



Direct vertical transmission of ESBL/pAmpC-producing *Escherichia coli* limited in poultry production pyramid

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ABSTRACT

Extended-spectrum beta-lactamase (ESBL) and plasmidic AmpC (pAmpC) producing *Escherichia coli* are found in the poultry production even without antibiotic use. The spread of these bacteria has been suggested to occur via imported parent birds, enabling transmission to production level broilers vertically via eggs. We studied transmission of ESBL/pAmpC-producing *E. coli* and *E. coli* without antibiotic selection by sampling imported parent birds (n = 450), egg surfaces prior to and after the incubation period (n = 300 and n = 428, respectively) and the laying house environment (n = 20). Samples were additionally taken from embryos (n = 422). To study the prevention of transmission, a competitive exclusion (CE) solution was added onto freshly laid eggs prior to incubation period (n = 150). Results showed carriage of ESBL/pAmpC-producing *E. coli* in parent birds (26.7%), the environment (5%) and egg surfaces before the incubation period (1.3%), but not from egg surfaces or embryos after the incubation period. Whole genome sequencing revealed ESBL/pAmpC-producing *E. coli* isolates belonging to clonal lineages ST429 and ST2040. However, the finding of *E. coli* cultured without antibiotic selection in two (2.2%) embryos strengthens the need to study *E. coli* transmission in poultry production in more depth. Since ESBL/pAmpC-producing *E. coli* seem not to persist on egg surfaces, there is no need to use CE solution *ex ovo* as a prevention method. The results indicate that other routes, such as for example transmission through fomites or horizontal gene transfer by other bacterial species, could be more important than vertical transmission in the spread of resistance in broiler production.

1. Introduction

Broilers are considered a reservoir for extended-spectrum beta-lactamase (ESBL) and plasmidic AmpC (pAmpC) producing *Escherichia coli*. Vertical transmission of ESBL/pAmpC-producing *E. coli* through the poultry production pyramid from pedigree stock downward has been suggested to be the origin of bacteria colonizing newborn chicks (Petersen et al., 2006; Bortolaia et al., 2010; Agersø et al., 2014; Nilsson et al., 2014; Zurfluh et al., 2014). In addition to bacterial clonal spread, the successful proliferation of resistance genes throughout poultry production is thought to be highly attributable to mobile genetic elements, in particular plasmids, which transfer resistance genes between bacteria (Carattoli, 2013; Mo et al., 2017). Plasmids may also play an important role in the spread of resistant traits between animals, food and humans (Bennett, 2008; Leverstein-van Hall et al., 2011; Börjesson et al., 2013).

Vertical transmission of pathogens may be defined as the transfer of bacteria or plasmids carrying resistance genes from the hen's

reproductive tract or cloaca to the developing or freshly laid egg. In addition to the vertical transmission route, bacteria can spread in poultry production via horizontal routes, i.e. the egg can be penetrated or eggshell contaminated from an environmental source (Messens et al., 2005; De Reu et al., 2006; Castellanos et al., 2017). A study by Projahn et al. (2017) has also suggested a pseudo-vertical transmission route of ESBL/pAmpC-producing *E. coli* into hatcheries through contaminated outer eggshell surfaces. However, the precise transmission routes remain unclear.

Broilers in Finland have the lowest prevalence of ESBL/pAmpC-producing *E. coli* among European Union (EU) countries (EFSA and ECDC, 2018). This is most likely attributed to the fact that the use of antibiotics in production animals in Finland is among the lowest in Europe (EMA, 2017) and production broilers have not been medicated with antibiotics after the year 2009 (Nauholz et al., 2014). Parent animals are, however, imported into the country as one-day-old chicks and treated occasionally with antimicrobials to treat infections. Therefore, the upper production levels might serve as the origin of these

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resistant bacteria in production broilers through vertical transmission.

The prevalence of ESBL/pAmpC-producing *E. coli* in broilers and broiler meat varies markedly between EU member states, and ESBL/pAmpC-producing *E. coli* have been found in broilers also in countries with limited antibiotic use (EFSA and ECDC, 2018). In the EU, in addition to beta-lactam resistance, the finding of carbapenemase-producing and colistin resistant *E. coli* in broilers and broiler meat is worrying (EFSA and ECDC, 2018).

In addition to the limited use of antimicrobials and high level of production hygiene, the use of competitive exclusion (CE) products are thought to contribute to the low levels of resistant bacteria. Originally developed to control *Salmonella* outbreaks in poultry, a CE product was developed in the 1970s (Nurmi and Rantala, 1973). The concept of CE is to administer intestinal bacteria of adult birds to newly hatched chicks to support the colonization of the chicks' intestines with beneficial bacteria and reduce the chance of colonization with pathogenic microorganisms (Nurmi and Rantala, 1973; Nurmi et al., 1992). One of the CE products is Broilact (Orion Corp., Orion Pharma, Espoo, Finland), which is a commercial freeze-dried CE product consisting of a healthy chicken's intestinal bacteria. In addition to controlling salmonellosis in poultry, CE products have been found to be effective in reducing ESBL-producing *E. coli* in broilers when administered to day-old chicks (Nuotio et al., 2013; Ceccarelli et al., 2017). To investigate the effect of the possible use of CE prior to hatching on the prevalence of ESBL/pAmpC-producing *E. coli* on egg surfaces and chick intestines after the incubation period we studied the effect of dipping freshly laid eggs into a Broilact solution. The hypothesis was that this procedure would cover the eggs with healthy gut bacteria and protect against the penetration of harmful, resistant bacteria. The effect of dipping eggs in CE products on the prevalence of resistant bacteria has not been studied before to the best of our knowledge. To study the possible transmission routes of ESBL/pAmpC-producing *E. coli* from parent birds to chicks, we sampled broiler parent birds, eggs, and chick embryos in a Finnish broiler production chain. To determine the presence of *E. coli* without antibiotic selection, a selection of the samples was also subjected to culturing without antibiotic supplement. We also took environmental samples from the laying house facilities. In order to study the transmission routes on a molecular level, whole genome sequencing methods were used to investigate the sequence type (ST), resistance genes, virulence factors and plasmids harboured by bacterial isolates.

2. Materials and methods

2.1. Sampling

Together 450 cloacal swabs were collected from broiler parent birds belonging to one flock composed of approximately 4000 birds housed in the same hall. Additionally 20 environmental samples were collected from the same farm. Sterile culture swabs (Copan Transystem, Copan Diagnostics, Italy) were used. Additionally, environmental samples were moistened in sterile buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK) prior to rubbing areas of 10 × 10 cm of the facilities and equipment. Environmental samples were taken from inside the layer house from the egg belt, drinking and feeding systems, laying houses and door knobs from the layer house and egg conveyor room. Samples were additionally taken from the egg conveyor and egg storage room. Additionally, newly laid eggs (n = 450) were collected (0–20 h from laying). Parent birds were 46 weeks old at the time of the study and had been medicated with amoxicillin at age 24 weeks.

To determine the presence of ESBL/pAmpC *E. coli* on the outer surface of the eggs, the eggs (n = 450) were divided into three groups, with 150 eggs in each group. Eggs in Group 1 were dipped into 20 ml sterile BPW. Eggs in Group 2 were dipped into 20 ml sterile BPW and subsequently dipped also into a solution of Broilact (2.5 l), prepared according to the manufacturer's instructions. Eggs in Group 3 were left

untreated to serve as controls and were not subjected to dipping in BPW or Broilact solution. All eggs (n = 450) were transported to the hatchery according to their normal schedule. Prior to transportation to the hatchery, the eggs were moved to a storage room and were sprayed twice daily with a disinfectant according to the laying house's normal disinfection methods. The disinfectant used was Teflex Plus (Oy Soft Protector, Espoo, Finland) consisting of polyhexamethylenebiguanide hydrochloride (PHMB) (CAS 32289-58-0). The different egg groups were kept in separate trays, avoiding contamination with other groups. Samples were transported to the laboratory at + 4 °C and analysed within 24 h.

After 17 days in the hatchery, the fertilized eggs (n = 428) were transported to the laboratory and resampled to determine the presence of post-incubation ESBL/AmpC-producing *E. coli* on their outer surfaces. Outer eggshell rinsing samples (n = 428) were obtained in the same manner as previously.

Subsequently, the eggs were cut open and the chick inside was mechanically euthanized (n = 422) prior to collecting a sample consisting of intestines and a part of the yolk sac. Samples were not obtained from six eggs as they had no visible embryo inside the egg due to putrefaction.

2.2. Isolation and confirmation of *Escherichia coli*

Parental bird cloacal samples (n = 450), chick intestinal samples (n = 422) and environmental samples (n = 20) were enriched in BPW (9 ml) and incubated at 37 °C overnight. Outer eggshell samples (n = 300 before incubation period, n = 428 after incubation period) were incubated at 37 °C overnight. After incubation, a loopful (10 µl) of the enrichment was streaked onto MacConkey agar plates (Lab M, Lancashire, UK; Scharlau Chemie s.a, Sentmenat, Spain) with 1 mg/l cefotaxime. The first 10 eggs from each egg group as well as the first 30 chick samples originating from each group (Groups 1–3) and the first 30 parent bird samples were also streaked onto MacConkey agar plates without antibiotic supplement to determine the presence of *E. coli* without antibiotic selection. The plates were incubated at 44 °C for 18–22 h. A typical colony from each sample was confirmed as *E. coli* by using an oxidase test (Thermo Fischer Scientific Inc., Waltham, MA, USA), Gram staining and an API20E biochemical test (Biomérieux, Marcy-l'Étoile, France).

2.3. Phenotypic identification of ESBL/AmpC-producing *Escherichia coli*

Susceptibility testing was performed with the disc diffusion method (EUCAST, 2017) to isolates grown with antibiotic supplement. Susceptibility to third-generation cephalosporins was tested with ceftazidime (10 µg) (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark) and cefotaxime (5 µg) (Oxoid Ltd, Basingstoke, Hampshire, UK), to cephamycin with cefoxitin (30 µg), and to carbapenem with meropenem (10 µg) (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). Synergism between third-generation cephalosporins and clavulanic acid was tested with a combination disc diffusion test with cefotaxime + clavulanic acid 30 µg + 10 µg and ceftazidime + clavulanic acid 30 µg + 10 µg (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). The epidemiological cut-off values were used as a reference (EUCAST, 2018). *E. coli* ATCC 25922 was included as a quality control. In addition to resistance to third-generation cephalosporins, resistance to cephamycin and < 5 mm difference in inhibition zones in the combination disc diffusion test were used as criteria for pAmpC production, whereas ESBL production was evidenced by resistance to third-generation cephalosporins and ≥ 5 mm difference in the combination disc diffusion test.

2.4. Whole genome sequencing and sequence analysis

A representative collection of *E. coli* isolates (n = 23) was chosen for

whole genome sequencing. Chosen isolates consisted of isolates from parent birds (8 resistant isolates, 4 isolates without antibiotic selection), outer eggshell samples (4 resistant isolates, 4 isolates without antibiotic selection), the environmental sample (1 resistant isolate), and chick intestines (2 isolates without antibiotic selection).

The collection of *E. coli* isolates was subjected to DNA extraction and purification with a PureLink Genomic DNA Mini Kit (Invitrogen by Thermo Fischer Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. Whole genome sequencing was performed with an Illumina Novaseq platform (Center for Genomics and Transcriptomics, Tuebingen, Germany) with paired end reads. Samples were sequenced with $100 \times$ coverage and 2×100 bp read length.

Analyses of samples were run on a web-based service (Center for Genomic Epidemiology, DTU, Denmark). Either raw reads or assembled contigs were used according to the service's recommendations. Resistance genes were determined using KmerResistance v 2.2 (Clausen et al. 2016) using raw reads, with a 70% identity threshold and depth correlation of 10%. Reads were assembled with SPAdes v 3.9 (Nurk et al., 2013). Species identification was confirmed with KmerFinder v 2.5 (Hasman et al., 2014; Larsen et al., 2014) using assembled contigs. Multilocus sequence typing (MLST) was determined with MLST v 1.8 (Larsen et al., 2012) using *E. coli* scheme 1 (Wirth et al., 2006) with assembled contigs. Virulence genes were determined with VirulenceFinder v 1.5 (Joensen et al., 2014) with assembled contigs, and an identity percentage threshold of 90% and a minimum length of 60% were used. Plasmids carried by the isolates were determined with PlasmidFinder v 1.3 (Carattoli et al., 2014) with assembled contigs, and an identity percentage threshold of 95% and a minimum length of 60% were used. CSI Phylogeny 1.4 (Kaas et al., 2014) was used with program default values to assess the single nucleotide polymorphism (SNP) differences between dominating ST types. One isolate of each ST group was used as the reference genome for both analyses, respectively.

2.5. Statistical analysis

Fisher's exact test was used to compare the prevalence of *E. coli* without antibiotic selection in the three different groups of egg surface and embryo samples. Analysis was carried out with SPSS version 24 (IBM, New York, NY, USA). *P*-values < 0.05 were regarded as significant.

3. Results

3.1. Confirmation of *Escherichia coli* and phenotypic tests

3.1.1. ESBL/pAmpC-producing *Escherichia coli*

ESBL/AmpC-producing *E. coli* was detected in 120 (26.7%) parent birds, 4 (1.3%) outer eggshells prior to the incubation period, and 1 (5%) environmental sample, whereas none of the embryo intestine samples or eggshells after the incubation period were positive for ESBL/AmpC-producing *E. coli*, as seen in Table 1. All of the isolates subjected to antimicrobial susceptibility testing were resistant to third-generation cephalosporins (cefotaxime and ceftazidime) and second-generation cephamycin (cefotaxime). None of the isolates were resistant to carbapenem (meropenem). According to the combination disc diffusion test, 33 parent bird isolates (27.5%) were phenotypically AmpC producers and 87 (72.5%) ESBL and AmpC producers. The difference in the inhibition zone diameter of the combination disc diffusion test with ceftazidime + clavulanic acid $30 \mu\text{g} + 10 \mu\text{g}$ varied from 2 to 8 mm, with most isolates ($n = 63$) having a result of 5 mm close to the threshold value. The difference in the inhibition zone diameter of the combination disc diffusion test with cefotaxime + clavulanic acid $30 \mu\text{g} + 10 \mu\text{g}$ varied from 0 to 5 mm, with most isolates ($n = 36$) having a result of 0 mm. All of the egg surface isolates and the environmental isolate were phenotypically ESBL + AmpC producers. Phenotypic results are shown in Table 1.

3.1.2. *Escherichia coli* without antibiotic supplement

E. coli was found on MacConkey plates without antibiotic supplement in 25 parent birds (83.3%), 17 outer eggshells prior to the incubation period (85%), and in two chick intestines (0.5%), whereas none of the outer eggshells after the incubation period were positive for *E. coli*. Positive chick intestine samples originated from Group 1 ($n = 1$) and Group 2 ($n = 1$), as seen in Table 1.

3.2. Statistical analysis

There was no significant difference found between chick intestine samples from Group 3 (no *E. coli* found) compared with Groups 1 and 2, as determined by Fisher's exact test ($P = 1.000$). There was also no significant difference in the presence of *E. coli* in egg surface samples in Groups 1 and 2 in Fisher's exact test ($P = 1.000$). No statistically significant differences were found in these analyses.

3.3. Whole genome sequencing and sequence analysis

The results of the whole genome sequenced isolates are shown in Table 2. All of the isolates were confirmed to be *E. coli*. Altogether 9 MLST types were identified: ST2040, ST429, ST106, ST428, ST88, ST906, ST1286, ST10 and ST453, with ST2040 and ST429 being dominant. Resistant isolates consisted of two MLST types (ST429 and ST2040), whereas isolates without antibiotic selection consisted of 7 MLST types. Of these resistant isolates, all ST429 isolates harboured the virulence genes *gad*, *iroN*, *iss* and *mchF*, whereas all ST2040 isolates harboured *cma*, *gad*, *lpfa*, *iroN*, *iss* and *tsh*. Phylogenetic analysis revealed 0–10 SNP differences within ST2040 isolates and 0–4 SNP differences within ST429 isolates.

From two to five different plasmid replicons were detected in each isolate (Table 2). All of the ST429 isolates harboured plasmids belonging to families of IncB/O/K/Z, IncFIB(AP001918) and IncFIC(FII), whereas four of the six ST2040 isolates harboured plasmids belonging to families of IncX1, ColpVC, IncFIB(AP001918) and IncFIC(FII).

All of the isolates grown on the plates with antibiotic supplement harboured the *bla*_{CMY-2} gene. No other beta-lactam resistance was detected in the isolates grown on plates with antibiotic supplement. ST2040 isolates carried solely *bla*_{CMY-2}, whereas ST429 isolates harboured in addition aminoglycoside resistance genes *aac(3)-Vla* and *aadA1*, sulphonamide resistance gene *sulI* and tetracycline resistance gene *tet(A)*. Two out of four of the parent bird isolates without antibiotic selection harboured resistance genes, one the sulphonamide resistance gene *sulI* and the other the beta-lactam resistance gene *bla*_{TEM-1B}. One out of two of the chick intestine samples without antibiotic selection harboured the beta-lactam resistance gene *bla*_{TEM-1C} and tetracycline resistance gene *tet(A)*. The phenotype matched the genotype in 14 samples, but 9 of the phenotypically ESBL + AmpC-producing isolates were genotypically only AmpC producers.

4. Discussion

The vertical spread of ESBL/pAmpC-producing bacteria from parent birds has been thought to be the main driver of the widespread occurrence of these bacteria in production level broilers, even in countries with limited antibiotic use. Recently, however, it has been suggested that the main transmission route of ESBL/pAmpC-producing *E. coli* is not strictly vertical from parent birds to offspring, but rather a mix of different transmission routes in the production chain (Dierikx et al., 2018; Projahn et al., 2018). Our results support these findings.

For the first time, the prevalence of ESBL/pAmpC-producing *E. coli* was studied in parent birds in Finnish poultry production. Our results showed a prevalence of 26.7% in ESBL/AmpC-producing *E. coli* in one flock of parent birds, which is in general higher than in Finnish production level broilers, which have been reported to have a 14% prevalence (EFSA and ECDC, 2018). ESBL/pAmpC-producing *E. coli*

Table 1Occurrence of extended-spectrum beta-lactamase (ESBL) / plasmidic AmpC (pAmpC) producing *Escherichia coli* and *E. coli* without antibiotic selection in broiler production.

| | No. of positive samples / total no. of collected samples (%) ^a | | | | |
|--|---|-------------------|---------------|---------------|---|
| Sampling | Total ESBL/pAmpC | ESBL ^b | pAmpC | ESBL + pAmpC | <i>E. coli</i> without antibiotic selection |
| Parent bird | 120/450 (26.7) | 0/120 (0.0) | 33/120 (27.5) | 87/120 (72.5) | 25/30 (83.3) |
| Egg surface before incubation period (total) | 4/300 (1.3) | 0/300 (0.0) | 0/300 (0.0) | 4/300 (1.3) | 13/20 (65) |
| Group 1 ^c | 2/150 (1.3) | 0/150 (0.0) | 0/150 (0.0) | 2/150 (1.3) | 6/10 (60) |
| Group 2 | 2/150 (1.3) | 0/150 (0.0) | 0/150 (0.0) | 2/150 (1.3) | 7/10 (70) |
| Group 3 | ND ^d | ND | ND | ND | ND |
| Egg surface after incubation period (total) | 0/428 (0.0) | 0/428 (0.0) | 0/428 (0.0) | 0/428 (0.0) | 0/30 (0.0) |
| Group 1 | 0/141 (0.0) | 0/141 (0.0) | 0/141 (0.0) | 0/141 (0.0) | 0/10 (0) |
| Group 2 | 0/144 (0.0) | 0/144 (0.0) | 0/144 (0.0) | 0/144 (0.0) | 0/10 (0) |
| Group 3 | 0/143 (0.0) | 0/143 (0.0) | 0/143 (0.0) | 0/143 (0.0) | 0/10 (0) |
| Embryo intestines (total) | 0/422 (0.0) | 0/422 (0.0) | 0/422 (0.0) | 0/422 (0.0) | 2/90 (2.2) |
| Group 1 | 0/138 (0.0) | 0/138 (0.0) | 0/138 (0.0) | 0/138 (0.0) | 1/30 (3.3) |
| Group 2 | 0/141 (0.0) | 0/141 (0.0) | 0/141 (0.0) | 0/141 (0.0) | 1/30 (3.3) |
| Group 3 | 0/143 (0.0) | 0/143 (0.0) | 0/143 (0.0) | 0/143 (0.0) | 0/30 (0.0) |
| Environmental samples | 1/20 (5) | 0/20 (0) | 0/20 (0) | 1/20 (5) | ND |

^a Based on phenotypic tests (EFSA Panel on Biological Hazards, 2011; EUCAST, 2017).^b ESBL = isolate expressed only ESBL phenotype; pAmpC = isolate expressed only pAmpC phenotype; ESBL + pAmpC = isolate expressed both ESBL and pAmpC phenotype.^c Group 1 = rinsing sample; Group 2 = rinsing sample followed by Broilact treatment; Group 3 = no treatment before incubation, rinsing sample after incubation.^d ND = not determined.

isolates and *E. coli* without resistance to third-generation cephalosporins were detected in 1.3% and 65% of egg surfaces soon after laying and before disinfection protocols in our study, respectively. However, after routine disinfection procedures at the laying house and the incubation period at the hatchery, all of the egg surfaces tested negative for *E. coli*. This finding suggests that bacteria colonize the surface of eggs immediately after laying, but bacterial counts greatly diminish after disinfection measures or during the incubation period. This indicates that there are also other routes of transmission from the parent birds to offspring than just direct vertical transmission.

It is noteworthy, however, that in our study no ESBL/pAmpC-producing *E. coli* were retrieved from unhatched embryos, but two (2.2%) of the embryos were positive for *E. coli* without antimicrobial selection. These *E. coli* strains did harbour plasmids, which indicates that also ESBL/pAmpC-carrying plasmids could possibly penetrate the egg and developing embryo. One of the penetrated strains harboured beta-lactam resistance gene *bla*_{TEM-1C} and tetracycline resistance gene *tet(A)*, which further demonstrates that bacteria carrying resistance genes in plasmids are able to penetrate the egg in certain circumstances. The presence of different virulence genes in these *E. coli* isolates further indicates that these virulence traits might be essential for penetration and deserves further study. The finding would further suggest that a direct vertical transmission route from the hen's reproductive tract into the developing egg is possible, although not recurrent.

There was no significant difference between the numbers of *E. coli* isolated from embryos belonging to Groups 1, 2 and 3, as was expected since the sample size per group was rather low (n = 30). Eggs belonging to Groups 1 and 2 were dipped into BPW at the laying house, while eggs in Group 3 were left untreated as controls. It has been demonstrated earlier, however, that subjecting eggs to fluids or water, especially when the water temperature is lower than that of the egg's, increases the penetration of bacteria into the egg (Berrang et al., 1999).

A pseudo-vertical transmission route from the hen to the offspring via the outer surface of eggs is also plausible, as suggested earlier (Projahn et al., 2017). Although no *E. coli* was recovered from the egg surfaces after the incubation period in our study, it has been proven earlier that bacteria can survive on egg surfaces after disinfection, although bacterial counts do shrink (Cadirci, 2009; Projahn et al., 2017). It is also possible that resistant bacteria remain in the eggshell pores, concealed from disinfectants, as suggested by Mezhoud et al. (2016). Differences in the prevalence of *E. coli* and other Enterobacteriaceae on egg surfaces in different studies might in part be caused by variable

disinfection protocols in different farms or countries, as well as other influencing factors such as humidity and temperature. The absence of *E. coli* on the shell surfaces after the incubation period in our study might in part have been caused by the eggs' own natural defence mechanisms, and the fact that a dry egg surface is unfavourable for bacterial growth. Disinfection measures after the collection of eggs at the laying house, during storage, and prior to entering the hatchery could also kill bacteria on the egg surfaces. The disinfectant compound PHMB used at the laying house in this study has been identified as being effective against ESBL-producing *E. coli* in vitro (Gorony-Bermes et al., 2013). The possible differences between ESBL/pAmpC-producing *E. coli* strains and non-resistant strains on different disinfectant susceptibility should be investigated in more depth, although Wieland et al. (2017) have found that quaternary ammonium compound inhibited ESBL/AmpC-producing *E. coli* from poultry even at lower concentrations, compared with non-ESBL/AmpC strains. Even though biocides are used, it is of vital importance to know, however, that no antibiotic wash is needed to achieve eradication of *E. coli* on egg surfaces.

Nevertheless, the same STs, plasmids and resistance genes were recovered from parent bird cloacal samples and egg surfaces before disinfection as well as one environmental sample from the laying house. ST2040 and ST429, with only a 0–10 and 0–4 SNP difference respectively, were the predominant STs found in ESBL/pAmpC-producing *E. coli* isolates in our study, implicating the presence of two different clonal lineages in the studied parent bird laying house. SNP differences of ≤ 10 in *E. coli* have been considered previously to be of the same origin (Schürch et al., 2018). These STs have been identified previously in samples of poultry origin (Pietsch et al., 2018). ST2040 harboured the resistance gene *bla*_{CMY-2}, whereas ST429 isolates carried other resistance genes in addition to *bla*_{CMY-2}.

Plasmid-borne transmission of ESBL/pAmpC resistance genes in ESBL/pAmpC-producing *E. coli* has been suggested to occur in poultry production (Dame-Korevaar et al., 2017). Four of the six ST2040 isolates in our study harboured plasmid IncX1, which has been previously linked to ESBL resistance genes *bla*_{SHV-12} and *bla*_{TEM-52} in broilers (Huijbers et al., 2014). All of the ST429 isolates harboured plasmid IncB/O/K/Z, IncFIB(AP001918) and IncFIC(FII). *bla*_{CMY-2} has been previously identified from IncB/O/K/Z-like and IncFIA/FIB replicon types from *E. coli* of poultry origin (Touzain et al., 2018). However, to determine which plasmids harboured *bla*_{CMY-2} in our study, plasmids should be subjected to long-read sequencing. Furthermore, research is needed to determine whether the transmission of ESBL/pAmpC-

Table 2
Genomic analyses of *Escherichia coli* (n = 23) isolated from the broiler production chain.

| Isolate name ^a | Origin | Species ^b | MLST ^c | Virulence genes | Plasmids | Resistance genes | Phenotype ^e |
|---------------------------|-----------------|----------------------|--------------------|--|--|---|------------------------|
| 1E61-R | Egg surface | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncFIC(FII), IncXI</i> | <i>bla_{CMV-2}</i> | ESBL + AmpC |
| 1E74-R | Egg surface | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncFIC(FII), IncXI</i> | <i>bla_{CMV-2}</i> | ESBL + AmpC |
| 2E151-R | Egg surface | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |
| 2E177-R | Egg surface | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncFIC(FII), IncXI</i> | <i>bla_{CMV-2}</i> | ESBL + AmpC |
| 1E5-S | Egg surface | <i>E. coli</i> | ST88 | <i>gad, iha, IpfA, iss, mchB, mchC, mchF</i> | <i>ColpVC, IncFII(pKSBI07)</i> | - | Sensitive |
| 1E10-S | Egg surface | <i>E. coli</i> | ST906 | <i>gad, IpfA</i> | <i>ColpVC, IncFII(29), p0111</i> | - | Sensitive |
| 2E153-S | Egg surface | <i>E. coli</i> | ST906 | <i>gad, IpfA</i> | <i>ColpVC, p0111</i> | - | Sensitive |
| 2E157-S | Egg surface | <i>E. coli</i> | ST1286 | <i>gad, iron, iss, mchF, ish</i> | <i>ColpVC, IncFIC(FII), IncFIB(AP001918), IncFII(29)</i> | - | Sensitive |
| P52-S | Parent bird | <i>E. coli</i> | ST10 ^d | <i>gad, iha, iss</i> | <i>ColpVC, IncFII(29), p0111</i> | - | Sensitive |
| P516-S | Parent bird | <i>E. coli</i> | ST1286 | <i>gad, iron, iss, mchF</i> | <i>IncFIC(FII), IncFIB(AP001918), IncFII(29)</i> | - | Sensitive |
| P519-S | Parent bird | <i>E. coli</i> | ST453 | <i>gad, IpfA, iss</i> | <i>IncFIB(AP001918), IncII, IncXI</i> | <i>bla_{TEM-1B}</i> | Sensitive |
| P530-S | Parent bird | <i>E. coli</i> | ST10 | <i>astA, astA, cha, ema, gad, ireA</i> | <i>IncFIB(AP001918), IncFII(29), IncXI, p0111</i> | <i>stII</i> | Sensitive |
| P5310-R | Parent bird | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | AmpC |
| P561-R | Parent bird | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncFIC(FII)</i> | <i>bla_{CMV-2}</i> | AmpC |
| P5174-R | Parent bird | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncI2</i> | <i>bla_{CMV-2}</i> | AmpC |
| P5371-R | Parent bird | <i>E. coli</i> | ST2040 | <i>ema, gad, IpfA, iron, iss, ish</i> | <i>ColpVC, IncFIB(AP001918), IncFIC(FII), IncXI</i> | <i>bla_{CMV-2}</i> | AmpC |
| P557-R | Parent bird | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColI56, ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |
| P5184-R | Parent bird | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |
| P5148-R | Parent bird | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |
| P5378-R | Parent bird | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |
| 1C14-S | Chick intestine | <i>E. coli</i> | ST106 ^d | <i>air, astA, eilA, gad, IpfA, iron, iss, mchB, mchC, mchF, mchF, mchA</i> | <i>Col(MG828), ColpVC, IncFIB(AP001918), IncFII, IncII</i> | <i>bla_{TEM-1C}, tet(A)</i> | Sensitive |
| 2C151-S | Chick intestine | <i>E. coli</i> | ST1428 | <i>gad, iron, iss, mchF, vat</i> | <i>Col(MG828), ColpVC, IncFIB(AP001918), IncFII,</i> | - | Sensitive |
| Y13-R | Environment | <i>E. coli</i> | ST1429 | <i>gad, iron, iss, mchF</i> | <i>ColpVC, IncB/O/K/Z, IncFIB(AP001918), IncFIC(FII)</i> | <i>aac(3)-Via, ant(3'')-Ia, bla_{CMV-2}, tet(A), stII</i> | ESBL + AmpC |

^a R = Extended-spectrum beta-lactamase (ESBL) / plasmidic AmpC (pAmpC) producing *E. coli*; S = *E. coli* without antibiotic selection; prefix 1 = Group 1, rinsing sample; prefix 2 = Group 2, rinsing sample followed by Broilact treatment.

^b Analysis tools by Center for Genomic Epidemiology: KmerFinder v 2.5 for species determination, MLST v 1.8 Server for multilocus sequence type, VirulenceFinder v 1.5 Server for virulence genes, PlasmidFinder v 1.3 Server for plasmids, KmerResistance v 2.2 for resistance genes, assembly with SPAdes v 3.9.

^c MLST = multilocus sequence type.

^d One or more loci do not perfectly match any previously registered MLST allele.

^e Based on phenotypic tests (EFSA Panel on Biological Hazards, 2011; EUCAST, 2017).

producing *E. coli* in poultry production is facilitated by other bacterial species harbouring ESBL/pAmpC gene-carrying plasmids which introduce these into *E. coli* again through conjugation.

The environmental sample positive for ST429 ESBL/pAmpC-producing *E. coli* was obtained from a scale pad located at the end of the egg conveyor belt in the sampled laying house. This implies that bacteria of the same origin can spread in the production environment from parent birds to egg surfaces, as well as indirectly through contact with contaminated equipment, facilities or workers. As animals and eggs move from one facility to another, special attention should be given to the hygienic handling of material.

In our study, the spread of bacteria via eggshell surfaces seemed to be limited, and as a consequence, no effect was seen when exposing freshly laid eggs to the CE solution. However, we did not sample live chicks after hatching, so it remains undetermined whether dipping eggs into CE solution would have an effect on bacterial findings later on in the chicks' life.

Although 72.5% of the isolates showing resistance to third generation cephalosporins displayed a phenotype of being both AmpC and ESBL producers, only pAmpC-type third-generation cephalosporin resistance was detected with whole genome sequencing. This might be caused by the fact that most of the results of combination disc diffusion test with ceftazidime + clavulanic acid 30 µg + 10 µg were close to the threshold value of 5 mm and might have made interpretation of the result difficult, although controls were always included. The overall level of resistance in Finnish poultry production is the lowest among EU countries (EFSA and ECDC, 2018). Many factors, such as an all-in all-out production method, use of CE products, and good production hygiene could contribute to this difference. Also, antibiotics are not used in production level broilers, which eliminates selective pressure for resistant bacteria via antibiotic use. The strength of our study is the fact that it was conducted in normal production facilities. Eggs were handled in a normal manner and followed normal production routines. This allows for a more realistic study of the transmission routes of resistant bacteria within the production system.

The current study suggests that the contamination pressure through egg surfaces or hatchlings into the production farms is low. It seems, however, that some ESBL/pAmpC resistance genes persist in broiler production. Certain virulence genes have been linked to the ability to persist in the production environment (Projahn et al., 2018). Plasmids, in addition to carrying resistance genes, have been linked to higher virulence in some *E. coli* strains (Schaufler et al., 2016; Touzain et al., 2018). To counteract the spread of ESBLs, as well as the worrying emergence of carbapenem and colistin resistance in poultry production, we suggest investigation of the role of plasmids in transmission dynamics in the poultry production environment as direct vertical spread does not seem to be the main route of resistance.

5. Conclusion

Our study strongly suggests that direct vertical transmission is not the main transmission route of ESBL/pAmpC-producing *E. coli* in poultry production. For the first time, the prevalence of ESBL/pAmpC-producing *E. coli* was determined in Finnish parent birds and their corresponding eggs and chick embryos, and isolates were further studied with whole genome sequencing methods. Also, the effect of the *ex ovo* application of a CE product on the prevalence of ESBL/pAmpC-producing *E. coli* was investigated. Our results show that certain *E. coli* strains are able to penetrate the egg and developing embryo, but this route does not seem to be common. Our results also highlight the fact that egg surfaces are free from *E. coli* after the incubation period even without the use of antibiotic washes. Certain ST types, resistance genes and plasmids persist in broiler birds as well as the production environment, and the reasons behind this persistence should be further investigated.

Conflicts of interest

None to declare.

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