotype in Norwegian clinical isolates of *Escherichia coli* is associated with an acquired ISEcp1-like ampC element or hyperproduction of the endogenous AmpC. J. Antimicrob. Chemother. 62, 694–702. https://doi.org/10.1093/jac/dkn257.

Horton, R.A., Randall, L.P., Snary, E.L., Cockrem, H., Lotz, S., Wearing, H., Duncan, D., Rabie, A., McLaren, I., Watson, E., La Ragione, R.M., Coldham, N.G., 2011. Fecal carriage and shedding density of CTX-M Extended-Spectrum β-Lactamase-Producing *Escherichia coli*in cattle, chickens, and pigs: implications for environmental contamination and food production. Appl. Environ. Microbiol. 77, 3715–3719. https://doi.org/10.1128/AEM.02831-10.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Käsbohrer, A., Kreienbrock, L., Roesler, U., 2013. Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing *Escherichia coli* at German broiler chicken fattening farms. Appl. Environ. Microbiol. 79, 4815–4820. https://doi.org/10.1128/AEM.00856-13.

Lazarus, B., Paterson, D.L., Mollinger, J.L., Rogers, B.A., 2015. Do human extraintestinal *Escherichia coli* infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. Clin. Infect. Dis. 60, 439–452. https://doi.org/10.1093/cid/ciu785.

Mo, S.S., Norström, M., Slettemeås, J.S., Løvland, A., Urdahl, A.M., Sunde, M., 2014. Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile. Vet. Microbiol. 171, 315–320. https://doi.org/10.1016/j.vetmic.2014.02.002.

Moreno, M.A., Teshager, T., Porrero, M.C., García, M., Escudero, E., Torres, C., Domínguez, L., 2007. Abundance and phenotypic diversity of *Escherichia coli* isolates with diminished susceptibility to expanded-spectrum cephalosporins in faeces from healthy food animals after slaughter. Vet. Microbiol. 120, 363–369. https://doi.org/10.1016/j.vetmic.2006.10.032.

NORM-VET, 2013. Usage of Antimicrobial Agents and Occurence of Antimicrobial Resistance in Norway. Norwegian Veterinary Institute, Tromso.

OIE, 2015. World Organisation for Animal Health. List of Antimicrobial Agents of Veterinary Importance. World Organisation for Animal Health, Paris.

Pacholewicz, E., Liakopoulos, A., Swart, A., Gortemaker, B., Dierikx, C., Havelaar, A., Schmitt, H., 2015. Reduction of extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* through processing in two broiler chicken slaughterhouses. Int. J. Food Microbiol. 215, 57–63. https://doi.org/10.1016/j.ijfoodmicro.2015.08.010.

Reich, F., Atanassova, V., Klein, G., 2013. Extended-spectrum ß-lactamase- and ampc-producing enterobacteria in healthy broiler chickens, Germany. Emerg. Infect. Dis. 19, 1253–1259. https://doi.org/10.3201/eid1908.120879.

Reich, F., Schill, F., Atanassova, V., Klein, G., 2016. Quantification of ESBL-*Escherichia coli* on broiler carcasses after slaughtering in Germany. Food Microbiol. 54, 1–5. https://doi.org/10.1016/j.fm.2015.10.020.

Simpson, E.H., 1949. Measurement of diversity. Nature 163https://doi.org/10.1038/163688a0. 688–688.

von Tippelskirch, P., Gölz, G., Projahn, M., Daehre, K., Friese, A., Roesler, U., Alter, T., Orquera, S., 2018. Prevalence and quantitative analysis of ESBL and AmpC beta-lactamase producing *Enterobacteriaceae* in broiler chicken during slaughter in Germany. Int. J. Food Microbiol. 281, 82–89. https://doi.org/10.1016/j.ijfoodmicro.2018.05.022.

WHO, 2017. World Health Organization. Critically Important Antimicrobials for Human Medicine – 5th Revision. World Health Organization, Geneva.