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Clonal spread of *Escherichia coli* resistant to cephalosporins and quinolones in the Nordic broiler production

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ABSTRACT

The intestinal flora of healthy broilers can contain *Escherichia coli* resistant to extended spectrum cephalosporins (ESC) and fluoroquinolones (FQ), representing a possible public health problem. We investigated the clonal epidemiology of *E. coli* with reduced susceptibility to ESC or FQ in broilers in three Nordic countries interconnected by a common source of breeding animals. Isolates (n = 319 and n = 132 non-wild type for ESC and FQ, respectively) from Norwegian, Swedish and Icelandic production originated mainly from the intestinal flora of broilers at the age of 20–35 days. Genetic relationships were investigated by ten loci multilocus variable number tandem repeat analyses (MLVA) and representative isolates of inter-Nordic clusters were subjected to multilocus sequence typing (MLST). Antimicrobial susceptibility data based on minimum inhibitory concentrations was compiled. Approximately one third of the ESC non-wild type isolates, including isolates from all three countries, clustered together. These isolates belonged to sequence type (ST) 38 and contained bla_{CMY-2}. The FQ non-wild type isolates were more genetically diverse, but related isolates occurred in more than one country. MLST typing showed clusters belonging to ST10, ST355, ST349, ST665 and ST93. Our study demonstrated inter-Nordic distribution of *E. coli* ST38 with bla_{CMY-2}, suggesting clonal proliferation as a contributing factor for spread of ESC resistance in the broiler production. The international trade in breeding material may explain introduction of resistant *E. coli*. The reason for their success and the success of certain clonal lineages in broiler production not exposed to antimicrobial selection pressure is currently unknown.

1. Introduction

The intestinal flora of healthy broilers can contain *Escherichia coli* resistant to antimicrobials categorized by the World Health Organization as critically important in human medicine, such as extended spectrum cephalosporins (ESC) and fluoroquinolones (FQ). Resistance to ESC via production of extended spectrum beta-lactamases (ESBLs) or plasmid mediated AmpC (pAmpC), has been reported in *Enterobacteriaceae* from poultry for more than a decade. Today, ESC resistant *E. coli* in poultry is globally dispersed (Ewers et al., 2012). Also, high prevalence of FQ resistance among intestinal *E. coli* from healthy broilers is common in most European countries (EFSA/ECDC, 2016). However, the situation among production animals in the three Nordic countries; Sweden, Norway and Iceland is favourable (NORM/NORM-VET, 2015; Swedres/Svarm, 2015; Thorsteinsdottir, 2009). In addition, the production of broilers in these countries is almost free from selection pressure caused by antimicrobial use (Borjesson et al., 2015; Mo et al., 2014). More importantly, cephalosporins have never been used in treatment of broilers (Mo et al., 2016a; Mo et al., 2014; Nilsson et al., 2014). Despite this, it is documented that a large proportion of intestinal *E. coli* from healthy broilers can have reduced susceptibility to ESC and FQ (MAST, 2015; NORM/NORM-VET, 2011,2014; Swedres/Svarm, 2011,2015; Thorsteinsdottir, 2009).

Marked geographical differences concerning the epidemiology of ESC resistance-encoding genes from broilers have been reported. Several countries report a multitude of genes to be involved (Ewers et al., 2012). However, in Sweden and Norway the bla_{CMY-2} gene encoding pAmpC has dominated until recently (Borjesson et al., 2013a; Mo et al., 2014). The genetic background for ESC resistance in Icelandic broiler production is so far unknown. Genes encoding ESC resistance from broilers are commonly located on self-transmissible plasmids (Agerso et al., 2014; Borjesson et al., 2013a; Mo et al., 2016b; Seiffert

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et al., 2017). These plasmids may be promiscuous and are able to disseminate into a broad range of hosts. Previous studies from Norway and Sweden have shown that bla_{CMY-2} in broiler isolates commonly resided on plasmids belonging to the incompatibility group IncK or IncI (Borjesson et al., 2013b; Mo et al., 2016b).

The genetic background for FQ resistance in *E. coli* from poultry in the three countries has not been extensively investigated. However, resistance profiles for nalidixic acid and ciprofloxacin indicate mutations in the chromosomally encoded *gyrA* and/or *parC* genes to be the main mechanism. In addition, such mutations are reported from recently performed smaller studies in Sweden and Norway. Here, a nucleotide alteration in *gyrA* was documented, yielding S83L amino acid substitution in quinolone resistant *E. coli* isolates from broilers (Borjesson et al., 2015; Slettemeas et al., 2017). As the main mechanism appears to be mutation in chromosomally located genes there is reason to believe that clonal spread may be important for dissemination of FQ resistance in *E. coli* among broilers.

Broiler production in the Nordic countries depends on import of breeding animals. This may have contributed to the introduction and subsequent spread of resistant bacteria (Borjesson et al., 2015; Mo et al., 2014; Nilsson et al., 2014). Moreover, the broiler production in Sweden, Norway and Iceland use the same lines of breeding stock, which implies the possibility of clonal spread between the three countries. If resistant bacteria are introduced to the three countries via imported birds, more knowledge is needed to understand why and how such bacteria can spread and persist in the bacterial populations and downwards in the production pyramid in the absence of antimicrobial selection pressure.

The low antimicrobial use indicates that factors other than selective pressure must have impact on the success of the resistant bacteria. Theoretically, a combination of bacterial fitness and antimicrobial resistance could lead to dissemination of successful clones contributing to emergence and persistence of resistance in *Enterobacteriaceae*. To identify successful clones and investigate their fitness properties, potential for transmission and colonization as well as their ability to persist and survive would give further insight into the epidemiology of these resistance traits.

The aim of this study was to investigate the molecular relationship of *E. coli* with reduced susceptibility to ESC or FQ in the broiler production in three Nordic countries using the same breeding stock and furthermore to identify possible successful clones with a wide geographical distribution.

2. Material and methods

2.1. Bacterial isolates

A total of 451 *E. coli* isolates (319 with reduced susceptibility to ESC and 132 with reduced susceptibility to FQ) from broiler production in Sweden, Norway and Iceland obtained during 2011–2014 were included. A minor proportion of the isolates (n = 63) originated from broiler meat (domestic production) whereas the remaining were from the intestinal flora of healthy animals. Isolates from Sweden and Norway were originally included in the national monitoring programmes for antimicrobial resistance in the two countries (Svarm and NORM-VET), and minimum inhibitory concentrations (MICs) to antimicrobial agents were known. Isolates from Iceland were collected in a recent screening study (MAST, 2015). In all three countries healthy animals were sampled at 20–35 days of age. An overview of the sampling and isolation procedures is briefly given below.

Norway: A total of 108 isolates exhibiting MICs above the epidemiological cut-offs (ECOFFs) for ESC defined by EUCAST (non-wild type) from 2011 were included. The isolates originated from boot swabs, were collected throughout the year and were isolated using a selective method (direct plating on MacConkey agar plates with 1 mg/L cefotaxime and plates with 2 mg/L ceftazidime). From each positive flock one *E. coli* was isolated and subjected to further investigations. All isolates exhibited AmpC phenotype and contained a gene belonging to the CIT group demonstrated by PCR (Perez-Perez and Hanson, 2002). Sequencing of amplicons from all isolates showed the presence of bla_{CMY-2} (NORM/NORM-VET, 2012). The 108 isolates represent all bla_{CMY-2} positive isolates from broilers in the NORM-VET 2011 programme.

A total of 50 isolates from 2014 exhibiting reduced susceptibility to FQ were included (all with ciprofloxacin MICs ≥0.12 mg/L, defined as ECOFF by EUCAST). The isolates originated from caecal samples and were collected throughout the year (one pooled sample per flock). Isolates were collected using both a selective, (n = 43) and a non-selective method (n = 7) (NORM/NORM-VET, 2015). The selective method included plating out on MacConkey agar plates containing 0.125 mg/L ciprofloxacin. Isolates appearing on the plates were confirmed as *E. coli* and susceptibility tested. In the non-selective screening one *E. coli* from each sample was randomly selected after growth on a non-selective agar plate and subjected to susceptibility testing. Isolates with MICs for ciprofloxacin ≥0.12 mg/L (n = 7), were included in this study. One *E. coli* isolate per caecal sample was included. In a previous study, the *gyrA* sequence of 29 of the isolates was determined (Slettemeas et al., 2017). In 28 isolates a GyrA S83L amino acid alteration was identified, the remaining isolate contained a D87N alteration. The *gyrA* sequence of the 21 remaining Norwegian FQ non-wild type isolates were determined in this study using a previously described method (Oram and Fisher, 1991).

Sweden: A total of 204 ESC non-wild type isolates collected in the period 2011–2014 were included; 165 from caecal content and 39 from Swedish produced meat (Swedres/Svarm, 2015). A selective method (direct plating on MacConkey agar with 1 mg/L cefotaxime) was used to collect the isolates (one isolate per flock/meat sample). All selected isolates contained a gene belonging to the CIT group (i.e. pAmpC) as demonstrated by PCR (Perez-Perez and Hanson, 2002). All amplicons were not sequenced, however, historically such isolates have always carried the bla_{CMY-2} gene as demonstrated by earlier studies (Borjesson et al., 2013b; Nilsson et al., 2014; Swedres/Svarm, 2015). To confirm the presence of bla_{CMY-2} a subset of isolates was in this study subjected to PCR and sequencing using a previously described method (Borjesson et al., 2013a). Isolates representing multilocus variable number tandem repeat analysis (MLVA) clusters with more than two Swedish isolates were investigated (Fig. 1). A few isolates containing genes encoding ESBLs were detected in the Svarm monitoring programme during these years, however these isolates (n = 11) were not included in this study.

A total of 52 isolates exhibiting reduced susceptibility to FQ obtained during 2012–2014 were included (49 from caecal content and 3 from Swedish produced meat). One *E. coli* per sample was randomly selected and subjected to susceptibility testing. Isolates with MICs for ciprofloxacin ≥0.12 mg/L were included in this study. A subset of isolates, belonging to the major clusters as demonstrated in Fig. 2 (one isolate pr cluster), were investigated for *gyrA* mutations using a previously described method (Oram and Fisher, 1991).

Iceland: A total of 7 and 30 ESC and FQ non-wild type isolates respectively were included. Isolates with reduced susceptibility to ESC originated from a screening programme for antimicrobial resistant *E. coli* in broilers and domestically produced broiler meat conducted for The Icelandic Food and Veterinary Authority in 2014 (MAST, 2015). Caecal and meat samples were screened selectively using the same methods as in the NORM-VET programme described above (NORM/NORM-VET, 2012). The ESC non-wild type *E. coli* isolates (3 from caecal samples and 4 from meat) had a beta-lactam resistance profile with an AmpC phenotype and were subjected to PCR and sequencing for confirmation of bla_{CMY-2} using a previously described method (Borjesson et al., 2013a). The same material was screened selectively for *E. coli* with reduced susceptibility to FQ by plating out on two MacConkey agar plates containing 0.125 mg/L and 0.250 mg/L ciprofloxacin. A total of 30 isolates, 13 from caecal samples and 17 from