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Agersø, Yvonne; Jensen, Jacob Dyring; Hasman, Henrik; Pedersen, Karl

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Spread of Extended Spectrum Cephalosporinase-Producing *Escherichia coli* Clones and Plasmids from Parent Animals to Broilers and to Broiler Meat in a Production Without Use of Cephalosporins

Yvonne Agersø, Jacob Dyring Jensen, Henrik Hasman, and Karl Pedersen

Abstract

**Objectives:** This study investigated the occurrence of extended spectrum cephalosporinase (ESC)–producing *Escherichia coli* in a broiler production with no cephalosporin use and a low use of antimicrobials in general. Furthermore, it investigated whether the current consumption of aminopenicillins selects for ESC-producing *E. coli* and whether certain clones or plasmids spread from imported parent flocks to the meat.

**Materials and Methods:** ESC-producing *E. coli* was isolated using MacConkey broth with 1 mg/L of ceftriaxone. ESC genes were identified using polymerase chain reaction and sequencing. Isolates with *bla*~*CMY*-2 were subtyped by pulsed-field gel electrophoresis (PFGE), phylotyping, and antimicrobial susceptibility testing. Selected isolates were used as donors in filter-mating experiments, multilocus sequence typing (MLST), and plasmid replicons were typed. Aminopenicillin use at the farm (not flock) level was obtained from VetStat, a database for mandatory registration of veterinary prescriptions in Denmark.

**Results:** ESC-producing *E. coli* occurred in 93% (27/29) of broiler parent farms in 2011, 27% (53/197) of broiler flocks in 2010, and 3.3% (4/121) of Danish retail broiler meat in 2009 and 8.6% (16/187) in 2010. The ESC producing *E. coli* contained *bla*~*CMY*-2, *bla*~*SHV*-2 or *bla*~*CTX-M*-1. Isolates with *bla*~*CMY*-2 represented 35 PFGE groups. One group dominated (39 isolates) and included isolates with indistinguishable PFGE patterns from parents, broilers, and meat. Most *bla*~*CMY*-2 isolates were susceptible to non-β-lactams, and *bla*~*CMY*-2 was mostly present on horizontally transferable incI1 or incK plasmids. Phylogroup D was most common and *E. coli* MLST types previously found in humans were observed. Broiler farms with registered aminopenicillin use had significantly higher occurrence of ESC *E. coli*.

**Conclusions:** ESC-producing *E. coli* from flocks of imported broiler parents spread clonally and horizontally to broiler meat (including potentially human pathogenic types) even in a country with no cephalosporin use. Use of aminopenicillins may influence the persistence of ESC-producing *E. coli* in the broiler production, but other factors should be investigated.

Introduction

Extended-spectrum-β-lactamase and plasmid-mediated AmpC resistance (extended-spectrum cephalosporinases [ESC]) are some of the fastest-emerging resistance problems worldwide (EFSA BIOHAZ, 2011). Production animals and their meat products (especially broiler meat) seem to be a source for ESC-producing bacteria causing infection in humans (Dutil et al., 2010; Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Kluytmans et al., 2013). Broiler production in Europe is characterized by having very few distributors of grandparent animals, and since ESC is widespread and often caused by the same types *bla*~*CMY*-2 or *bla*~*CTX-M*-1 present on horizontally transferable plasmids, ESC may have spread through the breeding chain from the very top of the breeding pyramid to the bottom (EFSA BIOHAZ, 2011). Moreover, the plasmids may have spread to other pathogen bacteria.

1Research Group of Bacterial Genomics and Antimicrobial Resistance, National Food Institute, Technical University of Denmark, Lyngby, Denmark.
2Diagnostic Engineering, National Food Institute, Technical University of Denmark, Søborg, Denmark.
The aim was to investigate the occurrence of ESC-producing *E. coli* in a broiler production with no use of cephalosporins and a low use of antimicrobial agents in general. Furthermore, this study investigated whether the current consumption of aminopenicillins selects for ESC-producing *E. coli* and whether certain clones or plasmids spread from imported parent flocks to retail broiler meat.

In Denmark, ESC-producing *Escherichia coli* in the Danish produced broiler meat was identified for the first time in 2009 (Agersø et al., 2012). The occurrence was low (less than 10%) in 2009 and in 2010 when compared to broiler meat imported into Denmark (DANMAP 2010, 2011). In 2011, ESC-producing *E. coli* increased significantly in the Danish-produced broiler meat to the same level as in imported broiler meat (44% and 48%, respectively) (DANMAP 2011, 2012). Cephalosporins have never been approved for use in poultry in Denmark, and no use of cephalosporins for poultry has been registered for at least a decade. In general, the consumption of antimicrobials in the Danish broiler production is low compared to other production animals, as only approximately 30% of the farms have had antimicrobials prescribed (414 kg, 182 prescriptions) corresponding to only approximately 30% of the farms. No flock was sampled more than once. This study was performed with a CHEF DR III System (Bio-Rad Laboratories, Hercules, CA) using 1% SeaKem Gold (Lonzas, Rockland, ME) agarose in 0.5× Tris-borate-EDTA. Running conditions were 6 V/cm and included angle 120°C in 14°C Tris-borate-EDTA buffer, with pulse times linearly increased from 12 s initial switch time to 40 s final switch time for 20 h.

Materials and Methods

**Sampling procedure**

Samples were collected from all conventional parent flocks catching broiler eggs, from randomly selected broiler flocks at slaughter, and from randomly selected Danish broiler meat. Boot swab samples (sock samples) were collected from the parent flock houses by the farm owners during September 2011. Sterile gauze socks were placed on clean boots, the sample collector walked around in the parent flock, and a house (flock) was sampled once. Up to four socks from different houses within the same herd were pooled and sent to the National Food Institute for analysis. Farms with one to four houses were examined as one pooled sample consisting of one sock from each house, while farms with more than four houses were examined as two pooled samples. No farm had more than six houses. In total, 29 farms were sampled, resulting in 70 sock samples pooled into 32 samples.

The broiler flocks were sampled weekly before slaughter at the five slaughterhouse in Denmark slaughtering conventional produced broilers from May through November as part of the DANMAP program, and the Central Husbandry Register (CHR) number of the farm was registered (DANMAP 2010, 2011). Cloacal swabs from five broilers of the same flock were collected from the parent flock houses by the farm owners during September 2011. Cloacal swabs before sending it to the regional laboratories for analysis.

The meat samples were collected randomly in retail stores and outlets in all regions of Denmark as part of the DANMAP program (Agersø et al., 2012; DANMAP 2010, 2011).

**Analysis of the samples**

Presumptive ESC-producing *E. coli* was isolated from meat as previously described (Agersø et al., 2012). From parent farms, 1 to 4 sock samples were added to 225 mL of MacConkey (Oxoid CM5a, Basingstoke, England) broth supplemented with 1 mg/L of ceftriaxone (Sigma C5793-1G, Steinheim, Germany) and incubated for 16–18 h at 44°C. Ten microliters of this enrichment broth was then streaked on a MacConkey agar supplemented with 1 mg/L of ceftriaxone incubated at 44°C, and a maximum of three colonies were subcultured and stored for further analysis (Agersø et al., 2012).

The samples of five pooled cloacal swabs from broiler flocks were mixed in 3 mL of saline. Thereafter, 1 mL of suspension was transferred to 9 mL of MacConkey broth supplemented with 1 mg/L of ceftriaxone. The same procedure as described for sock samples was followed. *E. coli* was identified on CHROM Orientation agar (Becton Dickinson A/S, Brøndby, Denmark).

**Detection of ESC genes and minimal inhibitory concentration (MIC) testing**

The genetic background for ESC-producing *E. coli* was examined as previously described (Agersø et al., 2012). In brief, all cephalosporinase-producing *E. coli* were initially tested by polymerase chain reaction (PCR) for bla*CMY-2*. If negative, the isolates were subsequently tested for *bla*CTX-M genes, *bla*SHV and *bla*TEM genes by PCR and sequencing (Agersø et al., 2012).

MIC were determined for *bla*CMY-2-positive isolates for the following non-β-lactam antimicrobial agents: tetracycline (2–32 mg/L), chloramphenicol (2–64 mg/L), florfenicol (2–64 mg/L), sulfamethoxazole (64–1024 mg/L), trimethoprim (1–32 mg/L), apramycin (4–32 mg/L), gentamicin (0.5–16 mg/L), neomycin (2–32 mg/L), spectinomycin (16–256 mg/L), ciprofloxacin (0.015–4 mg/L), nalidixic acid (4–64 mg/L) by use of Sensititre (Trek Diagnostic Systems Ltd., West Sussex, UK), and following Clinical and Laboratory Standards Institute guidelines as previously described (CLSI, 2008). Resistance was determined by use of European Committee on Antimicrobial Susceptibility Testing epidemiological cutoff values (EUCAST, 2012). The *E. coli* strain ATCC 25922 was used for quality control.

**Pulsed-field gel electrophoresis (PFGE), phylogrouping, and multilocus sequence typing (MLST) typing**

All isolates positive for *bla*CMY-2 (Table 1) were subtyped by use of PFGE with some modifications to the method described by Brolund et al. (Brolund et al., 2010). In brief, the DNA was digested with *XbaI* at 37°C. The electrophoresis was performed with a CHEF DR III System (Bio-Rad Laboratories, Hercules, CA) using 1% SeaKem Gold (Lonza, Rockland, ME) agarose in 0.5× Tris-borate-EDTA. Running conditions were 6 V/cm and included angle 120°C in 14°C Tris-borate-EDTA buffer, with pulse times linearly increased from 12 s initial switch time to 40 s final switch time for 20 h.
XbaI digested DNA from Salmonella Braenderup H9812 was included as normalization standard on every gel.

PFGE analysis was performed using BioNumerics v. 4.61 (www.applied-maths.com) with the following settings: Dice band analysis, unweighted-pair group method with arithmetic mean dendrogram, optimization: 0.00%, position tolerance: 1.10%. The isolates were grouped into different groups if the compared PFGE patterns had less than 80% similarity.

Phylogrouping was performed on all blaCMY-2-positive isolates as previously described (Clermont et al., 2000). MLST was performed on selected isolates by use of whole genome sequencing and the web-server MLSTfinder (www.genomicepidemiology.org) as previously described (Larsen et al., 2012).

Characterization of plasmid replicons and horizontal gene transfer

Selected isolates carrying blaCMY-2 were used as donors in filter-mating experiments to the recipient E. coli 1005RN, rifR, nalR (Table 2) as previously described (Agersø and Sandvang, 2005). Fifty microliters of the mating suspension was spread on one half of a Brain Heart Infusion agar supplemented with 1 mg/L ceftaxime and 100 mg/mL of rifampicin, and on the other half the suspension was further spread with a loop to ensure single colonies of presumptive transconjugants. Suspected transconjugants were subcultured and checked for resistance to nalidixic acid. The presence of blaCMY-2 in the transconjugants was verified by PCR.

PCR-based replicon typing was used to characterize plasmids in the isolates chosen for mating experiments (Carattoli et al., 2005). If no transconjugants were obtained, or if more than one replicon was found in a transconjugant, plasmids were purified and electroporated into E. coli 1005RN. Transformants were subjected to S1 nuclease PFGE to ensure the presence of a single plasmid as previously described (Bielak et al., 2011).

Consumption of aminopenicillin in the Danish broiler flocks

Data on consumption of aminopenicillins on the farm level was obtained from the VetStat database as previously described (Agersø et al., 2012). Information on date of sale,
amount of drug prescribed, animal species, and code for farm identity (CHR number) was extracted from VetStat on November 7, 2012. The consumption of aminopenicillins in the broiler farms was defined as the consumption registered in the VetStat database for use in poultry on the given CHR number. The consumption was analyzed in two ways: (1) consumption of aminopenicillins at the farm at least once within the preceding 3 months prior to sampling, and (2) consumption of aminopenicillins at least once within the preceding 6 months prior to sampling.

Statistical analysis

Statistical significance tests of difference between proportions of samples positive for ESC-producing \textit{E. coli} with or without use of aminopenicillins were calculated using chi-square, or Fisher exact test (two-tailed) when the number of positive samples was low (<5) (StatCalc in EpiInfo™ v. 6, Centers for Disease Control and Prevention, www.cdc.org). Estimation of exact 95% (two-sided) confidence intervals for proportions was based on binomial probability distributions as previously described (Armitage and Berry, 2001).

<table>
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<th>Donor ID</th>
<th>Origin</th>
<th>MLST type</th>
<th>PFGE type</th>
<th>Replicon type</th>
<th>Non-β-lactam resistance</th>
<th>Phylotype</th>
<th>Transfer (yes/no) of \textit{bla}_{CMY-2} to \textit{E. coli}</th>
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<td>D2</td>
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PFGE, pulsed-field gel electrophoresis; NT, nontypeable; ND, not determined; NAL; nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline.

Results and Discussion

The Danish conventional broiler production is almost exclusively based on import of day-old parent animals from Sweden. In Sweden, day-old grandparent animals are imported from Scotland (P. Johanssen, DanHatch Ltd., personal communication). In Sweden, batches of imported day-old grandparent animals were found positive for ESC-producing \textit{E. coli}, and the genotypes found were \textit{bla}_{CMY-2} and \textit{bla}_{CTX-M-1} (SVARM 2010, 2011).

In our study, cephalosporinase-producing \textit{E. coli} were isolated from 93% (27/29) of the parent farms, from 27% (53/197) of the broiler flocks, and from 3.3% (4/121) and 8.6% (16/187) of Danish broiler meat sampled in 2009 and in 2010, respectively. The use of selective enrichment with ceftriaxone revealed ESC-producing \textit{E. coli} in all the tested sample types. The method did not reveal the concentration of ESC-producing \textit{E. coli} in the samples, and the infective concentration to humans is also unknown. The high prevalence of ESC-producing \textit{E. coli} found in parent and broiler flocks was surprising, since cephalosporins have not been used in either the Danish or the Swedish broiler production, and in general the consumption of antimicrobials is low compared to other
production animals (DANMAP 2010, 2011; SVARM 2010, 2011). In January 2012, the British Poultry Association agreed to stop using cephalosporins in broiler production, but until then cephalosporins had been used in the United Kingdom (World Poultry, 2011; British Poultry Council, 2013). Therefore, the use of cephalosporins in the United Kingdom before 2012 may have selected for the ESC-producing *E. coli* occurring in the animals exported into Sweden and spread vertically from grandparents to parents imported into Denmark, but other (e.g., environmental) sources may also exist.

The most prevalent gene found to confer cephalosporinase-producing *E. coli* in our study was *bla*<sub>CMY-2</sub>. This gene was found in all ESC-producing *E. coli* from parent flocks, in 89% (47/53) from broiler flocks, and in 50% (2/4) and 75% (12/16) from broiler meat in 2009 and 2010, respectively. Other genes were found in six isolates from broiler flocks (*bla*<sub>SHV-2</sub>) and six isolates from broiler meat (*bla*<sub>CTX-M-1</sub>), but not in parent flocks. Either they are present at a low level in the parent flocks or other routes of transmission such as cross-contamination or the outer environment exist. In other countries, large proportions of ESC-producing *E. coli* from broiler and broiler meat carry *bla*<sub>CMY-2</sub> are found, and due to the wide distribution to many countries of animals from the top of the breeding pyramid, it is suspected that the occurrence of cephalosporinase-producing *E. coli* may be due to the continuous introduction of imported animals carrying these bacteria (MARAN-2009, 2010; EFSA BIOHAZ, 2011; SVARM 2010, 2011). In Sweden, the same *E. coli* clones with *bla*<sub>CMY-2</sub> were found in imported grandparent animals and in all levels of the Swedish broiler production, indicating spread (Nilsson et al., 2014).

PFGE was performed on all *bla*<sub>CMY-2</sub>-positive isolates in order to reveal whether the *bla*<sub>CMY-2</sub> isolates from parent flocks (29), broiler flocks (47), and broiler meat (14) were clonally related. Based on the PFGE patterns, the isolates grouped into 35 different PFGE groups with less than 80% similarity (Table 1, Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/fpd). One isolate from 29 of 35 groups was further MLST typed. Eleven groups consisted of 2 to 5 isolates with more than 80% similarity, and also subgroups with identical PFGE patterns were found within 4 of these groups. One group (PFGE type 21, Table 1, Supplementary Fig. S1) was interesting because this group contained 39 of 90 *bla*<sub>CMY-2</sub>-positive isolates and these originated from parent flocks, broiler flocks, and broiler meat. Moreover, seven subgroups with 100% similar PFGE patterns between at least 2 isolates were found, and 1 of the subgroups contained isolates from parent flocks, broiler flocks, and meat (Table 1, Supplementary Fig. S1). This suggests that *bla*<sub>CMY-2</sub> spreads both horizontally and clonally in the production chain, and that some clones are more common than others. Therefore, ESC-producing *E. coli* originating from the animals may potentially reach the consumer as described for ESC-producing *Salmonella* Heidelberg in Canada (Dutil et al., 2010). Similar clones and plasmids were also found in broilers, broiler meat, and patients in the Netherlands (Leverstein-van Hall et al., 2011; Kluytmans et al., 2013).

Phylogrouping of the *bla*<sub>CMY-2</sub>-positive isolates showed phylotypes previously associated with disease in humans such as urinary tract infections (D and B2) and followed the PFGE type except for a few nontypeable isolates (Table 1) (Jakobsen et al., 2010a, b; Johnson et al., 2005). In the Netherlands, similar ESC-producing *E. coli* in poultry meat and from human infections has been described (Leverstein-van Hall, 2011; Kluytmans et al., 2013). So it is likely that some ESC-producing *E. coli* clones from the Danish broiler meat production cause infection in humans and should be further investigated. *E. coli* blood infections are mandatory for reporting in Denmark and may be MLST typed. *E. coli* MLST types formerly involved in human infection were found, including ST131, a global-spread human clone; ST48, recently found with *bla*<sub>CMY-2</sub> causing human clinical infection in Denmark; ST88, causing human infection; ST10, suspected to cause foodborne human infections; and the most dominant clone (43% of the isolates) ST38, also a type found in clinical *E. coli* (Table 2) (Guillouzouic et al., 2009; Jørgensen et al., 2010; Poirel et al., 2011; Manges and Johnson, 2012). MLST types uncommon in human infections were also found, but these could be involved in horizontal spread of *bla*<sub>CMY-2</sub> to human pathogens.

**FIG. 1.** Occurrence of extended-spectrum cephalosporinase-producing *Escherichia coli* in broiler flock with and without registered use of aminopenicillins on farm level up to 3 and 6 months prior to slaughter. The numbers on the y-axis represent the percentage of samples positive for ESC-producing *E. coli.*
Twenty-eight isolates representing 27 different PFGE types were used as donors in mating experiments, and 25 could transfer blaCMY-2 to an E. coli recipient (Table 2). Moreover, blaCMY-2 was found to be located on two types of plasmids: IncI (20) and IncK (8), so the presence of different clones can be explained by horizontal transfer of such plasmids, but may also be due to other introduction routes (e.g., from the outer environment, and contact to humans, insects, or animals). A study from Sweden found E. coli from broilers carrying blaCMY-2 on IncK plasmids, and E. coli isolates with blaCMY-2 carried by IncI or IncK plasmids have previously been found in humans, meat, and production animals (Börjesson et al., 2013). Moreover, blaCMY-2 has also been found to be associated with other plasmids such as IncI2 and IncA/C (Verdet et al., 2009; Antunes et al., 2012; Börjesson et al., 2013).

Susceptibility testing of the blaCMY-2 positive isolates showed most isolates being pansusceptible to all other non-β-lactam antimicrobials tested (Table 2). However, 11% (10/90) of the isolates were resistant to tetracyclines, the second most used antimicrobials in Danish broiler production. Single isolates were resistant to nalidixic acid, sulfamethoxazole, trimethoprim, and neomycin, respectively. As ESC-producing E. coli are resistant to aminopenicillins, we investigated whether ESC-producing E. coli more often originated from broiler farms that used aminopenicillins. One hundred eighty-eight of 197 flocks had information on farm origin. The flocks originated from 99 different farms. Seventeen and 29 broiler farms that used aminopenicillins. One hundred eighty-eight of 197 flocks had information on farm origin. The flocks originated from 99 different farms. Seventeen and 29 broiler farms that used aminopenicillins. Therefore, the focus should be on reducing both use of cephalosporins and aminopenicillins. Even though E. coli carrying blaCMY-2 often is polyclonal, some clones, including MLST types involved in human infections, seem to establish better than others. Therefore, factors important for persistence and spread of ESC-producing E. coli should be investigated.

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[CLSI] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk and Dilution Susceptibility


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Yvonne Agersø, PhD
Research Group of Bacterial Genomics and Antimicrobial Resistance
National Food Institute
Technical University of Denmark
Kemitorvet Building 204
Lyngby 2800, Denmark
E-mail: yvoa@food.dtu.dk