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Clonal and plasmid-mediated flow of ESBL/AmpC genes in *Escherichia coli* in a commercial laying hen farm

Irene Aldea [a], Alicia Gibello [b], Marta Hernández [c], Pimlapas Leekitcharoenphon [d], Valeria Bortolaia [e], Miguel A. Moreno [a,b,*]

[a] Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense de Madrid, Madrid, Spain
[b] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain
[c] Laboratorio de Biología Molecular y Microbiología, Instituto Tecnológico Agrario de Castilla y León (ITACYL), Valladolid, Spain
[d] Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Lyngby, Denmark
[e] Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhaguen, Denmark

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ABSTRACT

Resistance to third- and fourth-generation cephalosporins in *Escherichia coli* is mainly due to extended-spectrum beta-lactamases (ESBL) and AmpC cephalosporinases, which have been increasingly reported, mainly in isolates from humans and poultry.

The aim of this study was to address the flow of antimicrobial resistance determinants in the full laying hen production cycle (four batches followed from day-old chicks to 83/84-week-old layers), using cephalosporin-resistant *E. coli* as a model and their characterization using whole genome sequencing (WGS).

Fifteen out of 22 samples analysed yielded growth on MacConkey agar with cefotaxime (1 mg/L). Of these, 141 isolates were identified as *E. coli* and 47 were characterized by WGS.

Genes detected were three ESBL ($bla_{CTX\text{-}M\text{-}1}$ (n = 19); $bla_{CTX\text{-}M\text{-}14}$ (n = 1); and $bla_{SHV\text{-}12}$ (n = 9)) and one AmpC ($bla_{CMY\text{-}2}$ (n = 13)). Some isolates only harboured $bla_{TEM\text{-}1B}$ (n = 2) or $bla_{TEM\text{-}1D}$ (n = 1).

IncI1 plasmids were the main platform for ESBL/AmpC genes. In addition, five clones were identified harbouring $bla_{CTX\text{-}M\text{-}1}$ (two), $bla_{SHV\text{-}12}$ (one) and $bla_{CMY\text{-}2}$ (two), drawing a clone-plasmid mixed flow model.

Gene $bla_{CTX\text{-}M\text{-}1}$ was found in the chromosomal DNA of clone 1 over 14 months, and in IncI1/ST3 plasmids over six months; over six months $bla_{SHV\text{-}12}$ was found harboured by clone 3 (IncI1/ST26 plasmids), and 15 months later in a non-replicon detected plasmid. Finally, $bla_{CMY\text{-}2}$ spread for at least 16 months, mainly by IncK2 (including clone 4) and IncI1/ST12 (clone 5) plasmids.

Proper use of antimicrobials should be combined with other farm management strategies for the effective control of cephalosporin-resistant *E. coli* isolates in commercial layer farms.

1. Introduction

Beta-lactams are a broad family of antimicrobials that include the cephalosporins. *Escherichia coli* resistance to third- and fourth-generation cephalosporins is mainly due to extended-spectrum beta-lactamases (ESBL) and AmpC cephalosporinases, which are generally plasmid-encoded. Resistance to cephalosporins in *E. coli* can also be mediated by chromosomal point mutations in the *ampC* promoter causing its overexpression.

Nearly 20 years ago, as part of the Spanish Antimicrobial Resistance Surveillance Programme, we reported the first detection of two ESBL ($bla_{SHV\text{-}12}$ and $bla_{CTX\text{-}M\text{-}14}$) and one AmpC ($bla_{CMY\text{-}2}$) genes in *E. coli* from broilers. Since then, ESBL/AmpC-producing *E. coli* have been increasingly detected, mainly from humans and healthy animals, especially poultry. Consequently, poultry (mainly broilers and to a lesser extent laying hens) became an important reservoir for these resistance traits (Saliu et al., 2017), posing a public health problem as a potential source of AMR bacteria in humans after consuming meat or eggs. Although studies of ESBL/AmpC *E. coli* in eggs are rare, both positive (presence of *E. coli* harbouring $bla_{CTX\text{-}M\text{-}2}$ in eggs (Grande Burgos et al., 2016) and

* Correspondence to: Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro, s/n, Madrid 28040, Spain.
E-mail address: mamoreno@ucm.es (M.A. Moreno).

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E. coli with ESBL phenotype in raw-egg surfaces (Rasheed et al., 2014) and negative (no detection Stewardson et al., 2014) results have been reported.

The types of ESBL-producing Enterobacteriaceae and their occurrence in poultry has been reviewed (Saliu et al., 2017), and many studies have reported the detection of cephalosporin-resistant *E. coli* in healthy poultry, including layers, and some in clinical isolates (Niero et al., 2018). Most of these studies are cross-sectional or surveillance studies focused on cephalosporin-resistant *E. coli* detection in healthy broilers (revised by Saliu et al., 2017), longitudinal studies following cephalosporin-resistant *E. coli* transmission across the broiler production pyramid (Apostolakos et al., 2019; Dame-Korevaar et al., 2017; Mo et al., 2014; Zurfluh et al., 2014), or longitudinal studies at the farm level on the dynamics of CTX-phenotypes (Baron et al., 2018). Although there are also some studies focused on or including laying hens (Baez et al., 2021; Blaak et al., 2015; Ceccarelli et al., 2019; Seo and Lee, 2019; among others), we found no longitudinal studies of the dynamics of different AMR-phenotypes at the farm level in layers.

The aim of this study was to address the flow of AMR determinants in the full laying hen production cycle using cephalosporin-resistant *E. coli* as a model. Having in mind that the use of antimicrobials throughout this commercial cycle is usually scarce, other additional factors should be involved if a flow could be detected.

2. Methods

Details about setting, sampling and sample preparation have been published elsewhere (Moreno et al., 2019). Briefly, four batches (B1, B2, B3 and B4) of hens raised in a commercial laying hen farm in Spain were followed from day-old chicks to 83–84-week-old hens, performing eight litter samplings (from S1 to S8) from March 2016 to October 2018 (Table 1). The farm had two facilities separated by about five kilometres: one grouped the two growing sites (GS-A and GS-B) and the other the four laying houses (LH-C, LH-D, LH-E and LH-F). No antibiotics were used during this longitudinal study, but two five—day colistin treatments were recorded, as previously reported (Moreno et al., 2019), in batches 1 (at week 24) and 2 (at week 23). Only samplings one to four, and seven or eight (only the last grew in MacConkey agar with cefotaxime [CTX]) have been included in this study.

3. Bacterial isolation, identification and characterization

Peptone water-diluted samples (10 g in 90 mL) were maintained at room temperature for one hour before being serially diluted tenfold with peptone water. Then, 0.1 mL of three dilutions (usually the first, second and third dilutions) were plated on MacConkey agar plates containing CTX at 1 mg/L and incubated at 37 ºC for 20–24 h. Ten lactose positive isolates of different morphology, if any, were taken from different plates and dilutions, and PCR bacterial identification was performed as described previously (Moreno et al., 2019).

The *E. coli* isolates were tested for susceptibility to antimicrobials by broth microdilution using EUVSEC commercial plates (Sensititre@, Thermofisher) (Moreno et al., 2019). EUCAST epidemiological cut-off values were used to interpret the minimum inhibitory concentration (MIC) values. Only no wild-type isolates for CTX (MIC >= 0.25 mg/L) or ceftazidime (MIC <= 0.50 mg/L) were selected for analysis.

4. Whole genome sequencing (WGS) and bioinformatic analysis

A subset of 46 isolates was sent to the Laboratorio Tecnológico Agrario de Castilla y León (ITACYL) for WGS analysis, which was performed as described elsewhere (Moreno et al., 2019) and summarized in Supplementary file 1. Forty-five isolates were chosen according to the number of different AMR phenotypic profiles observed in each sampling (at least half of the AMR phenotypic profiles of each sampling were represented in the sequenced isolates). The remaining was a CTX-resistant isolate (L3M1-CIP10) recovered in MacConkey agar plates supplemented with ciprofloxacin (0.125 mg/L). In addition, a previously sequenced CTX-resistant isolate (L1M3–09), recovered from the farm using MacConkey agar plates with no antibiotic (Moreno et al., 2019), was added to the study.

Multilocus sequence typing (ST) profiles were predicted using MLST

Table 1
Summary of data regarding samplings and *E. coli* isolation (MacConkey with cefotaxime 1 mg/L), AMR phenotyping and sequencing, from a commercial laying hen farm.

Batch	Sampling	Animal type	Sampling week	Site	Sampling date	Nº of isolates and (antibiograms performed)	Number of AMR phenotypic profiles and (sequenced isolates)
B1	S1	Day-old chicks	0	GS-A	18/03/2016	No growth	
B1	S2	Pullets	2	GS-A	05/04/2016	10 (10)	1 (1)
B1	S3	Pullets	14	GS-A	24/06/2016	10 (6)	2 (2)
B1	S4	Laying hens	24	LH-C	08/09/2016	10 (10)	1 (2)
B1	S8	Laying hens	83	LH-C	30/10/2017	6 (6)	3 (2)
B2	S1	Day-old chicks	0	GS-B	27/05/2016	No growth	
B2	S2	Pullets	2	GS-B	13/06/2016	7 (6)	1 (2)
B2	S3	Pullets	15	GS-B	14/09/2016	10 (10)	3 (5)
B2	S4	Laying hens	24	LH-D	15/11/2016	10 (10)	6 (5)
B2	S7	Laying hens	68	LH-D	25/09/2017	9 (9)	3 (2)
B2	S8	Laying hens	83	LH-D	09/01/2018	No growth	
B3	S1	Day-old chicks	0	GS-B	11/10/2016	7 (7)	3 (3)
B3	S2	Pullets	2	GS-B	25/10/2016	10 (10)	5 (5)
B3	S3	Pullets	14	GS-B	17/01/2017	10 (10)	3 (4)
B3	S4	Laying hens	23	LH-E	22/03/2017	3 (3)	2 (2)
B3	S8	Laying hens	85	LH-E	06/06/2018	9 (9)	3 (2)
B4	S1	Day-old chicks	0	GS-B	13/03/2017	No growth	
B4	S2	Pullets	2	GS-B	27/03/2017	No growth	
B4	S3	Pullets	13	GS-B	15/06/2017	10 (10)	–
B4	S4	Laying hens	23	LH-F	22/08/2017	10 (10)	5 (7)
B4	S7	Laying hens	67	LHS-F	02/07/2018	10 (10)	2 (1)
B4	S8	Laying hens	82	LH-F	15/10/2018	No growth	

GS: Growing site. LH: Laying house

v2.0. Non previously detected ST types were submitted to EnteroBase (https://enterobase.warwick.ac.uk/). AMR genes and chromosomal point mutations were studied using ResFinder software v.4.1 that contains PointFinder software, selecting as parameters 90% of threshold for %ID and 60% of minimum length for both applications. The search for integrases was performed with blastx. Plasmidfinder v2.1 and pMLST v2.0 were used to detect plasmid origin of replication and to predict plasmid multilocus sequence type, respectively.

The phylogenetic analysis was performed with CSI Phylogeny v.1.4 (Kaas et al., 2014), and the resulting phylogenetic trees were uploaded to iTOL v4 (Letunic and Bork, 2019) for annotation. Isolates differing by 40 or fewer single nucleotide polymorphisms (SNPs) were considered clones.

The CONTIGuator pipeline (Galardini et al., 2011) was used to map contigs against reference sequences of the putative plasmids and integrons detected. These reference sequences, when available, were chosen from poultry *E. coli*. Then, blastn was used for an additional comparison of the sequences obtained from the CONTIGuator using the previous reference sequences to check for identity and coverage.

5. Results

5.1. Detection of cephalosporin-resistant E. coli

Only samples from one of the four batches (batch 3) showed bacterial growth on CTX selective medium in samples from day-old chicks. Nevertheless, samples from all but one batch (batch 4) had growth on this medium in the second (two week pullets) and third samplings (14-week pullets).

All batches showed growth in CTX selective medium in samples from 23 to 24-week laying hens, but only batches 1 and 3 showed growth in the last sampling (82–85-week hens) (Table 1).

Overall, 15 out of 22 samples analysed yielded growth on CTX selective medium. From these, a total of 141 isolates were recovered and identified as *E. coli*.

6. Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed on 136 isolates. Fourteen isolates had CTX MIC values below 0.5 mg/L and were excluded from the study, except for one that had a ceftazidime MIC value of 1 mg/L. The remaining 122 isolates had CTX MIC values of over 2 mg/L and ampicillin MIC values of over 64 mg/L. In 89 of these 122 isolates, ceftazidime MIC values were equal to or higher than 1 mg/L, while in three of them MIC values below 1 mg/L. In all the 123 isolates, meropenem MIC values were below 0.12 mg/L. MIC values observed for colistin and tigecycline were below 2 mg/L and 16 mg/L, respectively.

In addition, 103 isolates presented a multidrug resistance profile, being resistant to at least three classes of antimicrobials.

Nineteen phenotypic AMR profiles were observed among these 123 isolates. The number of different AMR profiles per sample ranged from one to six, with the lowest variability found in batch 1 (Table 2). The AMR profile most frequently observed included resistance to quinolones (ciprofloxacin and nalidixic acid) and sulphonamides, in addition to resistance to beta-lactams (ampicillin, cefotaxime, and ceftazidime), and was detected in all batches and samplings, except sampling 8. At least 27 AMR phenotypic profiles were detected in two or more isolates from the same sample, and 10 isolates with the same AMR phenotypic profile were detected in batch 1 (samplings two and four) (Table 2).

7. Molecular characterization of cephalosporin-resistant *E. coli*

Among the 47 sequenced isolates we detected fifteen previously described STs, being ST155 (n = 9), ST93 (n = 8) and ST4243 (n = 6) the most frequent. In addition, two isolates were assigned to novel STs named ST13021 (close to ST155) and ST 13030 (close to ST170), whereas one isolate cannot be fully assigned and was recorded as close to ST1818.

The IncI1 replicon type was the most detected (IncI1–3, n = 10; IncI1–26, n = 7; IncI1–12, n = 3), followed by IncX1 (n = 13), IncK2 (n = 10), IncA/C2 (n = 5) and IncFII (n = 3). Seven isolates harboured IncI1–26 + IncX1 and five IncX1 + IncA/C2.

Table 2
Distribution by batch, sampling and antimicrobial resistance (AMR) profile of 124 *E. coli* isolated in medium containing cefotaxime (CTX) at 1 mg/L, from a commercial laying hen farm.

AMR profile	Batch	B1					B2					B3					B4				
	Sampling	S1	S2	S3	S4	S8	S1	S2	S3	S4	S7	S1	S2	S3	S4	S8	S1	S2	S3	S4	S7
AMP-CTX/TAZ-CIP/NAL-SMX-TET-TMP				4					3	1				4					9	3	
AMP-CTX/TAZ-CIP/NAL-SMX			10	2					4					3					1	1	
AMP-CTX/TAZ-SMX-TET					10	4						4	1								
AMP-CTX/TAZ								6			1		1			1					
AMP-CTX/TAZ-CIP-SMX-TET-CHL									3	3					2						
AMP-CTX/TAZ-CIP/NAL-SMX-TET												2	1		1						
AMP-CTX/TAZ-SMX-TET-TMP																5					1
AMP-CTX/TAZ-SMX-CHL										2										3	
AMP-CTX-SMX-TET						1															2
AMP-CTX/TAZ-SMX-TET-AZI						1										1					
AMP-CTX/TAZ-SMX													6								
AMP-CTX/TAZ-CIP/NAL														3							
AMP-TAZ-TET											3										
AMP-CTX-SMX-TET												1									
AMP-CTX/TAZ-SMX-TET-TMP													1								
AMP-CTX/TAZ-CIP/NAL-SMX-TET-CHL										2											
AMP-CTX/TAZ-CIP/NAL-SMX-TMP																				2	
AMP-CTX/TAZ-CIP/NAL-SMX-TET-TMP-GEN-AZI											2										
AMP-CTX/TAZ-CIP/NAL-SMX-TET-TMP										1											
AMP-CTX-CHL-CIP-SMX-TET-GEN										1											
AMP-CTX-CIP/NAL-SMX-TET-TMP																				1	

AMP: ampicillin; TET: tetracycline; SMX: sulfonamides; CIP: ciprofloxacin; NAL: nalidixic acid; TMP: trimethoprim; CHL: chloramphenicol; GEN: gentamicin; CTX: cefotaxime; TAZ: ceftazidime; AZI: azithromycin.

In the 47 isolates sequenced, cephalosporin resistance was mainly associated with ESBL/pAmpC-encoding genes. The genes detected in this study were three ESBL (*bla*$_{CTX-M-1}$ [n = 19]; *bla*$_{CTX-M-14}$ [n = 1]; and *bla*$_{SHV-12}$ [n = 9]) and one pAmpC (*bla*$_{CMY-2}$ [n = 13)]. In addition, three types of mutations (−18 and −1, always found together, [n = 14], and +24 [n = 8]) were found in the *ampC* gene and/or its promoter (n = 22). In all cases, these mutations were found in isolates harbouring ESBL/pAmpC genes. Three isolates only harboured *bla*$_{TEM-1B}$ (n = 2) or *bla*$_{TEM-1D}$ (n = 1), although these *bla* genes were also detected in 15 of the previously mentioned isolates: *bla*$_{TEM-1B}$ in combination with *bla*$_{CTX-M-14}$ (n = 1), *bla*$_{SHV-12}$ (n = 7) and *bla*$_{CMY-2}$ (n = 2), and *bla*$_{TEM-1D}$ in combination with *bla*$_{CTX-M-1}$ (n = 5). Into the remaining isolate we did not detect either *bla*/AmpC genes or AmpC mutations.

The gene *bla*$_{CTX-M-14}$ was identified in the chromosomal DNA of one ST2599 isolate (batch 2/S4, 67-week laying hens/laying house D). The isolate also carried *bla*$_{TEM-1B}$ and the sulphonamide- and tetracycline-resistance genes *tetA* and *sul2,* respectively, as well as a class 1 integron carrying *aadA5* (streptomycin/spectinomycin resistance) and *dfrA1* (trimethoprim resistance) genes.

8. Dynamics of bla$_{CTX-M-1}$ in the farm

Gene *bla*$_{CTX-M-1}$ was the most frequently detected ESBL gene (n = 19 isolates) in the farm, among which we identified two clones (Fig. 1). Clone 1 comprised eight ST93 isolates harbouring the gene in the chromosome, while clone 2 (three ST117 isolates) harboured the gene in IncI1/ST3 plasmids. Gene *bla*$_{CTX-M-1}$ was also located in IncI1/ST3 plasmids in eight additional isolates with other ST profiles (Table 3).

All the *bla*$_{CTX-M-1}$ (in plasmids IncI1/ST3 or chromosomes) containing isolates harboured an IS*Ecp1* element located in the upstream region of the *bla*$_{CTX-M-1}$ gene. CONTIGuator assembled contigs of 11 isolates containing *bla*$_{CTX-M-1}$ plus IS*Ecp1* (size between 110,613 and 121,618 bp) in IncI1/ST3 plasmids, were compared with the *bla*$_{CTX-M-1}$ plus IS*Ecp1* sequence of a published IncI1/ST3 plasmid (Accession no.

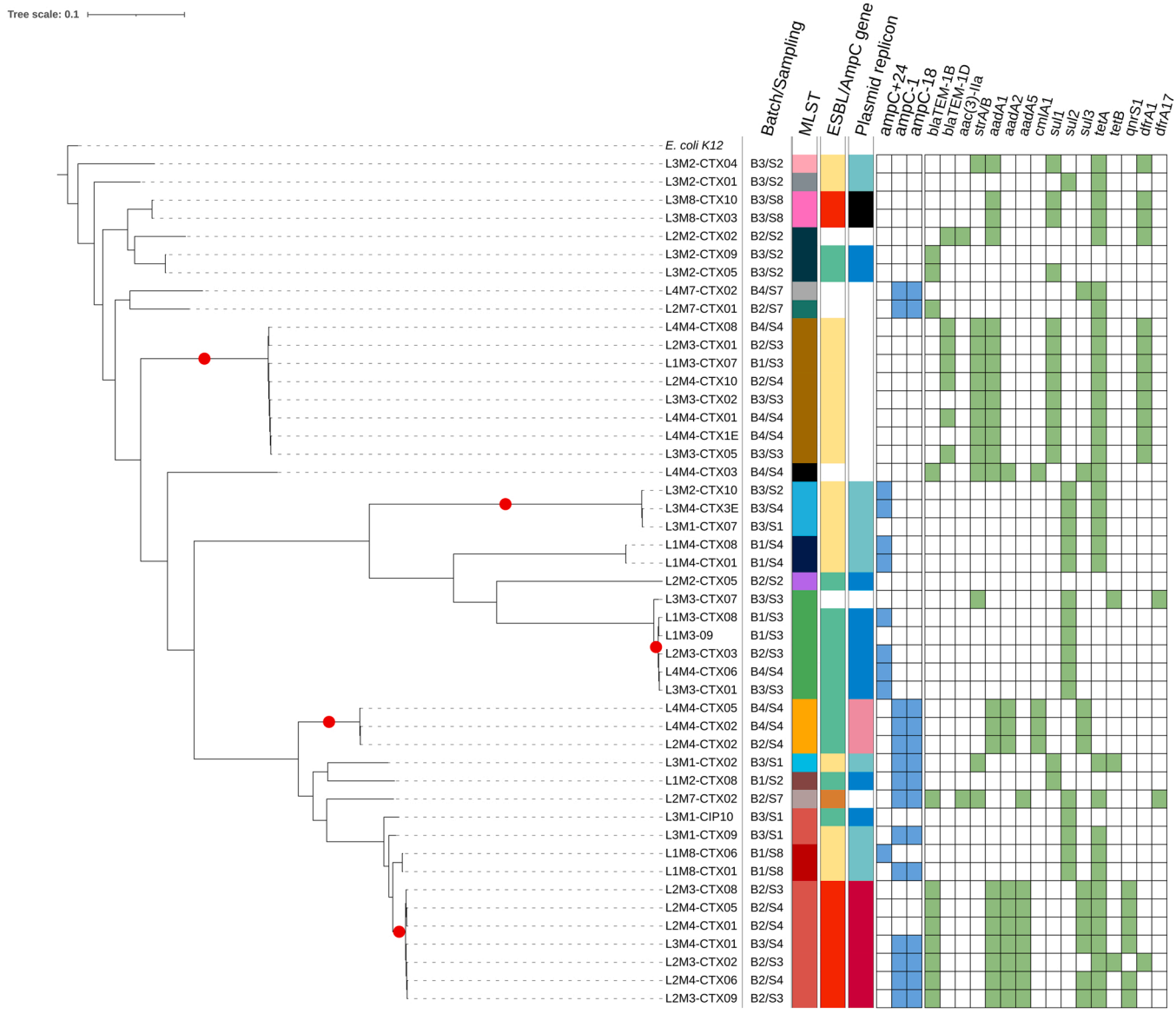

Fig. 1. Phylogenetic tree of the 47 *Escherichia coli* isolates from a commercial layer hen farm, *E. coli* K12 was used as reference genome, First column: batch and sampling; second colum: ST profile; third column: ESBL/pAmpC gene detected; fourth column: plasmid replicon; subsequent columns: presence (green)/absence (white) of point mutations in the AmpC promotor and antibiotic resistance genes, Isolates differing by 40 or fewer single nucleotide polymorphisms (SNPs) were considered clones and are represented with a red dot. Clone 1 corresponds to *bla*$_{CTX-M-1}$/ST93 isolates; clone 2 corresponds to *bla*$_{CTX-M-1}$/ST117 isolates; clone 3 corresponds to *bla*$_{SHV-12}$/ST155 isolates; clone 4 corresponds to *bla*$_{CMY-2}$/ST4243 isolates; and clone 5 corresponds to *bla*$_{CMY-2}$/ST4038 isolates.

Fig. 1. (*continued*).

KM377240) from broiler *E. coli* from Switzerland (Zurfluh et al., 2014), and 98%–100% were identical (93%–96% coverage).

All the 11 IncI1/ST3 plasmids also harboured *sul*2 and *tetA*. In addition, one of the isolates (ST1716) harbouring this plasmid also had a class 1 integron, located in a non-typeable plasmid, containing *dfrA1* and *aadA1* (streptomycin/spectinomycin resistance).

All the isolates of clone 1 had a class 1 integron, carrying resistance genes *dfrA1, aadA1* and *sul1* (sulphonamides resistance). This integron was detected in two different plasmid types (IncA/C2 and IncFII) (Table 3). Six of these isolates (from batches 1, 2 and 4) also had an IncX1 plasmid harbouring *tetA* and $bla_{\text{TEM-1D}}$.

The chronology of the platforms where $bla_{\text{CTX-M-1}}$ was detected in the farm can be followed in Table 3. Clone 1 was first identified in the farm in batch 1/S3 (14-week pullets/growing site A). Three months later, it was identified in batch 2/S3 (14-week pullets/growing site B), and two months later in the 24-week hens (S4) of this batch that were moved to laying house D.

This clone was again identified two months later in isolates of batch 3/S3 (14-week pullets/growing site B). Finally, it was identified in two isolates of batch 4/S4 (23-week hens/laying house F).

The IncI1/ST3 plasmid containing the gene $bla_{\text{CTX-M-1}}$ was firstly detected in the farm in ST5853 isolates from batch 1/S4 (24-week hens/laying house C) (Table 3). One month later, it was detected in isolates of three different ST profiles (ST155, 602 and 117/clone 2) of batch 3/S1 (day-old chicks/growing site B), and again in the subsequent sampling (S2) of this batch (two week pullets/growing site B) in three isolates (ST10, 1716 and 117/clone 2) (Table 3).

Five months later, the IncI1/ST3 plasmid containing the gene $bla_{\text{CTX-M-1}}$ was detected in the same batch 3/S4 in a ST117/clone 2 isolate (23-week laying hens/laying house E).

Finally, seven months later, it was identified in an isolate with an ST close to 155 in batch 1/S8 (83-week hens/laying house C).

In summary, $bla_{\text{CTX-M-1}}$ gene has been found in chromosomal DNA in clonal ST93 isolates over 14 months in the farm in the four batches, and in *IncI1/ST3* plasmids over six months (two batches) harboured by isolates with three different MLST profiles, including clone 2.

9. Dynamics of gene $bla_{\text{SHV-12}}$ in the farm

In the farm, the gene $bla_{\text{SHV-12}}$ was identified in nine isolates belonging to two different ST types (ST115 and 6616) and harboured by two different plasmids (Table 3). Seven ST155 isolates harboured the gene on *IncI1/ST26* plasmids and were grouped as clone 3 (Fig. 1), whereas the two remaining isolates belonged to ST6616 and harboured the gene on non-typeable plasmids. $bla_{\text{SHV-12}}$ in IncI1/ST26 plasmids was associated with an IS*26* insertion sequence and the CONTIGuator assembled contigs (size between 115,209 and 130,170 bp) of seven isolates showed 99% similarity (96%–98% coverage) with the same structure described in plasmid pCAZ590 from broiler *E. coli* (Alonso et al., 2017) (Accession no. LT669764).

The IncI1/ST26 plasmids also harboured *tetA* and a class 1 integron carrying *aadA1* and *aadA2*, the sulfonamide resistance gene *sul3*, and the chloramphenicol resistance gene *cmlA1*.

The sequence of the class 1 integron of six isolates was compared with a published isolate (Accession no. HQ875017) identified in IncI1 plasmids in ESBL *E. coli* (Curiao et al., 2011), showing 100% coverage and identity.

All the isolates belonging to clone 3 had an additional IncX1 plasmid, harbouring $bla_{\text{TEM-1B}}$ and the fluoroquinolone resistance gene *qnrS1*, and the two ST6616 isolates also had *tetA* and a chromosomal class 1 integron harbouring *dfrA1*, *aadA1* and *sul1*.

The chronology of $bla_{\text{SHV-12}}$ in the farm showed that clone 3 was first identified in batch 2/S3 (15-week pullets/growing site B), then at week 24 (S4) in the same batch moved to a laying house (D), and again four months later in batch 3/S4 (23-week laying hens/laying house E).

Therefore, the $bla_{\text{SHV-12}}$ gene was found in batches 2 and 3 over six months harboured by clone 3 in the same type of plasmids type and 15 months later in a no-typeable plasmid in ST 6616 isolates from 83-week

Table 3
Distribution by origin and features of the 42 *E. coli* isolates containing ESBL/pAmpC genes from a commercial layer hen farm in Spain.

Batch/ Sampling/ site Isolation date	ST	ESBL/ AmpC gene (n)	AmpC mutations (nn)	Plasmid replicon harbouring ESBL/AmpC (other AMR genes and/or integrons) (nn)	Plasmid replicons containing other AMR genes and/ or integrons
B1/S2/A 05/04/ 2016	937	bla_{CMY-2}	-18, −1	IncK2	
B2/S2/B 16/06/ 2016	1158	bla_{CMY-2}		IncK2	
B1/S3/A 24/06/ 2016	4243 (Clone 4)	bla_{CMY-2} (2)	+24 (1)	*IncK2*	
	93 (clone 1)	$bla_{CTX-M-1}$		Chromosomal	IncA/C2 *(int1-dfrA1-aadA1-sul1)* + IncX1 *(bla_{TEM-1D} + tetA)*
B1/S4/C 08/09/ 2016	5853	$bla_{CTX-M-1}$ (2)	-18, −1	IncI/ST3 (*sul2-tetA)*	
B2/S3/B 14/09/ 2016	155 (Clone 3)* *	bla_{SHV-12} (3)	-18, −1 (2)	IncI1/ST26 [*tetA* + (*IntI1-aadA1-aadA2-sul3-cmlA1*)]	IncX1 *(qnrS1-bla_{TEM-1B})*
	4243 (Clone 4)	bla_{CMY-2}	+24	IncK2	
	93 (Clone 1)	$bla_{CTX-M-1}$		Chromosomal	IncA/C2 *(int1-dfrA1-aadA1-sul1)* + IncX1 *(bla_{TEM-1D} + tetA)*
B3/S1/B 11/10/ 2016	155 *	bla_{CMY-2}		IncK2	
	155 * 117 (Clone 2) *	$bla_{CTX-M-1}$ (3)	-18, −1 (2)	IncI/ST3 (*sul2-tetA)*	
	602		-18, −1		
B3/S2/B 25/10/ 2016	48 **	bla_{CMY-2} (2)		IncK2	
	10	$bla_{CTX-M-1}$ (3)		IncI/ST3 (*sul2-tetA)*	
	117 (Clone 2) *		+24	No-replicon detected plasmid	
	1716				
B2/S4/D 15/11/ 2016	155 (Clone 3)	bla_{SHV-12} (3)	-18, −1 (2)	IncI1/ST26 [*tetA* + (*IntI1-aadA1-aadA2-sul3-cmlA1*)]	IncX1 *(qnrS1-bla_{TEM-1B})*
	93 (Clone 1)	$bla_{CTX-M-1}$		Chromosomal	IncA/C2 *(int1-dfrA1-aadA1-sul1)* + IncX1 *(bla_{TEM-1D} + tetA)*
	4038 (clone 5) *	bla_{CMY-2}	-18, −1	IncI1/ST12	
B3/S3/B 17/01/ 2017	93 (Clone 1)	$bla_{CTX-M-1}$ (2)		Chromosomal	IncFII *(IntI1-dfrA1-aadA1-sul2)*
	4243 (Clone 4)	bla_{CMY-2}	+24	IncK2	
		bla_{SHV-12}	-18, −1	IncI1/ST26 [*tetA* + (*IntI1-aadA1-aadA2-sul3-cmlA1*)]	*IncX1 (qnrS1-bla_{TEM-1B})*
	155 (Clone 3)				
	117 (Clone 2) *	$bla_{CTX-M-1}$	+24	IncI/ST3 (*sul2-tetA)*	
B4/S4/F 22/08/ 2017	93 (Clone 1) (3)	$bla_{CTX-M-1}$ (3)		Chromosomal	IncA/C2 *(int1-dfrA1-aadA1-sul1)* + IncX1 *(bla_{TEM-1D} + tetA)* (2) IncFII *(IntI1-dfrA1-aadA1-sul2)* (1)
	4243 (Clone 4)	bla_{CMY-2}	+24	IncK2	
	4038 (Clone 5)	bla_{CMY-2} (2)	-18, −1	IncI1/ST12	
B2/S7/F 25/09/ 2017	2599	$bla_{CTX-M-14}$	-18, −1	Chromosomal	
B1/S8/C 30/10/ 2017	13021 **	$bla_{CTX-M-1}$ (2)	-18, −1	IncI/ST3 (*sul2-tetA)*	
B3/S8/C 06/06/ 2018	6616	bla_{SHV-12} (2)		No-replicon detected plasmid	

n = number of isolates if higher than 1; nn = number of isolates if different than n; * / **= 1 or 2 isolates with plasmid replicon and ESBL/pAmpC gene into the same contig

layers (batch 3/S8/ laying house E).

10. Dynamics of bla_{CMY-2} in the farm

The AmpC beta-lactamase gene bla_{CMY-2} was the second most widely identified in the farm, and was mainly located in IncK2 plasmids harboured by five isolates of ST4243 (clone 4), two ST48 isolates and one isolate of ST1158, ST155 and ST973, respectively (Table 3). It was also was detected in IncI1/ST12 plasmids harboured by three ST4038 isolates (clone 5).

Gene bla_{CMY-2} in IncK2 plasmids was located downstream to an IS*ECp1* insertion sequence and upstream to genes *blc* and *sugE,* encoding a lipoprotein and a guanidinium exporter, respectively. The CONTIGuator-assembled sequences containing this arrangement in IncK2 plasmids (10 isolates) were compared with those of the plasmid pDV45 from Italian broiler *E. coli* (Apostolakos et al., 2020a) (Accession no. KR905384.1), obtaining 99% similarity (90%−100% coverage) with the IS*Ecp1*-bla_{CMY-2}-*blc*-*sugE* structure.

IncI1/ST12 plasmids harbouring bla_{CMY-2} had an IS*ECp1* element upstream and the CONTIGuator-assembled sequences (three isolates) were compared with a similar structure described by Roer et al. (2019) (Accession no. MH472638), obtaining 93% similarity (100% coverage).

The three ST4038 isolates (clone 5) also harboured non-typeable plasmids containing a class 1 integron with *aadA1, aadA2* and *cmlA1* genes (Fig. 1).

The chronology of bla_{CMY-2} showed that the IncK2 plasmids harbouring this gene were first detected in the farm in a ST973 isolate from batch 1/S2 (two week pullets/growing house A), and two months later

in an ST1158 isolate from batch 2/S2 (two week pullets/growing house B). In both batches, the plasmids were also detected in the following sampling (S3/14 and 15 weeks pullets, respectively), but now harboured by isolates belonging to clone 4 (ST4243) (Table 3).

Although this IncK2 plasmid was no longer detected in these two batches, *bla*$_{CMY-2}$ was detected again in batch 2/S4 (24-week hens/laying house D) harboured by the IncI1/ST12 plasmid in an isolate of clone 5. This clonal isolate was also detected eight months later in the farm in batch 4/S4 in 23-week laying hens, coexisting with clone 4.

In addition, the IncK2 plasmid harbouring *bla*$_{CMY-2}$ was detected in three consecutive samplings from batch 3 (from day-old chicks to 14-week pullets) harboured by three different ST types (ST155, 48 and 4233/clone 4) (Table 3). Therefore, the *bla*$_{CMY-2}$ gene had been spreading in the farm for at least 16 months, mainly harboured by IncK2 plasmids.

11. Discussion

When a non-selective medium was used, only one ESBL/pAmpC *E. coli* isolate was detected in this farm (Moreno et al., 2019), showing that this complex phenotype was not predominant in the farm over the 31 months covered by our study. Nevertheless, different isolates grew when selective screening with 1 mg/L of CTX was performed, proving that ESBL/pAmpC *E. coli* were present in the farm. A comparison between prevalence results using selective (CTX at 1 mg/L) and non-selective medium has been performed by Apostolakos et al. (2020b) showing "no bias towards particular ESBL/pAmpC-EC genotypes from the selective method or underestimation by the non-selective approach". Nevertheless, a comparison between 1 and 8 mg/L of CTX was performed in our laboratory (Moreno et al., 2007), showing higher prevalence and phenotypic diversity when using the lower CTX concentration.

Most of the studies on ESBL/AmpC *E. coli* used CTX at 1 mg/L as selective antibiotic concentration (Apostolakos et al., 2019; Chauvin et al., 2013; Dame-Korevaar et al., 2017; Dierikx et al., 2013; Wasyl et al., 2012), and this is the concentration recommended by the EU Reference laboratory for AMR (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/399_esbl-ampc-quantification-protocol-19-03-2018.pdf). Nevertheless, higher CTX concentrations, such as 2 mg/L (Mo et al., 2014) and even 8 mg/L (Baez et al., 2021), have been also used. Since the EUCAST epidemiological cut-off value for CTX is 0.25 mg/L, all these techniques are unable to detect isolates with low cefotaxime resistance levels.

12. ESBL and AmpC β-lactamase genes detected

All the identified genes in our study have previously been detected in poultry (Saliu et al., 2017) and in *E. coli* from laying hens: *bla*$_{CTX-M-1}$ (Baez et al., 2021; Blaak et al., 2015; Ceccarelli et al., 2019; Chauvin et al., 2013; Niero et al., 2018; Wasyl et al., 2012), *bla*$_{CTX-M-14}$ (Blaak et al., 2015; Seo and Lee, 2019; Shim et al., 2019), *bla*$_{SHV-12}$ (Blaak et al., 2015) and *bla*$_{CMY-2}$ (Ceccarelli et al., 2019; Chauvin et al., 2013; Seo and Lee, 2019; Shim et al., 2019; Wasyl et al., 2012). Other ESBL genes reported in *E. coli* from layers are *bla*$_{CTX-M-15}$ (Baez et al., 2021; Shim et al., 2019), *bla*$_{LAP-2}$ (Baez et al., 2021) and *bla*$_{TEM-52}$ (Blaak et al., 2015).

13. Plasmids harbouring ESBL/AmpC genes

At least in one isolate on each pattern we found a plasmid replicon and a ESBL/pAmpC gene in the same contig, proving that IncI1 is the main platform harbouring *bla*$_{CTX-M-1}$, *bla*$_{SHV-12}$ and *bla*$_{CMY-2}$. Our results confirm previous reports from poultry *E. coli* (reviewed by Saliu et al., 2017). Similarly, the presence of *bla*$_{CMY-2}$ in a plasmid belonging to incompatibility group IncK has also been previously reported in *E. coli* from poultry (Agerso et al., 2014; Apostolakos et al., 2020a; Seiffert et al., 2017). In fact, we detected *bla*$_{CMY-2}$ in the IncK subgroup designated IncK2 by Seiffert et al. (2017), and detected it from poultry, poultry meat and humans. Unfortunately, in the afore-mentioned studies, plasmid data from laying hens was only provided by Niero et al. (2018), who also detected *bla*$_{CTX-M-1}$ in an IncI1 plasmid, and Ceccarelli et al. (2019), whose results were similar since they detected *bla*$_{CTX-M-1}$ in IncI1 plasmids and *bla*$_{CMY-2}$ in both IncI1 and IncK plasmids.

A ESBL *bla*$_{SHV-12}$ gene (with a close class 1 integron gene) harboured by an IncI1/ST26 plasmid in ST isolates has been previously described in *E. coli* isolated from broilers (Alonso et al., 2017).

Nevertheless, there are no previous studies reporting the presence of *bla*$_{SHV-12}$ in *E. coli* ST6616 isolates.

13.1. Chromosomal location of *bla*$_{CTX-M}$ genes

Chromosomal integration of *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ genes has already been described in *E. coli* clinical isolates (Rodriguez et al., 2014), but not in isolates from poultry, such as *bla*$_{CTX-M-14}$ in our study.

In the case of *bla*$_{CTX-M-1}$, we found no reports of its chromosomal integration in *E. coli*, but only in clinical human strains of *Proteus mirabilis* (Song et al., 2011) and *Enterobacter cloacae* (Liu et al. (2015).

In our ST93 isolates, the chromosomal gene *bla*$_{CTX-M-1}$ was located near an insertion sequence IS*1380* (IS*Ecp1*), which has also been identified in the plasmid close to the same gene, suggesting it is involved in improving chromosomal integration of *bla*$_{CTX-M-1}$ (Hamamoto and Hirai, 2019). Although in our study the *bla*$_{CTX-M-14}$ gene was not found to be associated with an IS*ECp1* element, this transposase could also facilitate the insertion of other *bla*$_{CTX-M}$ genes.

14. Flow and persistence of ESBL/AmpC genes in the farm

The detection of *bla*$_{CTX-M-1}$ in different ST types of *E. coli* in a farm has been previously reported. Blaak et al. (2015), in a study with five laying hen farms, detected between four and 11 different ST *E. coli* isolates harbouring *bla*$_{CTX-M-1}$ in the same farm. Baez et al. (2021), also examining layers, detected *bla*$_{CTX-M-1}$ in at least 11 ST types. Among the ST types harbouring bla$_{CTX-M-1}$ in our study, only ST10 was also detected by these authors.

The dynamics of ESBL/pAmpC *E. coli* in poultry farms during the rearing period has been studied by Dierikx et al. (2013) and Baron et al. (2018). Dierikx et al. (2013), in a five-week longitudinal study at three commercial broiler farms, detected ESBL/AmpC *E. coli* from day-old chick to week five in three of four batches, whereas Baron et al. (2018), in the follow-up of several broiler flocks, detected IncI1/ST3 plasmids harbouring *bla*$_{CTX-M-1}$ from day two to seven (one flock) and from day two to 41 (two flocks). Although the authors did not discuss the clonal spread of *bla*$_{CTX-M-1}$ in these farms, isolates from days 2 and 41 only belonged to the same phylogenetic group in one flock, suggesting that *bla*$_{CTX-M-1}$ spread in these farms was mainly plasmid-mediated. Our results suggest longer survival periods (up to 14 months) in layers.

In the same study, Baron et al. (2018) also detected *bla*$_{CMY-2}$ in IncI1/ST12 plasmids from day zero to two (one flock) and from day two to seven (two flocks). All these isolates were of the phylogenetic group A. In our study, the IncI1 plasmids harbouring *bla*$_{CMY-2}$ were not detected in *E. coli* isolates from the same batch.

Dame-Korevaar et al. (2017) followed a broiler parent flock at an experimental farm and detected bla$_{CMY-2}$ from day 7 to week 19 during the rearing period, but not over the laying period. *bla*$_{CMY-2}$ persisted for longer in this farm than in our study, and it was related to an IncA/C type of plasmids. Curiously, the authors pointed out than an unsuccessful combination of *bla*$_{CMY-2}$ and IncA/C plasmids was the main reason for their inability to persist on animal level (Dame-Korevaar et al., 2017).

Several of the remaining studies of ESBL/pAmpC *E. coli* flow in the broiler production pyramid show that their vertical transmission is mainly due to plasmids, though several clues for clonal spread of *bla*$_{CMY-2}$, the most frequently detected *bla* gene in poultry, are also provided.

Zurfluh et al. (2014) found strong evidences that $bla_{CTX-M-1}$ spread to the French broiler production pyramid by an IncI1/ST3 plasmid, and Apostolakos et al. (2020a) also showed the same finding in the Italian broiler production pyramid, as well as the transmission of bla_{SHV-12} by IncX3 plasmids. In our study, bla_{SHV-12} was harboured by IncI1/ST26 and a non-replicon detected plasmids. In addition, Apostolakos et al. (2020a) also found IncFIB/FII plasmids harbouring $bla_{CTX-M-55}$.

Nilsson et al. (2014), using multiple-locus variable number tandem repeat analysis (MLVA), detected an *E. coli* clone carrying bla_{CMY-2} in the Swedish broiler production pyramid, and a similar conclusion about the transmission of this *bla* gene in the Danish pyramid was reached by Agerso et al. (2014) using pulsed-field electrophoresis (PFGE); they also detected bla_{CMY-2} in IncI1 and IncK plasmids. This Russian doll model (bla_{CMY-2} inside an IncK plasmid inside a clonal *E. coli* strain) was also detected by Apostolakos et al. (2020a) in the Italian broiler production pyramid.

Although the source of the ESBL/pAmpC *E. coli* in the studied farm was not examined, their isolation from day-old chick in one of the four batches studied suggested that an external source must be consider. Nevertheless it is impossible to rule out environmental contamination, as detected in other studies (Dame-Korevaar et al., 2017; Dierikx et al., 2013) as a reason for their persistence in the farm.

According to this and other studies, it is clear that, although antimicrobial use is a well-known driver of AMR, in the case of commercial farms of both layers and broilers it does not appear to be the only factor responsible for the persistence of ESBL/pAmpC *E. coli* at farm level. Therefore, other farm management strategies in addition to proper antimicrobial use should be implemented to control this spread.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2022.109453.

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