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Introduction of quinolone resistant *Escherichia coli* to Swedish broiler population by imported breeding animals

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ABSTRACT

During recent years a rapid increase of quinolone resistant *Escherichia coli* have been noted in the Swedish broiler population, despite the lack of a known selective pressure. The current study wanted to investigate if imported breeding birds could be a source for the quinolone resistant *E. coli*. The occurrence of quinolone resistant *E. coli* was investigated, using selective cultivation with nalidixic acid, in grandparent birds on arrival to Sweden and their progeny. In addition, sampling in hatcheries and empty cleaned poultry houses was performed. Clonality of isolates was investigated using a 10-loci multiple-locus variable number tandem repeat analysis (MLVA). To identify the genetic basis for the resistance isolates were also analysed for occurrence of plasmid-mediated quinolone resistance (PMQR) determinants and characterization of chromosomal mutations. *E. coli* resistant to nalidixic acid occurred in grandparent birds imported to Sweden for breeding purposes. Four predominant MLVA types were identified in isolates from grandparent birds, parent birds and broilers. However, resistant *E. coli* with identical MLVA patterns were also present in hatcheries and poultry houses suggesting that the environment plays a role in the occurrence. Nalidixic acid resistance was due to a mutation in the gyrA gene and no PMQR could be identified. The occurrence of identical clones in all levels of the production pyramid points to that quinolone resistant *E. coli* can be introduced through imported breeding birds and spread by vertical transmission to all levels of the broiler production pyramid.

1. Introduction

Quinolones are potent broad-spectrum antibiotics effective against both gram-positive and gram-negative bacteria. Due to their efficacy they are one of the most widely used antibiotic classes for treatment of bacterial infections in humans and have been deemed critically important for human health by the WHO (Kim and Hooper, 2014; Redgrave et al., 2014; WHO, 2007). The rapid emergence of fluoroquinolone and quinolone resistant bacteria in human clinical setting is therefore worrisome (Kim and Hooper, 2014; Pena et al., 1995; Zemkova et al., 2007). A direct correlation between the frequent use of fluoroquinolones and quinolones in humans and the development, occurrence and transmission of resistant bacterial strains in human healthcare has been described (Kim and Hooper, 2014; Pena et al., 1995; Zemkova et al., 2007).

Quinolones and fluoroquinolones are also used to animals, including farm animals, but in the European Union there is a large variation between countries in the amounts used (EMA, 2014). A similar association between use of fluoroquinolones and occurrence of resistant bacteria has also been observed in veterinary medicine (Garcia-Migura et al., 2014). Increased occurrence of quinolone resistance in bacteria from food animals is of concern from a zoonotic perspective (EFSA, 2008).

In Sweden, the occurrence of nalidixic acid and ciprofloxacin resistance among *Escherichia coli* from healthy broilers has previously been steady around 5% (SVARM, 2015). However, from 2007 to 2010 a rapid increase to 15% was noted and the occurrence has since then remained on these levels. This increase is puzzling as quinolones are generally not used in Sweden to broilers (Personal communication, the Swedish Poultry Meat Association

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(Svensk fågel)). Furthermore, since 2013 veterinarians in Sweden are generally only authorised to subscribe quinolones if susceptibility testing determine that no other antimicrobial will be effective (Swedish Board of Agriculture, 2013). Moreover, the overall use of antibiotics to broilers is limited and in 2014 only 4 of 3138 (0.1%) flocks were treated with antibiotics (SVARM, 2015). A potential source for the nalidixic acid and ciprofloxacin resistant *E. coli* might be breeding stock imported from the United Kingdom (UK) to Sweden, as previous studies in Denmark has suggested that vertical transmission of fluoroquinolone resistant *E. coli* can occur in the broiler production (Petersen et al., 2006). Import of breeding animals to the Swedish broiler production are dominated by two companies, and at the time of the current study breeding stock had only with a few exceptions been imported from UK. Furthermore, our group has previously described the potential of vertical transmission of extended-spectrum cephalosporin resistant *E. coli* from imported breeding stock through the Swedish broiler production pyramid (Nilsson et al., 2014).

The aim of this study was to identify if *E. coli* resistant to nalidixic acid could be detected from breeding stock imported from the United Kingdom at arrival in Sweden and if these *E. coli* can subsequently be disseminated by vertical transmission to broiler level. The occurrence of nalidixic acid resistant *E. coli* in hatcheries and broiler houses was also investigated. Furthermore, we wanted to determine the genetic basis for the quinolone resistance phenotype among isolates.

2. Material and methods

2.1. Sampling and identification of breeding animals positive for nalidixic acid resistant E. coli

In August 2011 paper linings from boxes in which grandparent (GP) animals arrived were collected on arrival to Sweden and screened for nalidixic acid resistant *E. coli*. When a shipment containing nalidixic acid resistant *E. coli* was identified, these animals and their progeny were sampled longitudinally as previously described (Nilsson et al., 2014), with the exception of inclusion of samples collected from parent (P) animals just before slaughter. Samples from GP- and P-animals as well as samples from broilers were taken at different ages and environmental samples were taken from empty houses before placement of birds as well as from the sorting bands at hatcheries by the end of the working day. Briefly, sampling of birds was performed using boot swabs ('Sterisocks humid'; Sodibox®, Nevez, France) and environmental samples were collected using sterile cloths (Sodibox®) that had been factory pre-impregnated with buffered peptone solution with 10% neutralizing agent.

2.2. Cultivation

Samples were treated by stomaching (Stomacher 400 circulator, Seward, UK) in saline for 1 min with 230 revolutions. Then, 0.1 mL was streaked on MacConkey agar (Difco, Hampshire, UK) with nalidixic acid (32 mg/L; Sigma–Aldrich, China) and incubated overnight at 37 °C. Furthermore, 10 mL was mixed 1:1 with 2× MacConkey broth (Lab M, Lancashire, UK) with nalidixic acid (64 mg/L) and incubated at 37 °C overnight before 0.1 mL was streaked on MacConkey agar with nalidixic acid and incubated as above. Colonies with morphology typical of *E. coli* were subcultured on horse blood agar (Oxoid, Basingstoke, UK) and indole tested. From the samples taken upon arrival of the traded grandparent animals, 1 isolate of quinolone resistant *E. coli* was collected per sample and from all the other samplings ≤10 isolates from each branch (fathers' fathers, fathers' mothers etc.) of the production pyramid were selected.

2.3. Genetic characterisation and antimicrobial susceptibility testing of isolates

To identify the genetic basis for fluoroquinolone and quinolone resistance a selection of isolates were analysed for occurrence of plasmid-mediated quinolone resistance (PMQR) determinants and characterization of mutations in the quinolone resistance determining-region (QRDR) of *gyrA* and *parC* genes.

DNA extraction was performed using Nuclisens Easymag (bioMérieux®). The *qnrA*,*qnrB*, *qnrC*, *qnrD* and *qnrS* genes were detected using multiplex real-time PCR performed on LightCycler 480 (Roche Diagnostics®) (Guillard et al., 2011, 2012). *qepA* genes detection was performed using simplex real-time PCR on LightCycler 480. *oqxAB* genes were detected using a two-step PCR-based strategy, allowing a specific assignment of *Tn*6010-associated *oqxAB*. Screening of *oqxAB* was performed by detection of *oqxB* using simplex real-time PCR on LightCycler 480 (Guillard et al., 2015). *aac(6′)-Ib*and*aac(6′)-Ib-cr* genes detection was performed on PSQ96 MA (Qiagen®) using pyrosequencing (Guillard et al., 2010).

QRDR mutation characterization, in hot spots, was performed using a multiplex pyrosequencing-based approach recently patented (patent FR1353627, PCT/FR2014/050939), which allow detection of mutation in QRDR for *Enterobacteriaceae* isolates. Briefly, after conventional PCR assays realized with the MyCycler thermocycler (Bio-Rad, Marnes-la-Coquette, France), pyrosequencing was performed using the PSQTM 96MA system (Qiagen, Courtaboeuf, France).

The relatedness of resistant isolates was investigated using multiple-locus variable number tandem repeat analysis (MLVA), as previously described (Lobersli et al., 2012).

Antimicrobial susceptibility was assessed using the VetMIC GN-mo plates (SVA, Uppsala, Sweden), *E. coli* ATCC 25,922 was used for quality control and MICs were interpreted with EUCAST epidemiological cut-off values.

3. Results

3.1. Cultivation from flocks, hatcheries and broiler houses

The first import of GP-animals screened in august 2011 contained *E. coli* resistant to nalidixic acid, so no additional imports were screened. In the following longitudinal sampling of the GP-animals, nalidixic acid resistant *E. coli* were identified on every occasion (Fig 1). Samples taken from the progeny of the GP-animals (P-flocks and broiler flocks) also contained nalidixic acid resistant *E. coli* on every sampling occasion (Fig 1).

Nalidixic acid resistant *E. coli* were identified in the empty houses for the GP-animals, showing that such bacteria occurred in the environment before the placing of the GP-animals (Fig 1). However, resistant *E. coli* could not be identified in the second empty GP-animal house where the GP-animals were moved at 18 weeks of age. In the empty P-house resistant *E. coli* could not be identified, but occurred in the empty house where P-animals were moved at 20 weeks of age. In the empty broiler houses, nalidixic acid resistant *E. coli* were identified in one out of five houses. In parent and broiler hatcheries, nalidixic acid resistant *E. coli* was also present in the environment (Fig 1).

3.2. Genetic characterization of isolates

A total of 95 of the collected isolates ($n = 273$) were subjected to MLVA and among these a total of 13 MLVA-profiles were identified (Table 1). The majority of isolates (75 out 95 isolates) belonged to one out of four MLVA-patterns, defined as clone 1 ($n = 10$), 2 ($n = 15$), 3 ($n = 16$) and 4 ($n = 34$). These four clones could all be