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Day-old chicks are a source of antimicrobial resistant bacteria for laying hen farms

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ABSTRACT

Antimicrobial resistant bacteria are rarely detected in laying hens and the objective of this longitudinal study was to test day-old chick as a source. Four different commercial batches raised on the same farm were monitored from day-old chick to laying hens using *Escherichia coli* as a model. Ten colonies from each of the eight samplings per batch were tested for antimicrobial susceptibility using 14 antimicrobials.

Overall (313 isolates), higher resistance percentages were detected for tetracycline (26.8%), followed by sulphonamides (16.3%), ampicillin (16.0%) and quinolones (10.9% and 9.3% for ciprofloxacin and nalidixic acid, respectively). Resistance percentages of bacteria from day-old chicks were higher than those of pullets and hens ($p < 0.05$) for tetracycline, sulphonamides, trimethoprim and chloramphenicol.

Forty different phenotypic resistance profiles were detected, led by fully susceptible (182 isolates; 58.1%), and followed by single tetracycline (28 isolates; 8.9%) and ciprofloxacin/ nalidixic acid (11 isolates; 3.5%) profiles.

By whole-genome sequencing, 17 genes and mutations of five chromosomal genes related to resistance were detected, the most frequent being *tetA*, bla_{TEM-1B} and *sul1*.

Using multilocus sequencing analysis, 58 different MLST types were detected, most of them only in a particular sample. The ST155 (27/142) was the most frequently detected, followed by ST10 (19/142) and ST48 (9/142).

The fate on the farm of the detected *E. coli* populations in old-day chicks was not clear, but our data suggest that they did not remain in the predominant faecal population of pullets and laying hens.

1. Introduction

The commercial table egg is one of the most important food production sectors. In 2015, the European Union member countries' share of global egg production was 10.3% (Windhorst, 2017). The structure of the egg production is pyramidal starting with grandparent and parent flocks (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014). The commercial egg production cycle begins with female day-old layer hen chicks supplied by a commercial hatchery that are raised until 16–18 weeks. The pullets are then transferred to the laying house to enter to the laying phase until the end of the production cycle at 60–72 weeks or until 72–120 weeks if a moulting procedure is used (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014).

The overall antimicrobial usage in egg-producing laying hens is relatively low compared to other food-producing animal species (Harisberger et al., 2011; van Hoorebeke et al., 2011), especially during the laying phase. Responsible factors are the improvement in animal health and sanitary conditions and the low number of veterinary medicinal products containing antimicrobials authorized for laying hens (due, among other reasons, to the restriction for many pharmacologically active substances of "not for use in animals from which eggs are produced for human consumption", Regulation 37/2010). Nevertheless, the mandatory monitoring of antimicrobial resistance (AMR) in laying hens in the EU, has detected in every biennial programme a variable number of antimicrobial resistant *Salmonella enterica* isolates, especially resistant against tetracycline and sulfamethoxazole (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018).

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Escherichia coli is a very good bacterial indicator of the current situation of AMR (Vila et al., 2016) in animals and humans due to both its gastrointestinal habitat and its ability to capture and horizontally transfer of genetic elements (like plasmids and integrons) containing AMR genes across bacteria. However, at the EU level, monitoring of commensal *E. coli* in laying hens is not mandatory so there are no harmonized AMR data and, in addition, there are few reports on this topic in laying hens (Harisberger et al., 2011; van Hoorebeke et al., 2011).

The sources of AMR bacteria in laying hen farms have not been explored previously, although there are several studies showing vertical transmission of different AMR *E. coli* profiles (cephalosporin and fluoroquinolone resistance) in the broiler pyramid (Bortolaia et al., 2010; Dame-Korevaar et al., 2017; Dierikx et al., 2013; Mo et al., 2014). Consequently, we tested the role of day-old chicks as a source of AMR bacteria for laying hen farms using *E. coli* as a model.

2. Material and methods

2.1. Setting

Four commercial batches of female day-old chicks (B1, size: 56,640; B2, size: 67,000; B3, size: 62,900; and B4, size: 57,600) from four different hatcheries and raised (from March 2016 to October 2018) by one commercial laying hen farm located in South-East of Spain were included in this longitudinal study. This farm had growing and production sites separated by about five kilometres. During the growing step, B1 was housed in a house and B2, B3 and B4 in the same house. During the laying step, the four batches were housed in different houses. Animals at both sites were housed in battery enriched cages where the manure was removed by a belt below the cages. Colistin was used in B1 (at week 24) and B2 (at week 23). Any other antimicrobials were used.

2.2. Sampling

Each batch was sampled eight times. Firstly, on arrival at the farm (S1), two times during the growing phase [pullets at weeks 2 (S2), and 13–15 (S3)] and five times during the laying phase [laying hens at weeks 24 (S4), 38 (S5), 53 (S6), 68 (S7) and 83 (S8)].

Farm samples were 15 transport box bottoms (between 15 and 50 g per bottom) each containing about 40 old-day chicks with fresh meconium droppings (S1) and 10 faecal samples (around 100 g per sample) from manure belts of the sites for pullets (S2 and S3) and laying hens (S5–S8). Consequently, no individual animal sample was taken. All samples were transported in a cool box to the laboratory and processed on the same day.

2.3. Sample preparation

Analytical samples were prepared by pooling the farm-samples. Transport box bottoms were individually weighed and 10 retained (discarding those of lowest and highest weights) and folded to take a piece of about 5 g per bottom. These 10 pieces were pooled (total weight between 45 and 50 g) and mixed with 225 ml of peptone water. Faecal samples from manure belts were individually weighed and mixed; then, a sample of 10 g was taken and diluted with 90 ml of peptone water.

2.4. Bacterial isolation and identification

Peptone water diluted samples were maintained at room temperature for one hour before being serially diluted tenfold with peptone water. Then, 0.1 ml of the last three dilutions were plated in duplicate on MacConkey agar plates, which were incubated at 37 °C for 20–24 h. Ten colonies with the typical *E. coli* morphology from several countable plates with visible growth were picked and identified by PCR (Cabal et al., 2013). Isolates of doubtful identification were further checked using the API® 20-E Enterobacteriaceae identification kit (bioMerieux). Identification of some isolates was checked by whole genome sequencing (WGS) as explained below.

2.5. Antimicrobial susceptibility test

Antimicrobial susceptibility was tested by broth microdilution using EUVSEC commercial plates (Sensititre@, ThermoFisher) containing 14 antimicrobials. Plates were incubated at 37 °C for 20–24 h and read by eye. Epidemiological cut-off values according to EU recommendation (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018) were used for interpretation of minimal inhibitory concentration (MIC) values. Isolates were considered multidrug resistant (MR) if they were simultaneously resistant to compounds belonging to three or more antimicrobial families and fully susceptible (FS) if they were susceptible to the 14 antimicrobials tested (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018).

2.6. DNA extraction

Bacterial DNA was purified from pure cultures with the QIAGEN DNeasy Blood and Tissue Kit using manufacturer instructions.

2.7. Whole genome sequencing

A subset of 152 isolates covering the four batches at four of the eight samplings (S1, S2, S4 and S6) was studied by WGS.

Sequencing libraries were prepared using the Nextera XT kit and sequenced on a MiSeq system (Illumina) using v3 reagents with 2 × 300 cycles. Raw reads were analysed by using our own developed bioinformatics pipeline TORMES® (Quijada et al., submitted). Briefly, it consists of quality filtering by Prinseq v.0.20.4 (Schmieder and Edwards, 2011) and Trimmomatic (Bolger et al., 2014). Genomes were assembled by using SPAdes v3.10 (Bankevich et al., 2012), and classified taxonomically and annotated by Prokka (Seemann, 2014).

2.8. Bioinformatics analysis

The multilocus sequencing typing (MLST) profiles were predicted by using mlst v2.10 (T. Seemann, https://github.com/tseemann/mlst) against the PubMLST database (Jolley and Maiden, 2010). Genes used for MLST typing were *adk, fumC, gyrB, icd, mdh, purA* and *recA* (Wirth et al., 2006).

Occurrence of resistance genes in the draft genome was analysed by blastn (Zhang et al., 2000) and ABRicate (T. Seemann, https://github.com/tseemann/abricate) searches against the ResFinder (Zankari et al., 2012), CARD (McArthur et al., 2013) and ARG-ANNOT (Gupta et al., 2014) databases. The presence and detection of point mutations in chromosomal genes was performed using PointFinder (Zankari et al., 2017).

2.9. Statistical methods

Fischer exact test and Cochran-Armitage test for trend analysis were performed with WinPepi version 11 (Abramson, 2011).

3. Results

3.1. Antimicrobial susceptibility

Of the 320 collected colonies, 313 isolates identified as *E. coli* were included into the longitudinal study. Seven presumptive *E. coli* colonies [batch 1 (two colonies from S2 and two from S6), batch 2 (one colony from S6) and batch 3 (two colonies from S2] were discarded because a non *E. coli* identification.

According to the current EFSA interpretative thresholds (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018), all the isolates were not

Table 1
Occurrence (%) of antimicrobial resistance in 313 *E. coli* isolates from four batches in a commercial laying hen farm in Spain.

Antimicrobials (ECOFF[a] values, mg/L)	Day-old chick (n = 40)	Pullets (n = 76)	Laying hens (n = 197)	All (n = 313)
Tetracycline[1, 2] (> 8)	75 (30)	21 (16)	19.3 (38)	26.8 (84)
Sulphonamides[1, 2] (> 64)	45 (18)	17 (13)	10.2 (20)	16.3 (51)
Ampicillin (> 8)	23 (9)	18 (14)	13.7 (27)	16.0 (50)
Ciprofloxacin[2, 3] (> 0.06)	30 (12)	17 (13)	4.6 (9)	10.9 (34)
Nalidixic acid[2, 3] (> 16)	30 (12)	14 (11)	3.0 (6)	9.3 (29)
Trimethoprim[1, 2, 3] (> 2)	20 (8)	1 (1)	7.6 (15)	7.7 (24)
Chloramphenicol[1, 2] (> 16)	10 (4)	1 (1)	2.5 (5)	3.2 (10)
Gentamicin (> 2)	5 (2)	(0)	3.0 (6)	2.6 (8)
Azithromycin (> 16)	(0)	(0)	3.6 (7)	2.2 (7)
Ceftazidime (> 0.5)	(0)	3 (2)	(0)	0.6 (2)
Cefotaxime (> 0.25)	(0)	3 (2)	(0)	0.6 (2)
Colistin (> 2)	(0)	(0)	(0)	(0)
Tigecycline (> 1)	(0)	(0)	(0)	(0)
Meropenem (> 0.12)	(0)	(0)	(0)	(0)

Statistically significant differences ($p < 0.05$): [1]day-old chicks/pullets, [2]day-old chicks/laying hens, [3]pullets/laying hens; Nº of isolates in brackets; [a] Epidemiological cut-off values.

resistant to meropenem, colistin and tigecycline (Table 1). Taking together all the isolates (Table 1), the highest resistance percentages were detected for tetracycline (26.8%), followed by sulphonamides (16.3%), ampicillin (16.0%) and quinolones (10.9% and 9.3% for ciprofloxacin and nalidixic acid, respectively).

Overall, the highest resistance levels against most antimicrobials were detected in bacteria from day-old chicks, although resistant isolates against ceftazidime/cefotaxime were only detected in pullets and against azithromycin only in laying hens (Table 1).

Resistance percentages in bacteria from day-old chicks were higher than those from pullets and hens ($p < 0.05$) for tetracycline, sulphonamides, trimethoprim and chloramphenicol (Table 1), and only higher than those of hens ($p < 0.05$) for ciprofloxacin and nalidixic acid. Differences between pullets and hens ($p < 0.05$) were detected for trimethoprim (higher resistance percentage in hens), ciprofloxacin and nalidixic acid (both quinolones showed higher resistance percentages in pullets).

A decreasing trend in antimicrobial resistance percentages against tetracycline, sulphonamides, ciprofloxacin and nalidixic acid in bacteria from day-old chicks to hens was detected.

Forty different phenotypic resistance profiles were detected, led by FS (182 isolates; 58.1%), and followed by two mono-resistant profiles: tetracycline (28 isolates; 8.9%) and ciprofloxacin/nalidixic acid (11 isolates; 3.5%). The highest MR profile (tetracycline – sulphonamides – ampicillin – ciprofloxacin/nalidixic acid – trimethoprim – chloramphenicol) was only detected on the first sampling of batch 1. Table 2 summarises the phenotypic resistance profiles according to batch and sampling. Overall, large differences were detected among *E. coli* from the four day-old chicks' batches, with those of batch 1 being the most resistant.

Temporal analysis of the batches from day-old chicks to laying hens regarding non-FS versus FS isolates showed a progressive decrease, especially from day-old chicks to pullets, in the four batches. In addition, phenotypic resistance profiles changed between batches and with time, suggesting a low ability of the predominant *E. coli* isolates detected in day-old chicks to persist as the dominant isolates in the faecal microbiota of pullets and laying hens or a loss or acquisition of mobile genetic elements carrying antimicrobial resistance traits. In any case, the increasing number of FS isolates during the production cycle, especially in laying hens, hindered the analysis of the dynamics of *E. coli* isolates on the farm using this phenotypic AMR feature.

3.2. Antimicrobial resistance genes

As mentioned before, a subset of 152 isolates was studied by WGS for detection of AMR genes and mutations of chromosomal genes mediating resistance. A short list of 17 AMR genes and mutations on five genes (*gyrA, gyrB, parC, parE* and *ampC* promoter) was detected (Table 3). The most frequently detected genes were *tetA,* $bla_{TEM\text{-}1B}$ and *sul1*. Some genes were only detected in *E. coli* from day-old chicks ($bla_{oxa\text{-}1}$, *floR*) or hens (*sul3,* $bla_{TEM\text{-}106}$, *qnrB19, dfrA5* and *cmlA1*). Mutations on the *ampC* promoter were only detected in isolates from pullets.

Generally, genotypic and phenotypic AMR data were well related, although some discrepancies were observed. At first, putative AMR genes were not detected in 21 phenotypically resistant isolates, related to resistance against tetracycline (six isolates), sulphonamides (four isolates), ampicillin (three isolates), sulphonamides/ampicillin (one isolate), trimethoprim (four isolates) and gentamicin (three isolates). Nevertheless, using a query in ResFinder 3.1 with less stringent conditions (threshold: 30%; minimum length: 20%) we detected genes associated with their respective phenotypes in six of these 21 isolates (Table 4).

The addition of genotypic AMR profiles scarcely improved the analysis of *E. coli* dynamics on the farm.

3.3. Multilocus sequence typing

The same subset of 152 isolates mentioned above was also studied by WGS for detection of the genes used for MLST. Table 4 summarizes MSLT data according to batch and sampling. Ten isolates were non-typeable, whereas the remaining 142 belonged to 58 different MLST types, most of them detected at a very low frequency (38 of the 58 ST types were unique) and usually only in a particular sample. Only 10 ST types (ST155, ST10, ST48, ST355, ST58, ST746, ST162, ST394, ST602 and ST1286) were detected in two or more samples. The ST155 (27/142) was the most frequently detected (nine of the 16 samples), especially in batch 2, followed by ST10 (19/142; seven samples) and ST48 (9/142; five samples).

The number of different MLST profiles per sampling ranged between 3 and 8, with a mean value of 5.

Associations were not detected between ST and AMR. For instance, the 71 FS isolates typed belonged to 30 different STs, whereas the 27 isolates of ST155 displayed seven different AMR profiles.

Very few isolates having the same ST were detected in two successive samplings in the same batch (Table 5), showing a noticeable change on the predominant *E. coli* isolates along the production cycle. Batch 2 showed the only detected event of persistence of an isolate from old-day chicks to laying hens, since we detected ST155/FS from S1 (three isolates), S2 (five isolates) and S4 (one isolate). Persistence of isolates from old-day chicks to pullets was detected in batch 1 (isolates in S1 and S2 belonging to ST355 and having the same chromosomal mutation on *gyrA* and a CIP/NAL-R phenotype). Equally, persistence in hens was detected in batch 4 (isolates in S4 and S6 belonging to ST10 and MR (*tetA-sul1-*$bla_{TEM\text{-}1B}$ *-dfrA1*). In addition, isolates belonging to

Table 2
Phenotypic antimicrobial resistance profiles of 313 *E. coli* isolates from four animal batches in a commercial laying hen farm in Spain.

Animals (sampling)	Batch 1	Batch 2	Batch 3	Batch 4
Day-old chicks (S1 – day 1)	TET-SMX-AMP-CIP/NAL-TMP-CHL (4) CIP/NAL (3) TET-SMX-AMP-CIP/NAL-TMP (2) TET-SMX (1)	FS (5) TET-SMX (2) TET (3)	TET (5) TET-SMX-GEN (2) AMP-CIP/NAL (2) TET-SMX-AMP-TMP (1)	TET-SMX (5) TET (4) TET-SMX-CIP/NAL-TMP (1)
Pullets (S2 – week 2)	FS (4) CIP/NAL (1) TET-AMP-CIP/NAL (1) TET-AMP-CIP/NAL-TAZ (1) TET-CIP/NAL (1)	FS (6) TET (2) SMX (1) SMX-CIP/NAL (1)	SMX-AMP (4) FS (3) TET-AMP-CIP-FOT (1)	FS (8) SMX (1) TET (1)
Pullets (S3 – week 13-15)	FS (4) TET (2) TET-AMP-CIP/NAL (2) SMX-AMP-CIP/NALT-FOT/TAZ (1) TET-SMX-TMP-CHL (1)	FS (4) TET-SMX-AMP (4) CIP/NAL (2)	FS (9) CIP (1)	FS (9) CIP/NAL (1)
Laying hens (S4 – week 24)	FS (4) TET (3) TET-AMP (2) AMP (1)	FS (4) AMP (2) SMX-CHL (1) CIP/NAL (1) TET-AMP-CHL-TMP (1) TET-SMX-GEN (1)	FS (8) SMX-AMP-TMP (2)	FS (5) TET-SMX-AMP-TMP (2) TET-AMP-TMP (1) SMX (1) TET-GEN (1)
Laying hens (S5 – week 38)	FS (6) SMX (1) AZI (1) SMX-CIP/NAL (1) TET (1)	FS (10)	FS (7) GEN (1) SMX (1) TET-AMP-AZI (1)	FS (8) SMX-TMP (1) TET-SMX-TMP-GEN (1)
Laying hens (S6 – week 53)	FS (7) GEN (1)	FS (7) TET-AMP (2)	FS (7) TET (1) AMP (1) TET-SMX-AMP-CHL (1)	FS (4) AMP-CIP (1) TET (1) TET-AMP-TMP (1) TET-CHL (1) TET-SMX-AMP-TMP (1) TET-TMP (1)
Laying hens (S7 – week 68)	FS (7) AZI (3)	FS (6) TET (3) TET-SMX-AMP-TMP (1)	FS (8) CIP/NAL (1) SMX-AMP-CHL (1)	FS (7) CIP/NAL (1) TET-CHL (1) TET-SMX (1)
Laying hens (S8 – week 83)	FS (6) AMP (1) AZI (1) TET (1) TET-SMX-AMP-TMP (1)	FS (7) CIP/NAL (1) CIP/NAL-AZI (1) GEN (1)	FS (6) TET-SMX (2) TET (1) TET-AMP-TMP (1)	FS (6) AMP (1) TET-AMP (1) TET-TMP (1) TET-AMP-CIP (1)

FS: fully susceptible; TET: tetracycline; SMS: sulphonamides; AMP: ampicillin; CIP: ciprofloxacin; NAL: nalidixic acid; TMP: trimethoprim; CHL: chloramphenicol; GEN: gentamicin; FOT: cefotaxime; CAZ: ceftazidime; AZI: azithromycin; Nº of isolates in brackets.

the same MLST but with different AMR profiles were detected in batch 3 from day-old chicks, pullets and laying hens (ST48) and in batch 4 from pullets and laying hens (ST10).

Consequently, MLST data improved the analysis of the dynamics of *E. coli* in this laying egg farm showing that predominant isolates changed during the production cycle.

4. Discussion

It is expected that the faecal microbiota of healthy animals would be composed, among many other bacteria, of different populations of *E. coli* of unknown abundance. Consequently, the number of isolates to be tested in the laboratory to encompass the *E. coli* diversity in the chicken gut microbiota remains unspecified and depends on the goals of the research. In this study, a dilution method was used to capture the most abundant *E. coli* populations in each pooled sample, taking 10 colonies from the most highly diluted plates. It is clear that this procedure is not exhaustive, and that the relative abundance of each *E. coli* population is the critical feature responsible for its detection. For this reason, we used the term "predominant population" when presenting the data. This procedure is well suited for the comparison of populations, but it would be less useful for tracking specific isolates if they do not maintain the same relative abundance over time, and could have reduced our ability to follow the isolates introduced by day-old chicks on the farm. In addition, tracking of antimicrobial resistant isolates is hampered by horizontal transfer of mobile genetic elements containing AMR traits.

The phenotypic analysis of the predominant *E. coli* populations of four different batches of day-old chicks clearly showed that day-old chicks were a source of AMR bacteria, as previously found in the broiler production pyramid (Dame-Korevaar et al., 2017; Huijbers et al., 2016; Mezhoud et al., 2016). Nevertheless, vertical transmission of bacteria from parental hens to day-old chicks was not been studied, precluding an in-depth analysis of the putative sources (parents or contamination in the hatchery environment).

To the best of our knowledge, AMR in *E. coli* from day-old chicks dedicated to commercial egg production has not been previously studied. Control groups composed of day-old chicks in two field trial studies in broilers (da Costa et al., 2009; Jimenez-Belenguer et al., 2016), showed high resistance percentages but against different antimicrobials. Jimenez-Belenguer et al. (2016), testing 22 isolates and using a disk diffusion methodology, reported the highest resistance levels against nalidixic acid, ampicillin and amoxicillin plus clavulanic acid. Da Costa et al. (2009), testing 26 isolates and also using disk diffusion, reported 22 of 26 resistant isolates, with tetracycline and ampicillin as the most frequently detected resistance phenotypes. Using EFSA terminology (EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2018) for describing the occurrence of AMR in the *E. coli* isolates of our study from day-old chicks, resistance was extremely high against tetracycline, very high against sulphonamides, and high against ciprofloxacin, nalidixic

Table 3
Genes (Resfinder and ARG-ANNOT) and chromosomal mutations (PointFinder) mediating antimicrobial resistance per sampling in 152 isolates of *E. coli* from a commercial laying hen farm (nº of isolates in brackets).

Resistance against	Day-old chicks (S1 – day 1) (n = 40)	Pullets (S2 – week 2) (n = 36)	Laying hens [S4 (week 24) + S6 (week 53)] (n = 76)
Genes			
Tetracycline	*tetA* (19) *tetB* (2)	*tetA* (3) *tetB* (3)	*tetA* (20)
Sulphonamides	*sul1* (11) *sul2* (2)	*sul1* (3) *sul2* (1)	*sul1* (5) *sul2* (3) *sul3* (2)
Ampicillin	bla_{TEM-1B} (8) bla_{OXA-1} (3)	bla_{TEM-1B} (2)	bla_{TEM-1B} (15) $bla_{TEM-106}$ (1)
Ciprofloxacin	*qnrS1* (1)	*qnrS1* (1)	*qnrS1* (2) *qnrB19* (1)
Trimethoprim	*dfrA1* (1) *dfrA14* (2)		*dfrA1* (4) *dfrA14* (2) *dfrA5* (1)
Chloramphenicol	*catA1* (4) *floR* (4)		*cmlA1* (2) *catA* (1)
Gentamicin	*aac(3)-Vla_1* (1)		
Streptomycin* / spectinomycin*	*aadA1* (2) *strA/strB* (7/8)	*strA/strB* (2/2)	*aadA2* (2) *strA/strB* (8/8)
Fosfomycin*	*fosA7* (2)		
Mutations			
Ciprofloxacin / nalidixic acid	*gyrA* S83*L* (12) *gyrB* H652R (1) *parC* E62 K (1) *parE* D475E (1)	*gyrA* S83L (4) *gyrA* D87Y (1) *gyrA* D87N (4) *parC* L440R (1) *parC* E62K (1) *parC* S80I (2) *parC* S80R (1) *parE* D475E (1)	*gyrA* S83 L (1)
Cefotaxime / ceftazidime		*ampC* n.-1C > T / *ampC* n.-18G > A (1)	

* Antimicrobials for which phenotypic susceptibility test was not performed.

acid, ampicillin and trimethoprim. There are also other studies of antimicrobial resistance in *E. coli* from day-old chicks for broiler production, but focused on the isolation of specific resistance phenotypes, mainly resistance to cephalosporins (Yossapol et al., 2017) or quinolones (Abdi-Hachesoo et al., 2013), precluding a comparative analysis with our results.

Regarding young pullets, da Costa et al. (2009) presented results of bacteria from four and nine day-old chicks, showing lower resistance percentages and different patterns of AMR than those of the bacteria from day-old chicks. These findings agree with our results when comparing bacteria from day-old chick and pullets. The decrease in antimicrobial resistant bacteria during the rearing period, when antimicrobials are not used, was also detected in both the above-mentioned studies (da Costa et al., 2009; Jimenez-Belenguer et al., 2016).

AMR in *E. coli* from laying hens has been studied in Switzerland (Harisberger et al., 2011; van Hoorebeke et al., 2011), and Belgium, Germany and Italy (van Hoorebeke et al., 2011). In Swiss laying hens (Harisberger et al., 2011), AMR occurrence in 371 *E. coli* isolates were high for sulfamethoxazole (22.4%), moderate for nalidixic acid, tetracycline, ciprofloxacin, trimethoprim and ampicillin, low for streptomycin, spectinomycin and neomycin, and very low or rare for the remaining nine antimicrobials tested. Taken together, the 1102 *E. coli* isolates from the four European countries studied by van Hoorebeke et al. (2011) showed a similar landscape led by tetracycline resistance (22.8%), followed by resistance to sulfamethoxazole (17.2%), ampicillin (15.8%), nalidixic acid (10.7%) and ciprofloxacin (10.4%). It is interesting that our results with laying hen *E. coli* isolates taken from four different batches on the same farm are quite similar to those reported by this European study.

The fate on the farm of the detected *E. coli* populations in old-day chicks was not clear, but our data suggest that they did not remain in the predominant faecal population of pullets and laying hens. The published longitudinal studies in poultry try to follow specific AMR phenotypes, most of them related to beta lactams resistance, in broiler production (Dame-Korevaar et al., 2017; Dierikx et al., 2013; Huijbers et al., 2016). Increased prevalence of an ESBL/AmpC *E. coli* phenotype at broiler farms has been detected independently of the use of antimicrobials (Dierikx et al., 2013; Huijbers et al., 2016), whereas Dame-Korevaar et al. (2017) found that CMY-2 *E. coli* did not persist in a broiler parent flock. Having in mind the differences in life-span in these studies among broilers (five to seven weeks until slaughter) and parents hens/chickens (49 weeks), data from parent flocks are more appropriate for comparison with those of laying hens. The need for longitudinal studies in laying hen farms has been highlighted (van Hoorebeke et al., 2011).

Our results also showed that MLST typing is useful for studying the dynamics of *E. coli* populations in egg production, where ST155 and ST10 have been the most frequently detected STs. Sequence type 48, a member of the clonal complex ST10, was also detected in our study and in laying hens by Dissanayake et al. (2014), who also detected ST10, ST155, ST1662 and ST101 in poultry. Sequence types 10 and 429, as well as several of the unique STs in our study (ST23, ST88, ST95 and ST141), had been previously detected among avian pathogenic *E. coli* in broilers by Pires-dos-Santos et al. (2013). Dame-Korevaar et al. (2017) reported that the most widespread STs among CMY-2 *E. coli* were ST48 and ST155, which were both detected at six of the eight

Table 4
Antimicrobial resistance genotype-phenotype discrepancies detected in *E. coli* isolates from a commercial laying hen farm in Spain with a putative explanation.

Batch/ sampling	Isolate nº	Phenotypic profile (MIC mg/L)	Detected gene*	Identity	Query[1]/HSP[2]	Contig position/ length
B2-S4	10	GEN-R (> 32)	*aac(3)-Via aac(3)-VIa*	100 99.54	900/337 900/431	1031..1367 /1367 1..431/1278
B3-S1	10	AMP-R (> 64)	bla_{TEM-47}	99.79	861/474	16..489/11053
			bla_{TEM-1A}	100	861/434	1..434/2088
B3-S1	2	TET-R (> 64)	*tetB*	100	1206/484	63..546/546
			tetB	100	1206/678	82..759/759
B3-S1	9	TET-R (> 64)	*tetB*	100	1206/611	1..611/2101
B3-S4	2	SUL-R (> 1024)	*sul2*	100	816/248	1..248/2155
			sul2	100	816/313	1..313/862
B4-S1	4	TET-R (64)	*tetA*	99.26	1275/544	1822..2365/2376
			tetA	98.84	1200/862	18320..19181/19181

corresponding sequence in the genome.
GEN: gentamicin; AMP: ampicillin TET: tetracycline; SUL: sulphonamides.
* ResFinder-3.1: selected threshold: 30%; selected minimum length: 20%.
[1] Query length is the length of the best matching resistance gene in the database.
[2] HSP is the length of the alignment between the best matching resistance gene and the.

Table 5
Distribution of MLST types of 152 *E. coli* isolates from four animal batches in a commercial laying hen farm in Spain.

Animals (Sampling)	Batch 1	Batch 2	Batch 3	Batch 4
Day-old chicks (S1)	**355 (1)** ***gyrA83****/parE475* (1) 58 (4) . *tetA-sul2-Bla*$_{TEM-1B}$ */bla* $_{oxa1}$*-gyrA83*-TMP-*floR/catA1* 429 (2) / 2253 (2) / 1304 (1)	**155 (7)** **. FS (4)** **. *tetA* (3)** 602 (1) . *tetB* (1) 135 (1) / 2557 (1)	**48** (1) . *tetA-sul1-bla*$_{TEM-1B}$ *-dfrA1* (1) 155 (2) . *tetA-sul1/2-aadA1/aac(3)* (2) 602 (2) . *tetB*-strB-fosA7* (1) . *tetB*-strA/strB-fosA7* (1) 1276 (3) / 1828 (2)	155 (3) . *tetA-sul1* (3) 2276 (2) / 88 (1) / 141 (1) / 2726 (1) / 7546 (1) / NI (1)
Pullets(S2)	**355 (1)** **. *gyrA83*** (1) 10 (4) . FS (4) 38 (1) / 154 (1) / 4456 (1)	**155 (7)** **. FS (5)** **. *tetA* (2)** 162 (1) . *sul2-gyrA83/ gyrA87/parC80* (1) 69 (1) / 770 (1)	**48** (4) . *sul1- bla*$_{TEM-1B}$ (2) . *sul1*-AMP (1) . SMX-AMP (1) 5826 (2) 23 (1) 1286 (1)	**10** (4) . FS (3) . TET (1) 7547 (2)/ 95 (1) / 2329 (1) / 7548 (1) / 7549(1)
Laying hens (S4)	155 (2) . FS (1) . bl*a*$_{TEM-1B}$ (1) 1308 (2) / 13 (1) / 226 (1) / 1011 (1) / 1629 (1) / 1706 (1) / 4481 (1)	**155 (2)** **. FS (1)** . bl*a*$_{TEM-1B}$ (1) 10 (2) . FS (1) . *gyrA83* (1) 746 (1) / 1850 (1) / NI (3)	355 (5) . FS (5) 162 (2) . FS 101 (2) / 278 (1)	**10 (2)** **. *tetA-sul1-bla*$_{TEM-1B}$ *-dfrA1 (2)*** **155 (1)** **. FS (1)** 48 (1) . *tetA*-GEN (1) 58 (1) . *tetA-sul1-bla*$_{TEM-1B}$ *-dfrA5* 394 (1) / 3076 (1) / NI (3)
Laying hens (S6)	1403 (5) / 1079 (1) / 7550 (1) / NI (1)	10 (3) . FS (3) 165 (1) / 205 (1) / 1303 (1) / 2614 (1) / 5797 (1)	**48** (1) . FS 10 (3) . FS (1) . bl*a*$_{TEM-1B}$ *b* (1) . *tetA* (1) 155 (1) . *tetA-sul3-bla*$_{TEM-1B}$*-cmlA* (1) 394 (1) / 746 (1) / 1246 (1) / 2935 (1)	**10 (1)** **. *tetA-sul1/sul2-bla*$_{TEM-1B}$ *-dfrA1*** **155 (1)** **. FS** 48 (2) . *tetA-dfrA1* (1) . *tetA-catA* (1) 1286 (1) / 3270 (1) / 3998 (1) /NI (2)

FS: fully susceptible; when genes where not detected, the phenotypic resistance profile was annotated as: TET: tetracycline; SMX: sulphonamides; AMP: ampicillin; TMP: trimethoprim; GEN: gentamicin; * = see Table 4; MLST types detected in consecutive samplings in each batch are market in bold and annotated with the AMR genes and/or chromosomal mutations detected.

broiler parent farms.

In our study, *E. coli* from laying hens showed the lowest AMR occurrence, considering both resistance percentages and resistance profiles. Nevertheless, some genes (like *sul3, qnrB19, dfrA5, cmlA1* and *bla* $_{TEM-106}$) were only detected in laying hens. Although this fact could be explained by the higher number of sequenced isolates from laying hens in our study, this would also indicate other sources for AMR bacteria. Dierikx et al. (2013) detected ESBL/AmpC producing *E. coli* from the floor of the poultry house before the start of the production period, suggesting an environmental source. According to Bortolaia et al. (2010) regarding farms of closed broiler production and high biosecurity, the farm environment, the feed and the animals are the scarce sources of bacteria. This would be also applicable to egg production.

Although AMR detected genes are widespread and abundantly reported in *E. coli* isolates from different animal species, there are very few reports of their detection in laying hens. Lanz et al. (2003) in a collection of 122 *E. coli* isolates from septicemia in Swiss laying hens detected *sul*1, *sul*2, *strA/strB*, *aadA*, *tetA* and *tetB*. Niero et al. (2018) studied 22 clinical *E. coli* isolates from Italian layer hen farms looking for genes related to resistance to the third-generation cephalosporins and quinolones and found *bla*$_{TEM-1b}$, *bla*$_{CMY-2}$ and *qnrS*1. Wasyl et al. (2012), looking also for cephalosporin resistance, detected *bla*$_{CTX-M-1}$ and *bla*$_{CMY-2}$ in nonpathogenic *E. coli* isolates from Polish laying hens. Cavicchio et al. (2015), looking for integrons in 31 avian pathogenic *E. coli* isolates from laying hens, identified *aad*A1, *dfr*A1 and *est*X. To the best of our knowledge, this is the first report of 11 AMR genes (*sul3, bla*$_{OXA-1}$, *bla*$_{TEM-106}$, *qnrB19, dfrA14, dfrA5, catA1, floR, cmlA1, aadA2* and *fosA7*) in *E. coli* from laying hens production. If these AMR genes were detected in isolates from eggs, a public health risk should be considered.

Discordant results in *E. coli* between AMR phenotype and genotype [established by microarray assay (Davis et al., 2011) or by WGS (Tyson et al., 2015], have been reported previously. It is to be noted that we were not able to detect genetic resistance in 15 isolates showing phenotypic resistance. Negative results for genetic resistance by WGS in isolates showing phenotypic resistance could be due to gene sequences fragmented during sequencing, resulting in two gene fragments in different contigs (like the genes *aac(3)VIa*, *tetB*, and *sul2* in our study; Table 4). Discordant result for sulphonamides would be related to the well-known difficulties in reading their MIC values; since two of these 15 isolates showed sulphonamides MIC values of 128 and 256 g/L but we do not detect resistance genes, we would reasonably reclassify them as non-resistant. In four indistinguishable trimethoprim resistant isolates from the same sample, we did not detect trimethoprim resistance genes. Trimethoprim resistance has been experimentally related to different mutations in the dihydrofolate reductase gene *fol*A (Toprak et al., 2011). One of these mutations, L28R, was detected by Moran et al. (2017), in an *E. coli* isolate showing phenotype-genotype discrepancy, but we did not detect any of them in our isolates. The in-depth analysis of these isolates in underway.

Isolates phenotypically resistant to azithromycin (MIC > 16 mg/L) have only been detected in hens in our study, but these isolates belonged to samplings where the isolates were not included in the WGS (S5, S7 and S8; Table 2). Tyson et al. (2015) and Moran et al. (2017)

detected the *mphA* gene (macrolide resistance) by WGS in *E. coli* isolates resistant to azithromycin and erythromycin, respectively. This finding will be checked in these isolates of our study.

Finally, bearing in mind that the old-day chicks from four hatcheries differed significantly in *E. coli* regarding AMR phenotypes/genotypes, MLST types and their relative abundances, the choice of a provider of day-old chicks according to this information would improve the occurrence of antimicrobial resistant bacteria in layer farms.

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