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ORIGINAL ARTICLE

Extensive dissemination of CTX-M-1- and CMY-2-producing *Escherichia coli* in poultry farms in Tunisia

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Significance and Impact of the Study: This study is the first detailed documentation of a high occurence of extended-spectrum β-lactamases and plasmidic cephalosporinases in *E. coli* at the poultry farm level in Tunisia. Moreover, this is the first description of plasmid-mediated quinolone resistance (PMQR) in Tunisian animals. This study highlights that Tunisian poultry are a reservoir of antibiotic resistance genes which may be transferred to humans.

Keywords
bla_{CMY}, bla_{CTX-M}, *Escherichia coli*, poultry.

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Abstract

We characterized 67 *Escherichia coli* isolates with reduced susceptibility to cefotaxime obtained from 136 samples of healthy broilers housed in 36 Tunisian farms. All these isolates harboured $bla_{CTX-M-1}$ and/or bla_{CMY-2} genes located mostly on self-conjugative IncI1 plasmids. *qnrS1*, *qnrA6* and *aac(6′)-Ib-cr* were detected in six isolates. Considerable genetic diversity was detected among isolates from different farms. To our knowledge, this is the first detailed documentation of a high occurrence of $bla_{CTX-M-1}$ and bla_{CMY-2} in *E. coli* at the poultry farm level in Tunisia as well as the first description of plasmid-mediated quinolone resistance in food animals in Tunisia which may contribute to the dissemination of these genes throughout Tunisia.

Introduction

Since the beginning of the 1990s, the increase in the prevalence of extended-spectrum β-lactamases (ESBLs) among clinical *Escherichia coli* isolates in human medicine have been a cause of great concern. The earliest ESBLs, TEM-1, TEM-2 and SHV-1 derivatives were detected mostly in hospital-acquired pathogens. However, recently, CTX-M enzymes have taken over as the main ESBL type and had spread across the world, particularly in both hospital and community *Escherichia coli* strains (Pitout and Laupland 2008; Cantón *et al.* 2012). Plasmidic class C beta-lactamase (AmpC) have also taken their entry and CMY-2 appears to be the most commonly detected AmpC beta-lactamse found in *E. coli* causing human infections (Doi *et al.* 2010). Different reports have alerted in the last few years about the dissemination of ESBL/AmpC-producing *E. coli* in healthy food-producing animals in different countries (Girlich *et al.* 2007; Smet *et al.* 2010; Randall *et al.* 2011; Zheng *et al.* 2012). Previous studies carried out in Tunisia reported the presence of different ESBLs in food products but not in farm animals (Jouini *et al.* 2007; Ben Slama *et al.* 2010). The diversity and prevalence of ESBL and plasmidic AmpC among *E. coli* at the poultry farm level in Tunisia are still unknown.

The purpose of our study was to evaluate the faecal carriage of plasmidic AmpC- and ESBL-producing *E. coli* in broilers from different Tunisian farms and to detect the presence of other antimicrobial resistance markers in these bacteria.

Results and discussion

A total of 67 cefotaxime (CTX)-resistant *E. coli* were recovered from 57 of the 136 chicken faecal samples (42%) and from 24 of the 36 investigated farms (66%). The double-disc synergy test revealed synergy between clavulanate and cefotaxime or ceftazidime-containing discs for 43 isolates from 41 samples, suggesting production of an ESBL in 30% of the samples. The 24 remaining CTX-resistant isolates had an AmpC-phenotype. All the 67 CTX-resistant *E. coli* isolates were multidrug-resistant

and showed resistance to more than two non-beta-lactam antibiotics, including tetracycline (94%), nalidixic acid (89·5%), norfloxacin (71·6%), trimethoprim–sulfamethoxazole (73·1%), gentamicin (6%), amikacin (6%). All the isolates were susceptible to imipenem.

All ESBLs belonged to CTX-M group 1: 39 CTX-M-1 and 4 CTX-M-15. All isolates with AmpC phenotype harboured the *bla*$_{CMY-2}$ gene. Only one isolate carried *bla*$_{CTX-M-1}$ and *bla*$_{CMY-2}$ genes. *bla*$_{TEM-1}$ was detected in 26 isolates (38·8%). QnrS1 was detected in 2 CTX-M1 producing *E. coli* and QnrB5 in one CMY-2 isolate and the *aac(6′)-Ib-cr* gene in 2 CTX-M-15 and one CTX-M-1 producing isolates.

The determination of the phylogenetic group of the ESC resistant *E. coli* revealed that group A was dominant (34, 50·7%) followed by group D (21, 31·3%) and group B1 (11, 16·4%). Only one isolate belonged to group B2 and did not belong to clone O25b-ST131. Clonal relationships among the *E. coli* isolate within each farm assessed by ERIC genotyping revealed that clonality within the same farm was often observed (18 cases, Table 1). One isolate per ERIC profile was selected for further studies. Consequently, a total of 44 nonrepetitive isolates (27 ESBL and 17 AmpC) were included in the following experiments (Table 1). Pulsed-field gel electrophoresis analysis of XbaI-digested genomic DNA revealed a high diversity among the 44 studied isolates as the obtained patterns displayed less than 80% similarity (Fig. 1). Thus, the spread of the *bla*$_{CTX-M-1}$ and *bla*$_{CMY-2}$ did not result from the dissemination of a single clone. In fact, there were no common clones between farms except for two cases where we have identified the same clones between farms belonging to different governorates (Fig. 1).

Resistance to ESCs was transferred from 29 of the 44 selected isolates (66%) by conjugation for 26 isolates (59%) and by transformation for three isolates. *bla*$_{CTX-M-1}$ genes were transferred to recipient by conjugation for 14 of 27 isolates (51%) and by transformation for three isolates. *bla*$_{CMY-2}$ genes were transferred from 12 of 17 (70%) isolates by conjugation. Depending on the strain, other resistances were cotransferred, mostly tetracycline (55%) and rarely trimethoprim–sulphamethoxazole (8%; Table 1). Quinolone resistance was not cotransferred in any case. *bla*$_{TEM-1}$ was cotransferred with *bla*$_{CMY-2}$ in two strains and with *bla*$_{CTX-M-1}$ in one strain (Table 1). PCR-based replicon typing of the major plasmid incompatibility group showed that all *bla*$_{CTX-M-1}$ and eight *bla*$_{CMY-2}$ carrying plasmids belonged to the IncI1 incompatibility group, and four *bla*$_{CMY-2}$ genes were located on IncK plasmids (4). None *bla*$_{CTX-M-1}$ or *bla*$_{CMY-2}$ gene was located on incF plasmid. However, PCR-based replicon typing of the total plasmid content of the parental strains showed that most strains contained an IncF-type plasmid (31 of 44 strains; Table 1), consistent with other reports (Pitout and Laupland 2008; Randall *et al.* 2011; Zheng *et al.* 2012). All but 7 of the 44 donor strains contained multiple plasmids (Table 1).

Since their first description in 1989, different studies have reported the dissemination of CTX-M *E. coli* isolates among the intestinal flora of healthy humans, as well as of food-producing animals and also in food products (Pitout and Laupland 2008; Doi *et al.* 2010; Smet *et al.* 2010; Cantón *et al.* 2012). *bla*$_{CTX-M-1}$ is the ESBL encoding gene mostly detected in poultry especially in France, Great Britain, Belgium and Portugal (Girlich *et al.* 2007; Pitout and Laupland 2008; Doi *et al.* 2010; Smet *et al.* 2010; Randall *et al.* 2011; Zheng *et al.* 2012). However, a wide range of additional *bla*$_{CTX-M}$ subtypes (*bla*$_{CTX-M-2}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-17/18}$, *bla*$_{CTX-M-20}$, *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-53}$) have been detected in food-producing animals and food worldwide (Girlich *et al.* 2007; Pitout and Laupland 2008; Doi *et al.* 2010; Smet *et al.* 2010; Randall *et al.* 2011; Zheng *et al.* 2012). In Tunisia, before this study, no CTX-M *E. coli* had been reported from live broiler chickens, although they had been isolated from chicken meat and food samples of animal origin in Tunis, including CTX-M-1, CTX-M-14 and CTX-M-8 (Jouini *et al.* 2007; Ben Slama *et al.* 2010). A previous study carried out by Ben Slama *et al.* on *E. coli* isolates recovered from food samples in Tunisia during 2007 demonstrated that 26·9% of chicken meat was colonized by CTX-M-1 (Jouini *et al.* 2007). So, the high faecal carriage rate of CTX-M-1-producing *E. coli* in Tunisian poultry and contamination of food derived from these animals may contribute to transmission of *bla*$_{CTX-M-1}$ genes, from poultry to humans in Tunisia. In fact, recently, it was demonstrated that 7·3% of Tunisian healthy humans are faecal carrier of CTX-M-1 producing *E. coli* (Ben Sallem *et al.* 2012). However, like other studies, the most prevalent ESBL genotype in clinical isolates in humans in Tunisia, *bla*$_{CTX-M-15}$, was found only in 4 of the 67 isolates. Moreover, the distribution of poultry ESBL types found in the present study is similar to other European countries such as France, the Netherlands, Portugal and England where CTX-M-1 is the dominant type and the major replicon type is Inc I1 (Girlich *et al.* 2007; Smet *et al.* 2010; Randall *et al.* 2011; Zheng *et al.* 2012). From the current literature, the prevalence of IncI1 plasmids seems to be linked to a particular reservoir of *E. coli* and *Salmonella* from poultry (García-Fernández *et al.* 2008). IncI1 has also been recently observed from human strains of *E. coli* and *Salmonella* isolated in UK, German, the Netherlands, Spain and France and were found to be associated mainly with CMY-2, CMY-7, CTX-M-1, CTX-M-15 and TEM-52 suggesting a high prevalence of this plasmid in Europe and

Table 1 Characteristics of CTX-M- and CMY-2- producing *Escherichia coli* isolates

Farm no. /isolate no.	Nb of isolates*	PG	PBRT: donors	β-lactamase types and PMQR	PBRT: TC/Tf	Non-β-lactam associated resistances
B1/1	2	B1	I1	CTX-M-15, TEM-1	–	NA, NOR,TE
B2/1B	1	D	F, K	CMY-2	–	NA, NOR, G,Tb,Net
B2/1J	2	A	I1, K	CMY-2, TEM-1	I1	NA, NOR, SXT, TE
B2/2B	1	A	I1, K	CMY-2, TEM-1	–	NA, NOR, SXT
B3/1	2	B1	I1	CTX-M-1	I1	NA, NOR, TE
B4/1	2	A	I1, FIA	CTX-M-1	I1	SXT, TE
B5/1	2	A	F, FIB, N	CTX-M-1	–	NA, NOR, SXT, G,Tb,Net,TE
B6	1	A	F, FIA, FIB, N	CTX-M-1, TEM-1, Aac-6′-Ib-cr	I1	NA, NOR, SXT,G,Tb,Net,TE
B9/1	1	B1	I1	CMY-2	I1	NA, NOR, TE
B9/3	1	A	F, FIA, I1	CTX-M-1, TEM-1, QnrS1	I1	NA, NOR, TE
B9/4	2	B1	I1	CTX-M-1	I1	NA, NOR, SXT, TE
B10/1	2	A	F, FIA, FIB	CTX-M-15, Aac-6′-Ib-cr	–	NA, NOR, SXT,G,Tb,Net,TE
B10/2P	1	B2	F, K, I1	CTX-M-1, CMY-2	–	NA, NOR, TE
B11/1	1	B1	FIA, I1	CTX-M-1, TEM-1	I1	NA, NOR, SXT, TE
B11/2	1	A	I1, K	CMY-2	I1	NA, NOR,TE
B12/1G	1	A	I1, K	CMY-2	I1	NA, NOR,TE
B12/1P	2	D	F, K	CMY-2	–	NA, NOR, SXT
B12/3	1	B1	I1	CMY-2	I1	NA, NOR, SXT
B13/2	1	B1	F, I1	CTX-M-1, TEM-1	I1	NA, NOR, TE
B14/1P	1	A	F, I1, K	CMY-2, TEM-1, QnrB5	K	NA, NOR, TE
B14/1G	1	A	F, I1, K	CMY-2, TEM-1	K	NA, NOR,TE
B16/1	2	D	FIA, FIB, I1	CTX-M-1, TEM-1	I1	NA, SXT, TE
B16/2	1	A	FIA, I1	CTX-M-1, TEM-1, QnrS1	I1	NA, SXT, TE
B16/1S	1	A	FIB, I1, K	CMY-2, TEM-1	I1	NA, NOR,TE
B17/1	1	A	F, FIB, I1	CTX-M-1	I1	NA, SXT,TE
B18/2	5	A	I1	CTX-M-1	I1	SXT, TE
B22/2J	1	D	F, FIB, K, I1	CMY-2	K	NA, NOR, SXT
B22/3	1	D	F, FIB, I1	CTX-M-1	–	NA, NOR, TE
B23/4	1	D	F, I1	CTX-M-1	I1	NA, NOR, TE
B24/2B	1	D	F, FIB, I1	CTX-M-1, TEM-1	I1	NA, NOR, SXT, TE
B24/3	2	A	F, FIB, I1	CMY-2, TEM-1	–	NA, NOR, SXT
B25/7J	1	D	F, FIB, I1	CTX-M-1	I1	NA, NOR,TE
B25/8	1	A	F, FIB, I1	CTX-M-1, TEM-1	–	NA, NOR,TE
B25/4J	2	D	K, N	CMY-2, TEM-1	K	NA, NOR,TE
B25/5J	3	D	F, FIB, K	CMY-2, TEM-1	–	NA, NOR, SXT, TE
B27/1	3	D	F, FIB, I1	CTX-M-1, TEM-1	–	NA, SXT, TE
B28/2	2	D	F, I1	CTX-M-1	–	NA, SXT, TE
B28/3	1	A	F, FIA, FIB, I1	CMY-2	I1	NA, SXT, TE
B29/2	2	A	F,FIA	CTX-M-1, TEM-1	–	NA, NOR,TE
B30/1	2	A	FIB,FIA,I1	CTX-M-1, TEM-1	–	NA, NOR, SXT,TE
B30/4	1	D	I1	CTX-M-1	I1	NA, SXT, TE
B30/5	1	B1	FIA, FIB, K, I1	CTX-M-1, TEM-1	I1	NA, NOR, SXT,TE
B30/2	2	A	K, I1	CMY-2, TEM-1	I1	NA, NOR, SXT, TE
B36	1	A	FIA, FIB, I1	CTX-M-1, TEM-1	I1	NA, SXT, TE

Patterns transferred by conjugation or transformation are underlined.

PG, phylogenetic group; PBRT, PCR-based replicon typing; PMQR, plasmid-mediated quinolone resistance: QnrA, QnrB, QnrC, QnrS, Aac(6′)-Ib; G, gentamicin; AN, amikacin; Tb, tobramycin; Net, netlimicin; NA, nalidixic acid; NOR, norfloxacin; SXT, sulfamethoxzole/trimethoprim; TE, tetracycline; TC, transcpnjugants; Tf, transformants.

*Number of isolates having the same ERIC2 profiles.

perhaps in North Africa (Girlich *et al.* 2007; Smet *et al.* 2010; Randall *et al.* 2011; Zheng *et al.* 2012). bla_{CMY-2} is the most prevalent type of plasmid AmpC β-lactamases in members of the *Enterobacteriaceae* of both animal and human origin all over the world particularly in the USA (Pitout and Laupland 2008; Doi *et al.* 2010). In Tunisia, CMY-2 has already been detected in food samples and the closely related enzyme CMY-4 in human clinical

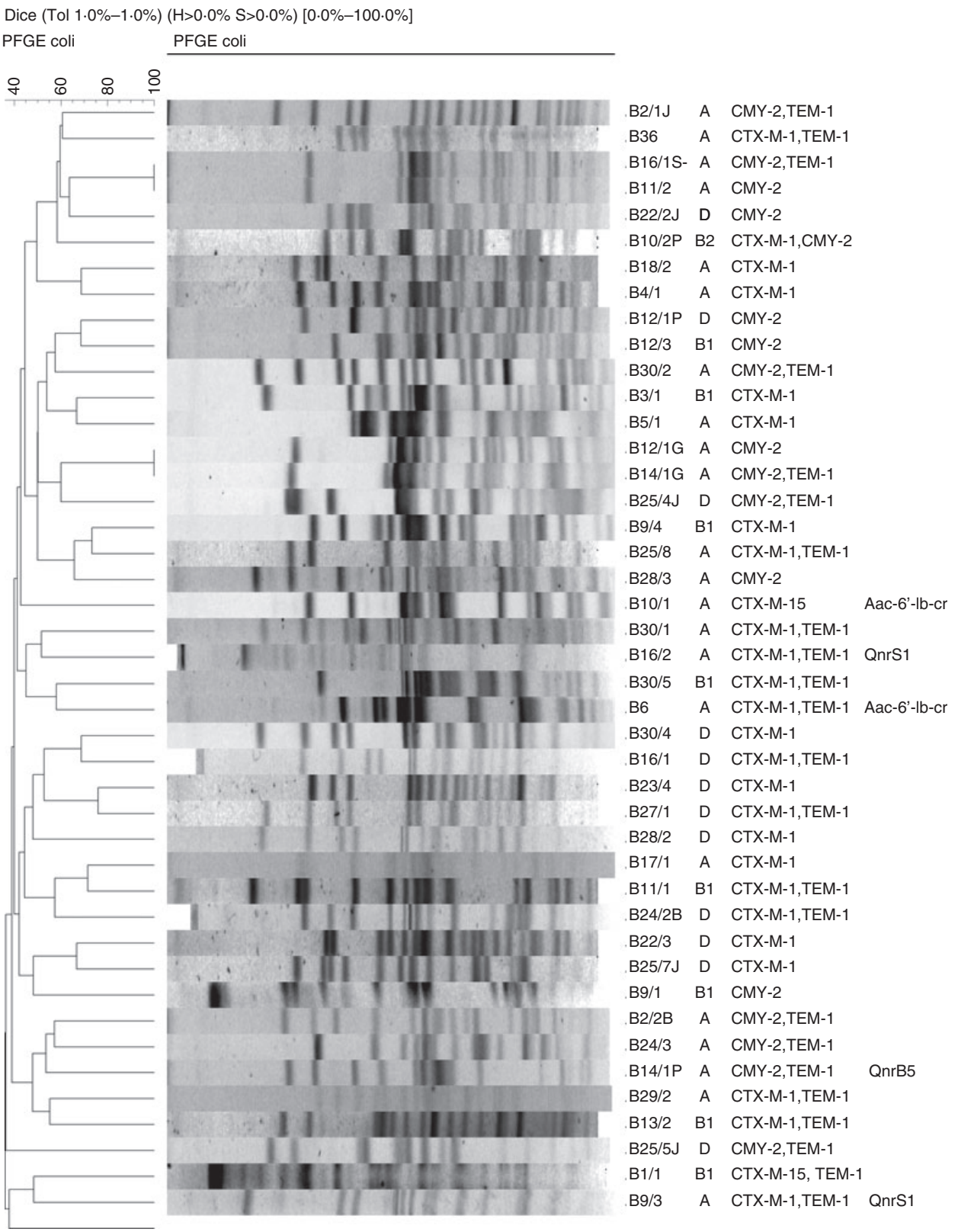

Figure 1 *Xba*I-PFGE dendrogram for 44 *Escherichia coli* isolates.

isolates of *Klebsiella pneumoniae* and *Proteus mirabilis* (Ktari *et al.* 2006; Jouini *et al.* 2007). The occurence of ESBL-producing *E. coli* at the poultry farm level in Tunisia is higher than the findings of other investigators. In a survey of chickens in France in 2005, of the 112 faecal samples examined, 32 (28·5%) yielded ESC resistant *E. coli* and 12 isolates (10·7%) were CTX-M-1 producers (Girlich *et al.* 2007). Randall *et al.* (2011) reported that CTX-M-1-producing *E. coli* was isolated from 54·5% of the United Kingdom broiler abattoirs and from 6·7% of pooled broiler caecal samples. In china during 2007–2009, 14% of healthy food animals were colonized by ESC-resistant *E. coli* and 12·3% were CTX-M producers (Zheng *et al.* 2012). In Tunisia, we did not identify the O25b-ST131 clone in the faecal poultry samples, which is reassuring at this time. However, other studies have recently identified ST131 clone in poultry and retail meat confirming that this clone can colonize different hosts (Mora *et al.* 2010). Moreover, Leverstein-van Hall *et al.* (2011) recently reported that Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains, indicating transmission of ESBL genes from poultry to humans through the food chain.

In conclusion, in this study, a high occurrence of ESC resistance has been detected in faecal samples of poultry in Tunisia. This ESC resistance among a high clonal diversity of *E. coli* from healthy poultry was often mediated by $bla_{CTX-M-1}$ and bla_{CMY-2} harboured by the self-conjugative IncI1 plasmid. To our knowledge, this is the first detailed documentation of a high occurrence of ESBL and plasmidic AmpC in *E. coli* at the poultry farm level in Tunisia. In addition, this is the first time that PMQR, QnrS1, QnrB5 and Aac(6′)-Ib-cr have been detected in poultry in Tunisia. So, more studies should be carried out in the future to track the origin of these types of resistance among faecal *E. coli* and to analyse the relationship between human and animal resistant *E. coli* isolates.

Materials and methods

Bacterial strains and sampling

A total of 136 faecal samples of healthy chickens were recovered from 36 farms located in six different governorates of Tunisia during 4 months from February 2010 to May 2010. On each farm, faecal samples were obtained from different flocks that contained from 2000 to 10 000 animals. Fresh dropping faecal samples were recovered from crates. Samples were processed immediately after collection. Samples were plated onto MacConkey-medium supplemented with cefotaxime at 2 mg l^{-1} and incubated for 24 h at 37°C. Samples were also seeded on nonsupplemented medium to control faecal *E. coli* colonization of chickens. Isolates that grew on the selective plates with typical *E. coli* morphology were selected and identified by classical biochemical methods. One colony per plate was taken, except for ten samples two colonies with different morphologies per plate were selected for further identification and studies.

Antibiotic susceptibility testing

Susceptibility to 17 antibiotics (amoxicillin, amoxicillin + clavulanic acid, ticarcillin, ticarcillin + clavulanic acid, cefalothin, cefoxitin, ceftazidime, cefotaxime, cefepime, gentamicin, amikacin, tobramycin, netilmicin, nalidixic acid, norfloxacin, sulfamethoxzole/trimethoprim and tetracycline) was tested by the disc diffusion method according to the CLSI guidelines and interpreted according to EUCAST criteria. ESBLs were detected using the double-disc synergy test between clavulanic acid and ceftazidime, cefotaxime or cefepime.

Molecular analysis of antibiotic resistance genes

Detection of several beta-lactamase genes, including bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, bla_{CMY}, bla_{FOX}, bla_{ACC-1} and plasmid-mediated quinolone resistance (PMQR) genes *qnrA, qnrB*, *qnrS*, *qepA* and *aac(6′)-Ib-cr* were carried out by PCR as described previously (Kim *et al.* 2009; Dallenne *et al.* 2010). PCR products were sequenced on ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were edited using BIOEDIT software (ver. 7.0.9.0; T. Hall, http://www.mbio.ncsu.edu/BioEdit/bioedit) and than the NCBI BLAST program was used for resistance gene identification. (http://www.ncbi.nlm.nih.gov/).

Strain typing

The phylogenetic group of the extended-spectrum cephalosporin (ESC)-resistant *E. coli* was determined by a multiplex PCR assay (Clermont *et al.* 2000). Isolates belonging to phylogenetic group B2 were screened with a previously established PCR-based method to identify the O25b-ST131 clone (Clermont *et al.* 2009). Clonal relationships among the *E. coli* isolates within each farm were assessed by studying ERIC genomic DNA profiles, as generated using the primer ERIC2 5′-AAG TAA GTG ACT GGG GTG AGC G-3′ (Versalovic *et al.* 1991). Pulsed-field gel electrophoresis of chromosomal DNA digested with the restriction enzyme *Xba*I was carried out according to a standard protocol using a GenePath system (Bio-Rad, Marnes-la-Coquette, France) to determine the genetic relatedness of selected isolates (Ribot *et al.* 2006).

Transfer of resistance determinants and plasmid analysis

Transfer of resistance genes by conjugation was performed by mating-out assays using the *E. coli* J53-2 Rf^r or HB101 strain as recipients. Transconjugants were selected on MH agar containing rifampin (250 mg l^{-1}) or streptomycin (50 mg l^{-1}) plus ceftazidime or cefotaxime (2 mg l^{-1}). When plasmids were not transferable by conjugation, a transformation assay was carried out. Plasmid DNA obtained using the QIAprep Spin Miniprep kit (Qiagen) was electroporated into *E. coli* DH10B (Invitrogen). Transformants were selected on MH agar plates supplemented with ceftazidime (2 mg l^{-1}) or cefotaxime (2 mg l^{-1}). Plasmid replicons were determined for the parental strains and the transconjugants and transformants using the PCR-based replicon-typing scheme described previously (Carattoli *et al.* 2005).

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Conflicts of Interest

No competing financial interests exist.

Transparency Declarations

None to declare.

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