High genetic diversity among extraintestinal *Escherichia coli* isolates in pullets and layers revealed by a longitudinal study

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Abstract

**Background:** Various information about the genetic diversity of *Escherichia coli* isolates from chickens are available but a detailed epidemiological investigation based upon isolates obtained from interrelated pullet and layer flocks is still missing. Therefore, in the course of a longitudinal epidemiological study on pullets and layers, 144 *E. coli* isolates from chickens with or without pathological lesions of the reproductive tract were serotyped and genotyped with pulsed-field gel electrophoresis (PFGE). These isolates were collected during rearing, peak and at the end of production. The actual study is the first of its kind so as to elucidate genetic relatedness among extraintestinal *E. coli* isolated from chickens with varying pathological conditions in interrelated layer farms/flocks at different stages of rearing.

**Results:** Serotyping revealed that 63.19 % of the isolates could not be assigned to any of the three serotypes tested whereas 30.55 % of the isolates belonged to serotype O1:K1, 4.86 % to O2:K1 and 1.38 % to O78:K80. After macrorestriction digest with *XbaI*, 91.66 % of the isolates were typeable resulting in 96 distinct PFGE profiles. Among them, five PFGE types included isolates collected from diseased chickens as well as from birds without pathological lesions. This finding shows that pathogenicity of *E. coli* in layers seems to be largely influenced by concurrent susceptibility factors. Furthermore, in six out of eight cases where two isolates were collected from each of eight birds, different PFGE types were found in the same or different organs of the same bird. The existence of predominant or persistent *E. coli* genotypes was only observed in two cases.

**Conclusions:** It is concluded that extraintestinal *E. coli* genotypes and serotypes in pullets and layers are heterogenous and also do not maintain a single clonality within the same bird. The facts that *E. coli* strains did not show any definite clonal population structure based on geographical region, age of the host and pathological lesions should have relevance in further epidemiological studies and control strategies.

**Keywords:** *Escherichia coli*, Longitudinal epidemiology, Pullets, Layers, Pulsed-field gel electrophoresis
Background

*Escherichia coli* isolates that are extraintestinal in nature are associated with the disease named colibacillosis that can infect all aged groups of chickens [1]. In layers, the pathogen is able to cause a systemic infection leading to fibrinous polyserositis, pericarditis, perirepitis, salpingitis, peritonitis, salpingoperitonitis and a decrease in egg production ultimately leading to severe economic losses [2–8]. Despite serological diversities, serogroups such as O1, O2 and O78 are mostly implicated in disease conditions [9–11]. Until now, the pathogenicity of *E. coli* infection in chickens is not well understood. Several putative virulence and virulence-associated genes have been reported in avian pathogenic *E. coli* (APEC) [1, 11, 12]. However, the fact that a single genetic trait cannot separate disease-associated *E. coli* from commensal intestinal isolates raised certain concern on the definition of APEC as a single pathotype [13, 14].

From an epidemiological point of view, understanding the clonal population structure of extraintestinal *E. coli* involving a longitudinal sampling scheme in interrelated rearing and laying flocks has a high priority. Thus we performed a longitudinal study in order to characterize the relatedness among *E. coli* isolates from systemic organs of pullets and layers kept in alternative housing systems in Austria. Beside the determination of the serotype, pulsed-field gel electrophoresis (PFGE) was applied for genetic fingerprinting which has higher discriminating power compared to other methods such as multilocus sequence typing [15]. PFGE is more applicable to investigate large-scale genomic diversity within a distinct population and has also been previously applied to infer molecular relatedness among APEC isolates in other geographical locations [16–18].

Methods

Flock history, sampling and *E. coli* isolation

The present investigation was focused on extraintestinal *E. coli* isolates from pullets and laying hens kept in alternative husbandry systems that were located in different provincial states of Austria. Six rearing and eight related layer farms comprising 15 layer flocks were included in the longitudinal study. Rearing farms are designated with letter “R” along with farm numbers as R1 – RVI (e. g. R1 is rearing farm 1). The layer flocks are designated with letter “L” along with the flock number and the corresponding rearing farm (e. g. L1/I indicates for layer flock 1 that comes from rearing farm 1). Detailed information on farms and flocks is provided in Table 1.

In total, 188 birds were sampled for extraintestinal *E. coli* based on the sampling scheme as shown in Fig. 1. Sampling was performed during rearing (age of birds: 16–19 weeks), at the peak of production (age of birds: 37–42 weeks) and at the end of production (age of birds: 64–80 weeks). In each of the sampling events, five birds per rearing farm/layer flock were necropsied and sampled for extraintestinal *E. coli*. In two flocks of one layer farm (L2/IV and L3/IV), additional samplings were included.

Table 1 Farms and flocks included in the study

| Rearing farm | Layer farm/flock |
|--------------|------------------|
| Farm identification | Location | Layer farm/flock | Farm | Flock identification | Location | Housing system | Flock size |
| R1 | Lower Austria | 1 | L1/I | Styria | FR | 7500 |
| R2 | Salzburg | 2 | L1/I | Carinthia | ORG | 3000 |
| R3 | Styria | 3 | L2/I | Lower Austria | ORG | 3000 |
| R4 | | 4 | L2/I | Burgenland | ORG | 6000 |
| R5 | | 5 | L2/I | Burgenland | ORG | 6000 |
| R6 | | 6 | L3/I | Lower Austria | DL | 9030 |
| R7 | | 7 | L3/I | Lower Austria | DL | 10890 |
| R8 | | 8 | L3/I | Styria | DL | 14950 |

*aSix rearing farms are indicated as R1 - R8 (all birds were kept in deep litter system)*

*bLayer flocks are designated with letter “L” along with flock number/corresponding rearing farm number*

*cHousing system: FR – conventional free range, ORG – organic free range, DL – deep litter*

*dNumber of birds in each layer flock*
at 30–33 weeks of age (eight birds in total) because of increased mortality and drop in egg production. The sampling scheme was focused on the isolation of *E. coli* from the reproductive organs (ovary and oviduct). Where *E. coli* could not be isolated from the reproductive tract, isolates from liver, heart or lung were chosen for further investigation. For isolation of *E. coli*, organ samples were aseptically streaked on McConkey agar (Scharlau, Vienna, Austria) and incubated at 37 °C for 24 h aerobically. On the following day, subcultures were made on Columbia agar supplemented with 5 % sheep blood (COS agar, BioMérieux, Vienna, Austria) and incubated at 37 °C for 24 h aerobically. Most of the isolates were collected from ovary or oviduct (number of isolates *n* = 106) followed by liver (*n* = 25), lung (*n* = 10) and heart (*n* = 3). Details on *E. coli* isolates included in the present study are shown in Table 2. Isolates from rearing farms are marked with letter “R” along with farm number/corresponding rearing farm number – age of birds during sampling (weeks) – number of *E. coli* isolates in parenthesis. Isolates from layer flocks are labelled with letter “L” along with flock number/corresponding rearing farm number – age of birds during sampling (weeks) – number of *E. coli* isolates in parenthesis.

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**Fig. 1** Sampling scheme for the longitudinal study of extraintestinal *Escherichia coli* in pullets and layers. Each box represents an individual sampling event and includes the following information: farm/flock identification (rearing farms are indicated as R₁ - R₆ and layer flocks are designated with letter “L” along with flock number/corresponding rearing farm number) – age of birds during sampling (weeks) – number of *E. coli* isolates in parenthesis.
| Isolate identification | Age (weeks) | Reproductive lesions | Serotype | PFGE type |
|------------------------|-------------|----------------------|----------|-----------|
| R_{I1} RI-1            | 18          | no                   | nt       | LA23      |
| R_{I1} RI-2            | no          | nt                   |          |           |
| R_{I1} RI-3            | no          | nt                   |          | LA20      |
| R_{I1} RI-4            | no          | nt                   |          | LA19      |
| L_{I1A} L_{I1A}-1      | 42          | no                   | nt       | S31       |
| L_{I1A} L_{I1A}-2      | no          | nt                   |          | S37       |
| L_{I1A} L_{I1A}-3      | no          | nt                   |          | S37       |
| L_{I1A} L_{I1A}-4      | no          | nt                   |          | S6        |
| L_{I1A} L_{I1A}-5      | no          | nt                   |          | S6        |
| L_{I2A} L_{I2A}-1      | 42          | no                   | O1:K1    | S2        |
| L_{I2A} L_{I2A}-2      | no          | O2:K1,     |          | S15       |
| L_{I2A} L_{I2A}-3      | no          | O1:K1    |          | S15       |
| L_{I2A} L_{I2A}-4      | no          | O1:K1    |          | S15       |
| L_{I2A} L_{I2A}-5      | no          | O1:K1    |          | S15       |
| L_{I3A} L_{I3A}-1      | 77          | degeneration of oviduct | O1:K1, O2:K1, O78:K80 | Sa3 |
| L_{I3A} L_{I3A}-2      | no          | O1:K1, O2:K1, O78:K80 |          | Sa3       |
| L_{I3A} L_{I3A}-3      | no          | O1:K1, O2:K1, O78:K80 |          | Sa3       |
| L_{I3A} L_{I3A}-4      | no          | O1:K1, O2:K1, O78:K80 |          | Sa3       |
| L_{I3A} L_{I3A}-5      | no          | O1:K1, O2:K1, O78:K80 |          | Sa3       |
| L_{I3B} L_{I3B}-1      | 77          | oophoritis   | O2:K1    | S19       |
| L_{I3B} L_{I3B}-2      | oophoritis  | O2:K1    |          | S19       |
| L_{I3B} L_{I3B}-3      | oophoritis  | O2:K1    |          | S19       |
| L_{I3B} L_{I3B}-4      | oophoritis  | O2:K1    |          | S19       |
| L_{I3B} L_{I3B}-5      | oophoritis  | O2:K1    |          | S19       |
| R_{I2} R_{I2}-1        | 19          | no                   | O1:K1,O2:K1, O78:K80 | Sa3 |
| R_{I2} R_{I2}-2        | no          | O1:K1,O2:K1, O78:K80 |          | Sa3       |
| R_{I2} R_{I2}-3        | no          | O1:K1,O2:K1, O78:K80 |          | Sa3       |
| R_{I2} R_{I2}-4        | no          | O1:K1,O2:K1, O78:K80 |          | Sa3       |
| R_{I2} R_{I2}-5        | no          | O1:K1,O2:K1, O78:K80 |          | Sa3       |
| L_{I2B} L_{I2B}-1      | 40          | no                   | O1:K1    | nt        |
| L_{I2B} L_{I2B}-2      | no          | O1:K1    |          | nt        |
| L_{I2B} L_{I2B}-3      | no          | O1:K1    |          | nt        |
| L_{I2B} L_{I2B}-4      | no          | O1:K1    |          | nt        |
| L_{I2B} L_{I2B}-5      | no          | O1:K1    |          | nt        |
| L_{I2A} L_{I2A}-1      | 38          | degeneration of oviduct | O1:K1,O2:K1, | nt        |
| L_{I2A} L_{I2A}-2      | no          | O1:K1,O2:K1, |          | LA10      |
| L_{I2A} L_{I2A}-3      | no          | O1:K1,O2:K1, |          | LA10      |
| L_{I2A} L_{I2A}-4      | no          | O1:K1,O2:K1, |          | LA10      |
| L_{I2A} L_{I2A}-5      | no          | O1:K1,O2:K1, |          | LA10      |
| L_{I2B} L_{I2B}-1      | 80          | egg peritonitis       | nt       | Ca4       |
| L_{I2B} L_{I2B}-2      | egg peritonitis | nt       |          | Ca4       |
| L_{I2B} L_{I2B}-3      | egg peritonitis | nt       |          | Ca4       |
| L_{I2B} L_{I2B}-4      | egg peritonitis | nt       |          | Ca4       |
| L_{I2B} L_{I2B}-5      | egg peritonitis | nt       |          | Ca4       |
| R_{I3} R_{I3}-1        | 18          | no                   | O1:K1,O2:K1, | S4        |
| R_{I3} R_{I3}-2        | no          | O1:K1,O2:K1, |          | S4        |
| R_{I3} R_{I3}-3        | no          | O1:K1,O2:K1, |          | S4        |
| R_{I3} R_{I3}-4        | no          | O1:K1,O2:K1, |          | S4        |
| Rearing farm | L_{1/IV-A} | L_{1/IV-A-1} | 41 | no | O1:K1 | LA16 |
|--------------|-------------|---------------|----|----|-------|------|
|              | L_{1/IV-A-2} | no            |    |    |       |      |
|              | L_{1/IV-A-3} | no            |    |    |       |      |
|              | L_{1/IV-A-4} | no            |    |    |       |      |
|              | L_{1/IV-A-5} | no            |    |    |       |      |
|              | L_{2/IV-A}  | L_{2/IV-A-2}  | 41 | no | O1:K1 | LA13 |
|              |              | L_{2/IV-A-1}  |    |    |       |      |
|              | L_{3/IV-A}  | L_{3/IV-A-1}  | 41 | degeneration of ovary and oviduct | nt | LA21 |
|              |              | L_{3/IV-A-2}  |    |    |       |      |
|              |              | L_{3/IV-A-3}  |    |    |       |      |
|              |              | L_{3/IV-A-4}  |    |    |       |      |
|              |              | L_{3/IV-A-5}  |    |    |       |      |
|              | L_{4/IV-B}  | L_{4/IV-B-1}  | 73 | degeneration of ovary and oviduct | nt | LA25 |
|              |              | L_{4/IV-B-2}  |    | oophoritis | nt | LA1  |
| Rearing farm | L | isolate | 73 | | pathological findings | nt | nt |
| L2/V-B | L2/V-B-1-ovary | oophoritis | nt | | LA25 |
| L2/V-B-2-liver | oophoritis | O1:K1 | LA25 |
| L2/V-B-3 | oophoritis | O1:K1 | LA25 |
| L2/V-B-4 | oophoritis | O1:K1 | LA25 |
| L2/V-B-5 | oophoritis | nt | LA28 |
| L3/V-B | L3/V-B-1 | 73 | oophoritis | O1:K1 | LA3 |
| L3/V-B-2 | oophoritis | O1:K1 | LA14 |
| L3/V-B-3 | oophoritis | O1:K1 | LA25 |
| L3/V-B-4 | oophoritis | nt | LA8 |
| L3/V-B-5 | oophoritis | O1:K1 | LA25 |
| L2/V-Z1 | L2/V-Z1-1 | 31 | oophoritis | O1:K1,O2:K1,O78:K80 | LA22 |
| L2/V-Z1-2 | no | O1:K1,O2:K1,O78:K80 | LA22 |
| L2/V-Z1-3 | egg peritonitis | O1:K1,O2:K1,O78:K80 | LA22 |
| L3/V-Z1 | L3/V-Z1-2 | 30 | degeneration of ovary and oviduct | O1:K1,O2:K1 | LA24 |
| L3/V-Z2 | L3/V-Z2-1 | 33 | no | nt | LA12 |
| Rearing farm 6 | L1/V-A | L1/V-A-2 | 37 | no | O1:K1 | nt |
| L1/V-A-3-oviduct | no | O1:K1 | S7 |
| L1/V-A-4-oviduct | egg peritonitis | O1:K1 | S18 |
| L1/V-A-5-oviduct | egg peritonitis | S7 |
| L1/V-A-6 | egg peritonitis | O1:K1 | nt |
| L1/V-B | L1/V-B-1 | 64 | egg peritonitis | O2:K1 | S8 |
| L1/V-B-2 | egg peritonitis | O2:K1 | S9 |
| L1/V-B-3 | egg peritonitis | nt | S25 |
| L1/V-B-4 | egg peritonitis | O2:K1 | S9 |
| L2/V-A | L2/V-A-3 | 39 | degeneration of ovary | nt | S27 |
| L2/V-A-4 | degeneration of ovary | O78:K80 | S29 |
| L2/V-A-5 | no | O1:K1 | nt |
| L2/V-B | L2/V-B-1 | 74 | no | O1:K1 | S10 |
| L2/V-B-2 | no | O1:K1 | S10 |
| L2/V-B-3 | oophoritis and salpingitis | O1:K1 | S10 |
| L2/V-B-4 | oophoritis | nt | S20 |
| L2/V-B-5-ovary | egg peritonitis | O1:K1 | S34 |
| L2/V-B-5-heart | egg peritonitis | O1:K1 | S34 |
| Rearing farm 6 | L1/V-A | L1/V-A-1 | 38 | no | nt | S36 |
| L1/V-A-2 | no | nt | S21 |
| L1/V-A-4 | oophoritis | nt | S11 |
| L1/V-A-5 | no | nt | S21 |
reproductive tract comprised egg peritonitis in one bird, inflammation of ovary and/or oviduct in two birds and degeneration of ovary and oviduct in one bird.

**Subtyping of *E. coli* isolates**

Serotyping was performed on 144 *E. coli* isolates applying a slide agglutination test to *Escherichia coli* O1:K1, O2:K1 and O78:K80 antisera following supplier’s guidelines (Animal Health and Veterinary Laboratory Agency, Weybridge, Surrey, UK).

For PFGE, *E. coli* isolates were grown on COS agar at 37 °C for 24 h. The plug preparation and PFGE was performed according to the standardized PulseNet International protocol for *E. coli* O157:H7, *E. coli* non-O157, *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri* (http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL05_Ec-Sal-ShigPFGEprotocol.pdf; accessed on 18.12.2015). The macrorestriction digest was performed applying XbaI (500U/sample; Thermo Fisher Scientific, Fermentas; Waltham, Massachusetts, USA) at 37 °C for 2–3 h. Restricted samples were separated in a 1 % (w/v) SeaKem Gold agarose gel (Lonza Group AG, Basel, Switzerland) in 0.5 × TBE buffer at 6 V/cm on a Chef DR μL system (Bio-Rad Laboratories, Inc.). A linear ramping factor with pulse times from 2.2 to 54.2 s at 14 °C and an inclined angle of 120° was applied for 22.5 h. The gels were stained with ethidium bromide (Sigma Aldrich, Vienna, Austria), digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.) and normalized as TIFF images (BioNumerics 6.6 software Applied Math NV, Sint-Martens-Latem, Belgium) applying the PFGE global standard *Salmonella* ser. Braenderup H9812. In order to identify indistinguishable PFGE types, a Dice coefficient similarity of 100 % was used.

**E. coli confirmation of non-typeable genotypes**

Partial sequencing of 16S rRNA gene was done in PFGE non-typeable isolates (n = 12) as described previously [19]. For this purpose, strains were grown on COS agar plates at 37 °C for 24 h. DNA extraction was done from two to three colonies using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following manufacturer’s recommendation. PCR was performed with a set of primers: 16S F 5′-GGCGGCRKGCCTAAYACATGC-AAGT-3′ and 16S R 5′-GACGACARCCATGCACCTGT-3′. Amplification was carried out in 25 μl reaction volume consisting of 12.5 μl of HotStarTaq Master Mix (Qiagen, Hilden, Germany), 8 μl of nuclease free distilled water, 1 μl of each forward and reverse primers (10 pmol/μl) and 2.5 μl of DNA template. The PCR thermocycler was programmed as: initial denaturation at 95 °C for 15 min followed by 40 cycles of heat denaturation at 94 °C, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min. Final elongation was performed at 72 °C for 10 min. The PCR products were visualized by agarose gel electrophoresis. The gel slices were cut and purified using QIAquick* gel extraction kit (QIAGEN, Germany). Samples were then dispatched to LGC genomics GmbH (Berlin, Germany) for sequencing. The data obtained were processed with software Accelrys Gene v2.5 (Accelrys Inc) and analyzed with BLAST search in NCBI database.

### Table 2 *E. coli* isolates and pathological findings in reproductive tract (Continued)

| Isolates | Age | Lesions in reproductive tract | Serotypes | PFGE Types |
|----------|-----|--------------------------------|-----------|------------|
| L1/VI-B | 80  | degeneration of ovary and oviduct | O1:K1     | S33        |
| L1/VI-B-1-ovary | 80  | degeneration of ovary and oviduct | nt        | S12        |
| L1/VI-B-2 |     | degeneration of ovary and oviduct | O1:K1     | S33        |
| L1/VI-B-3 |     | oophoritis                      | O1:K1     | S26        |
| L1/VI-B-4 |     | degeneration of ovary and oviduct | nt        | S24        |
| L1/VI-B-5 |     | egg peritonitis                 | O1:K1     | S35        |
| L2/VI-A | 38  | no                             | nt        | S17        |
| L2/VI-A-1 |     | oophoritis                      | nt        | S13        |
| L2/VI-A-4 |     | oophoritis, degeneration of oviduct | nt        | S17        |
| L2/VI-B | 80  | oophoritis                      | O1:K1     | S30        |
| L2/VI-B-2-ovary | 80  | oophoritis                      | nt        | S28        |
| L2/VI-B-2-oviduct |     | oophoritis                      | O1:K1     | S30        |
| L2/VI-B-4 |     | degeneration of ovary           | nt        | S14        |
| L2/VI-B-5 |     | egg peritonitis                 | O1:K1     | S32        |
Antimicrobial resistance (AMR)

Sixteen *E. coli* isolates originating from eight birds (two isolates per bird from the same or different organs) were investigated for the potential difference in AMR among strains isolated from the same organ (2 birds) or from different organs of the same bird (6 birds). The antimicrobial susceptibility test was performed using the disk diffusion method on Mueller-Hinton Agar (BioMérieux, Vienna, Austria) according to Bauer et al. [20]. The following antimicrobials were tested: aminopenicilline (amoxicillin and ampicillin (each 10 μg)), aminoglycoside (gentamicin (10 μg), neomycin (30 μg)), tetracyclines (tetracycline and doxycycline (each 30 μg)), co-trimoxazole (sulphamethoxazole and trimethoprim (25 μg)), macrolide (tylosin 30 μg), quinolone (oxolinic acid 2 μg, enrofloxacin (5 μg)), cephalosporine (cefotiofur (30 μg)), polymyxin (colistin (10 μg)) and aminocyclitol (spectinomycin (100 μg)). Multidrug resistance (MDR) among avian *E. coli* was defined as resistance to three or more classes of antimicrobial agents.

Results

**Subtyping of *E. coli* isolates**

Serotyping revealed that 44 isolates (30.55 %) were grouped as O1:K1 while 7 (4.86 %) and 2 (1.38 %) strains belonged to O2:K1 and O78:K80, respectively. Furthermore, 91 isolates (63.19 %) could not be assigned to a definite serotype using these three antisera as they did not show agglutination (n = 79) or reacted positive with more than one anti-serum used (n = 12). Isolates that did not show agglutination with any or reacted positive with more than one anti-serum were assigned as non-typeable (Table 2). The PFGE analysis of 132 *E. coli* isolates resulted in a heterogenous PFGE cluster: 96 *E. coli* profiles were obtained after macrorestriction digest applying XbaI while 12 isolates were non-typeable. The dendrogram obtained from the cluster analysis is shown in Fig. 2.

The most abundant *E. coli* PFGE-profile was LA25 (n = 8) which included strains from three layer flocks (L1/III-A, L2/IV-B and L3/IV-B) that originated from a single rearing farm (R1V). All these isolates were associated with lesions in the reproductive tract. Likewise, B1 included four isolates from birds with inflammation of ovaries in the same flock (L3/III-A). Furthermore, *E. coli* genotypes which caused reproductive tract lesions in more than one laying bird at one sampling occasion from the same flock were: B9 (n = 2), LA11 (n = 2), LA18 (n = 3), LA21 (n = 2), S19 (n = 2), S33 (n = 2), S34 (n = 2) and S9 (n = 2).

Interestingly, *E. coli* genotypes B7 (n = 2), B12 (n = 3), LA22 (n = 3), S10 (n = 3) and S17 (n = 2) included isolates from both normal and diseased chickens. S5 (n = 2), Ua1 (n = 2), B4 (n = 2), B8 (n = 2), LA15 (n = 2) and S21 (n = 2) were present in pullets or layers without clinical signs. PFGE type S7 included three *E. coli* isolates that were collected from two pullets without pathological lesions and one laying hen with egg peritonitis originating from the same rearing farm.

DNA sequencing

The non-typeable isolates were confirmed by partial sequencing of 16S rRNA gene as *E. coli* (99-100 % identity). Accession numbers of the isolates to the European Nucleotide Archive are as follows: R1-V: LT548255, L1/III-A: LT548253, L1/III-A-2: LT548254, L1/III-A-3: LT548256, L2/III-A-1: LT548257, L3/III-B-5: LT548258, R1-V: LT548251, L1/IV-A-2: LT548250, L1/IV-A-6: LT548252. Following three isolates had 100 % identity with the existing database: L1/III-A-4: JQ975905.1, L1/III-A-5: JQ975905.1, L1/IV-B-3: KU560507.1.

Antimicrobial resistance (AMR)

The results of antibiotic resistance tests are shown in Table 3. These *E. coli* isolates were considered for the test in order to investigate similarities or differences in antibiotic sensitivity profiles between two strains collected from the same bird. All isolates were resistant to tylosin. Additionally, MDR was observed in three isolates originating from different birds. Two of these were resistant to five antibiotic substances [aminopenicilline (amoxicillin and ampicillin), tetracycline, doxycycline, sulphamethoxazole and trimethoprim] and the other to three antibiotic substances (oxolinic acid, doxycycline and neomycin). The following pair of isolates had non-identical pattern of resistance towards several antimicrobials used: 1) L1/III-B-2-ovary1 and L1/III-B-2-ovary2: amoxicillin; 2) L1/III-B-3-ovary and L1/III-B-3-oviduct: ampicillin, amoxicillin, doxycycline and sulphamethoxazole + trimethoprim; 3) L1/III-B-4-oviduct1 and L1/III-B-4-oviduct2: ampicillin, amoxicillin, doxycycline, tetracycline and sulphamethoxazole + trimethoprim; 4) L2/IV-B-1-ovary and L2/IV-B-1-liver: amoxicillin; 5) L1/IV-A-4-oviduct and L1/IV-A-4-ovary: oxolinic acid; 6) L2/IV-B-5-ovary and L2/IV-B-5-heart: doxycycline, enrofloxacin, neomycin; 7) L1/VT-B-1-oviduct and L1/VT-B-1-ovary: oxolinic acid; 8) L2/VT-B-2-ovary and L2/VT-B-2-oviduct: amoxicillin and doxycycline.

Discussion

An infection with *E. coli* in layers is regarded as one of the major problems in global poultry industry that might cause reproductive disorders referred as salpingitis/peritonitis/salpingoperitonitis and peritonitis syndrome ultimately leading to severe economic losses on commercial farms [6]. In this regards, an epidemiological knowledge of the disease and disease causing agent is fundamental in order to develop effective control and prophylactic strategies. Here, we studied molecular
| FPD/E-type | Isolates (x) | Source | Lesions | Serotype |
|------------|--------------|--------|---------|----------|
| S1         | 1            | oviduct | 1       | n.a.     |
| S2         | 1            | liver   | 1       | 01:R1    |
| S3         | 1            | oviduct | 1       | n.a.     |
| LA1        | 1            | liver   | 2       | n.a.     |
| B1         | 4            | oviduct | 2       | n.a.     |
| B4         | 1            | oviduct | 1       | n.a.     |
| B5         | 1            | oviduct | 2       | 02:R1    |
| B6         | 1            | oviduct | 1       | n.a.     |
| LA6        | 1            | oviduct | 2       | 02:R1    |
| LA7        | 1            | oviduct | 2       | n.a.     |
| S16        | 1            | oviduct | 1       | n.a.     |
| LA8        | 1            | oviduct | 2       | n.a.     |
| LA9        | 1            | oviduct | 2       | n.a.     |
| LA10       | 1            | oviduct | 1       | n.a.     |
| S17        | 2            | oviduct, oviduct | 1, 2   | n.a.     |
| S42        | 1            | oviduct | 1       | n.a.     |
| Ua2        | 1            | oviduct | 1       | n.a.     |
| LA11       | 2            | oviduct, oviduct | 2      | n.a.     |
| S18        | 1            | oviduct | 2       | n.a.     |
| B6         | 1            | oviduct | 1       | 01:R1    |
| B7         | 2            | oviduct | 1, 2    | 01:R1    |
| Co2        | 1            | oviduct | 1       | 02:R1    |
| B8         | 2            | oviduct | 1       | n.a.     |
| T9         | 2            | liver   | 2       | 01:R1    |
| B10        | 1            | oviduct | 2       | n.a.     |
| LA12       | 1            | oviduct | 1       | n.a.     |
| BA         | 2            | oviduct | 1       | n.a.     |
| Co3        | 1            | oviduct | 1       | n.a.     |
| S19        | 2            | oviduct | 2       | 02:R1    |
| LA14       | 1            | oviduct | 2       | 01:R1    |
| LA15       | 2            | liver   | 1       | n.a.     |
| LA16       | 1            | liver   | 1       | n.a.     |
| La2        | 1            | oviduct | 1       | n.a.     |
| SA4        | 1            | oviduct | 1       | n.a.     |
| S20        | 1            | liver   | 2       | n.a.     |
| S22        | 1            | oviduct | 2       | n.a.     |
| S23        | 1            | oviduct | 2       | n.a.     |
| S24        | 1            | oviduct | 2       | n.a.     |
| S25        | 1            | oviduct | 1       | 01:R1    |
| LA17       | 1            | oviduct | 2       | n.a.     |
| LA18       | 3            | oviduct, oviduct | 1, 2  | n.a., O1:K1 |
| B1         | 1            | oviduct | 1       | n.a.     |
| S25        | 1            | oviduct | 2       | n.a.     |
| S42        | 1            | oviduct | 2       | n.a.     |
| B14        | 1            | liver   | 1       | 01:R1    |
| B15        | 1            | liver   | 2       | n.a.     |
| LA23       | 1            | oviduct | 1       | n.a.     |
| S13        | 1            | oviduct | 2       | 02:R1    |
| B16        | 1            | liver   | 1       | 01:R1    |
| LA15       | 1            | oviduct | 1       | n.a.     |
| LA16       | 1            | liver   | 1       | n.a.     |
| La2        | 1            | oviduct | 1       | n.a.     |
| La2        | 3            | oviduct, heart | 1, 2  | n.a., O1:K1 |
| S29        | 1            | oviduct | 2       | 01:R1    |
| LA20       | 1            | oviduct | 2       | n.a.     |
| S11        | 1            | oviduct | 1       | n.a.     |
| Bl3        | 1            | oviduct | 2       | 01:R1    |
| S12        | 2            | oviduct | 2       | n.a., O1:K1 |
| LA21       | 2            | liver   | 2       | n.a.     |
| S22        | 2            | oviduct, heart | 1, 2  | n.a.     |
| S14        | 2            | oviduct | 1       | 01:R1    |
| B14        | 1            | liver   | 1       | 01:R1    |
| B15        | 1            | liver   | 2       | n.a.     |
| LA23       | 1            | oviduct | 1       | n.a.     |
| S15        | 1            | oviduct | 2       | 01:R1    |
| LA6        | 1            | oviduct | 1       | n.a.     |
| S36        | 1            | oviduct | 1       | n.a.     |
| La4        | 1            | liver   | 1       | n.a.     |
| LA24       | 1            | oviduct | 2       | n.a.     |
| Co5        | 1            | oviduct | 2       | n.a.     |
| S37        | 1            | liver   | 1       | n.a.     |
| LA25       | 1            | liver, oviduct | 2      | n.a., O1:K1 |
| LA26       | 1            | oviduct | 2       | 01:R1    |
| B17        | 1            | oviduct | 2       | n.a.     |
| LA27       | 1            | oviduct | 1       | n.a.     |
| LA28       | 1            | oviduct | 2       | n.a.     |
epidemiology of \textit{E. coli} isolates collected from pullets and layers in a longitudinal sampling study in Austria. Data obtained from genetic fingerprinting by PFGE were analyzed together with serotypes, geographical regions of isolation, and concurrent pathological lesions in each of the sampled birds.

In total, more than half of the \textit{E. coli} isolates \((n = 91/144)\) could not be assigned to a single serotype using antibodies against O1:K1, O2:K1 and O78:K80. Furthermore, for those isolates that could be assigned to one of the named serotypes, no correlation was found between a specific serotype and the occurrence of lesions in birds. In previous studies, it was also shown that \textit{E. coli} isolates collected from diseased birds display a high serological diversity \cite{16, 21, 22}, demonstrating as high as 62 different O serogroups \cite{21}. Thus classifying \textit{E. coli} strains into a definite serotype might sometimes be somewhat challenging. Hence, our finding is in agreement with a previous notion that serotyping alone might not be helpful as a tool for characterization of \textit{E. coli} \cite{16}.

In this study, the PFGE subtyping of \textit{E. coli} isolates \((n = 132)\) resulted in 96 XbaI profiles. Exclusively in two events, the same PFGE profile was seen in isolates from different sampling dates in mutually related farms/flocks, indicating potential \textit{E. coli} persistence. The PFGE-type S7 \((n = 3)\) included isolates from pullets \((n = 2,\) rearing farm R\textsubscript{V}) without pathological lesions and from one layer in the corresponding flock L\textsubscript{1/V} suffering from egg peritonitis and fibrinous oophoritis at the peak of production. In the second case, PFGE type S32 contained two isolates from the same layer flock \((L_{2/V1})\) at the peak and end of production. One bird sampled at the peak of production showed inflammation of the ovary whereas egg peritonitis was diagnosed in the other birds necropsied at the end of production. These results indicate that some \textit{E. coli} genotypes may retain in certain flocks at different stages of rearing but the associated pathological outcomes in birds can vary.

The genomic profile of extraintestinal \textit{E. coli} with PFGE further revealed that strains collected from birds with pathological lesions can have 100 \% genetic identity with strains that were collected from healthy birds. For instance, in PFGE type S10 \((n = 3)\) in flock \(L_{2/V1}\) two birds did not have any lesions while one had oophoritis and salpingitis. Likewise in PFGE type B7 \((n = 2)\) in \(L_{2/III}\), one bird showed no lesions while in contrast, the other had egg peritonitis. Also, remaining isolates could not be grouped into distinct clonal clusters based on presence or absence of pathological lesions in sampled birds. This finding is in agreement with a previous study in broilers where authors have reported a high heterogeneity of \textit{E. coli} isolates in broilers \cite{13, 23}. It can be hypothesized that pathogenicity of extraintestinal \textit{E. coli} in chickens is highly dependent on concurrent environmental and host susceptibility factors. Providing a suitable opportunity in certain circumstances, \textit{E. coli} residing in clinically healthy chickens might turn up into pathogenic. The hypothesis is further supported by an earlier finding in broiler that many colibacillosis associated isolates might not be clearly distinguished solely on the basis of presence of virulence associated genes as compared to intestinal commensal \textit{E. coli} \cite{13}.

In the present study, we found no evidence for clonality of \textit{E. coli} with respect to geographical locations of farms. Previously, Ewers et al. (2004) found only a limited number of \textit{E. coli} clones to be distributed in poultry production in Germany \cite{16}. In another study, it was reported that chickens with peritonitis in a single flock were likely to be infected by the same \textit{E. coli} strain \cite{24}. Different to this, we did not find clonality of \textit{E. coli} isolates in birds from the same flock showing gross pathological lesions in the reproductive tract thus maintaining a high heterogeneity of PFGE types. Interestingly, we further noticed that a single bird can harbour two different PFGE types of \textit{E. coli} in the same or different organs. Thus, the study demonstrated that a layer can be infected simultaneously by different \textit{E. coli} genotypes. A similar finding was previously reported in broilers \cite{18}. However, in another study in layers, one PFGE type was found to be present in bone marrow of an individual bird \cite{17}. It might be that in some organs \textit{E. coli} isolates possess less or no genetic diversity due to an adaptation process, which should however be further elucidated. In the present study, we also tested antibiotic susceptibility of 16 isolates that were collected from eight birds. All the isolates were sensitive to ceftiofur, colistin, gentamicin and spectinomycin but the resistant rate to tylosin was found 100 \%. Mixed results were obtained for other antibiotics tested. MDR was seen in 3/16 isolates showing resistance to as high as five different antibiotics used. Although the number of isolates included for antimicrobial susceptibility test in the actual study is
Table 3 Antibiotic resistance test of 16 *Escherichia coli* isolates collected from 8 birds (two isolates per bird)

| Antibiotics       | Isolates          |
|-------------------|-------------------|
|                   | L1/III-B-2-ovary1 | L1/III-B-2-ovary2 | L1/III-B-3-ovary | L1/III-B-4-oviduct1 | L1/III-B-4-oviduct2 | L2/IV-B-1-ovary | L1/V-A-4-oviduct | L1/V-A-4-oviduct | L2/V-B-5-ovary | L2/V-B-5-heart | L1/VI-B-1-ovoiduct | L2/VI-B-2-ovoiduct |
| Ampicillin        | I                 | I                 | R                 | R                 | S                 | S                 | S                 | I                 | S                 | S                 | I                 | I                 | I                 | I                 |
| Amoxicillin       | I                 | R                 | I                 | R                 | R                 | I                 | S                 | I                 | I                 | I                 | I                 | I                 | I                 | R                 | I                 |
| Ceftiofur         | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 |
| Colistin          | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 |
| Doxycycline       | I                 | I                 | S                 | R                 | R                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | R                 | S                 | S                 | S                 |
| Enrofloxacin      | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | I                 | S                 | S                 | I                 | S                 | S                 | S                 |
| Gentamicin        | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 |
| Neomycin          | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | R                 | S                 | S                 | S                 |
| Oxolinic acid     | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | R                 | R                 | R                 | R                 | S                 | S                 | S                 |
| Tetracycline      | S                 | S                 | S                 | R                 | R                 | R                 | R                 | S                 | S                 | S                 | S                 | I                 | S                 | S                 | S                 | S                 | S                 | S                 |
| Tylosin           | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 | R                 |
| Spectinomycin     | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 | S                 |
| Sulphamethoxazole + trimethoprim | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

Each isolate ID is designated with letter "L" along with the number of layer flock and rearing farm – A (sampling at the peak of production) or B (sampling at the end of production) – number of sampled bird – organ of isolation – isolate number (in case when two isolates were collected from the same organ). Antibiotic resistance pattern of two isolates from the same bird are in bold letter and highlighted if they showed different sensitivity to antimicrobials used. S: sensitive, I: intermediate, R: resistant
isolate per bird might not be enough to decide the most appropriate treatment.

**Conclusions**

Serotyping, antibiotic resistance test and genotypic fingerprinting of extraintestinal *E. coli* revealed that isolates exhibit high diversities within and between birds. As one bird can harbour different *E. coli* types an appropriate number of isolates should be considered for epidemiological studies and antibiotic sensitivity test.

**Abbreviations**

AMR: Antimicrobial resistance; APEC: Avian Pathogenic *Escherichia coli*; COS: Columbia agar supplemented with 5 % sheep blood; MDR: Multidrug resistance; PCR: Polymerase chain reaction; PFGE: Pulsed-field gel electrophoresis

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**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article and partial 16S RNA gene sequence data are deposited in the European Nucleotide Archive.

**Authors’ contributions**

SP, CH, AZ, MH designed the study, CH and AZ were involved in necropsy and sampling, SP and BS performed PFGE. SP drafted the manuscript and BS, CH, AZ, MH contributed with their inputs. All authors have read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Sampling was performed during post mortem investigations and complies with national legislation (Tierversuchsgesetz – TVG 2012, §1). Furthermore, the study was performed in co-operation with veterinarians in charge of the respective farms who have agreements with farm owners for applying veterinary procedures.

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