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High Prevalence of *Escherichia coli*-Producing CTX-M-15 Extended-Spectrum Beta-Lactamases in Poultry and Human Clinical Isolates in Romania

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Use of antibiotics in food animals may contribute to development and spread of resistant organisms, particularly so in some countries. The aim of this study was two-fold; first, to establish the prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in chicken production in a region within Romania. Second, to study the relatedness of ESBL-producing *E. coli* isolates recovered from broilers, abattoir workers where the chickens were slaughtered and from the human clinical specimens from two regional hospitals. The results indicated a very high (69%) rate of carriage of ESBL and AmpC-producing *E. coli* in chickens with 36% CTX-M producers. Sequencing showed that chickens in Romania have the highest worldwide prevalence (53%) of $bla_{CTX\text{-}M\text{-}15}$ reported in poultry *E. coli* isolates. The majority (53%) of the extended-spectrum cephalosporin-resistant *E. coli* carried plasmid-mediated bla_{ampC} genes, mostly $bla_{CMY\text{-}2}$ type, one of the highest prevalences reported in Europe. The predominant CTX-M type found in the human clinical *E. coli* isolates was $bla_{CTX\text{-}M\text{-}15}$ and most isolates coharbored $bla_{OXA\text{-}1}$, bla_{TEM}, and *aac(6′)-ib-cr*. The majority (60%) of the human clinical isolates belonged to the pandemic virulent clone B2-ST131. The clonal relationship between broiler and the human CTX-M-producing *E. coli* isolates was assessed by macrorestriction pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), which indicated strain diversity with no common STs found between human and poultry isolates. Moreover, IncI1 was the most prevalent replicon found in broiler ESBL-producing *E. coli* isolates and also in transconjugants, indicating that plasmids and not clonal spread may play a role in the transfer of $bla_{CTX\text{-}M}$ genes. This study identifies a high prevalence of ESBL-producing *E. coli* from broiler chickens in Romania with a high occurrence incidence of $bla_{CTX\text{-}M\text{-}15}$, which reflects the main ESBL type found in human *E. coli* infections in this country.

Introduction

Antimicrobial resistance has emerged as a problem in both veterinary and human medicine, and recent studies have recognized food production animals as a potential reservoir of resistant bacteria or resistance determinants.[7,44,45] Resistance to beta-lactam antimicrobials is of special interest because of their critical importance for human as well as for veterinary medicine. *Escherichia coli* has the potential to cause a variety of intestinal and extraintestinal infections in both humans and animals, and the development of resistance to extended-spectrum cephalosporins (ESCs) has become a serious public health problem worldwide.[32]

The most important mechanism of resistance to beta-lactam antimicrobials is based on production of enzymes that inactivate these compounds. The genes encoding these enzymes are mostly located on mobile genetic elements, such as plasmids and transposons, which can easily be transferred horizontally to other bacteria, including other bacterial species.[44] Of particular concern is the emergence over the past 10 years of CTX-M beta-lactamases, of which CTX-M-15 and CTX-M-14 are the most prevalent type found in human isolates worldwide[6] and have also been identified in animal isolates. Very often the $bla_{CTX\text{-}M}$ genes are located on genetic elements, which also harbor plasmid-mediated *ampC* beta-lactamase genes, or plasmid-mediated quinolone resistance genes (PMQR) and genes encoding resistance to other drug classes giving rise to multidrug-resistant isolates.[47]

The use of antimicrobials and especially of ESCs in food production animals is of concern, with surveillance studies

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highlighting the importance of poultry as a source of foodborne pathogens and antimicrobial-resistant organisms.[9] In Canada, the use of ceftiofur in chickens has been associated with emergence of ceftiofur-resistant *E. coli* and *Salmonella enterica* serovar Heidelberg in poultry and humans.[21] In addition, a recent study in the Netherlands has shown a high degree of similarity of resistance genes and multilocus sequence types (MLST) between *E. coli* isolates obtained from retail chicken meat and from humans, suggesting interspecies transmission.[39] The prevalence of extended-spectrum beta-lactamase (ESBL)/AmpC-producing *Enterobacteriaceae* isolated from broilers at the abattoir level has been studied in Western European countries, such as the Netherlands, France, and Portugal, and has provided further evidence of chicken meat as a potential zoonotic source of ESBL-producing bacteria.[3,20,24,34] Many Eastern European countries export chicken meat to the global food market and hence chicken-to-human transmission of resistant *E. coli* strains could occur both nationally and internationally. Romania is a major producer (80 million chickens per annum) and a significant exporter (250 million Euros in 2012) of chicken meat.[37] This involves a range of intensive and extensive production systems, although most chickens are intensively reared.

Currently, there have been no studies to establish the epidemiology of transmission between poultry and those involved in processing poultry meat on production lines and human clinical isolates. To determine the potential for transmission between poultry, their meat and people, longitudinal sampling at two Romanian broiler abattoirs was undertaken to screen for the presence of ESBL-producing *E. coli* in the cecum of broilers; the staff working at one broiler abattoir were also screened. We aimed to establish if ESBL-producing *E. coli* isolated from the abattoir workers shared the same resistance genes, mobile genetic elements carrying such genes, or *E. coli* phylogenetic groups with those in the broiler meat they handle. Molecular characterization of *E. coli* isolates from broiler cecal contents, abattoir workers, and human clinical isolates from two major local hospitals was also performed to establish commonality of ESBL and other resistance genes between these groups of isolates.

Materials and Methods

Bacterial isolates

Broiler cecal samples were collected from two abattoirs (A1 and A2) in the northeast of Romania. The two abattoirs operated different systems; abattoir A1 was a slaughterhouse for four local poultry farms, while abattoir A2 represented a small integrated broiler system, which included a hatchery, broiler house, and a slaughter unit. Sampling took place from October 2011 to October 2012. For abattoir A1, 4–5 cecal samples/flock were collected from 3 flocks every 2 months, totaling 87 samples from different farms over a year. From abattoir A2, which only served one farm, 2–3 cecal samples/flock were collected following the same sampling schedule as for abattoir A1, resulting in 40 samples collected over a year. At collection, the gastrointestinal tract was ligated at each end, wrapped individually, and transported to the laboratory where they were cultured within 2–4 hours.

Human fecal samples were also collected from staff working at the slaughterhouses of abattoir A1. Sample collection was performed by the Epidemiology Department of the National Institute of Public Health, Iasi (NIPHI), Romania, as part of a routine *Salmonella* screening protocol; from these samples, 55 *E. coli* isolates were available for inclusion in this study. To screen for ESC-resistant *E. coli*, all chicken cecal samples and abattoir worker isolates were streaked onto eosin methylene blue agar (EMBA) containing 1 μg/ml of ceftazidime and EMBA containing 1 μg/ml of cefotaxime (all antibiotics from Sigma-Aldrich Ltd.). In addition, twenty-five human clinical *E. coli* isolates obtained from different patients from two local major hospitals from the northeast of Romania between 2008 and 2011, previously confirmed as ESBL producers in a different study, were included for comparison.[48]

Antimicrobial sensitivity testing

Any cefotaxime or ceftazidime-resistant isolates obtained on the EMBA selective media, producing a characteristic *E. coli* green metallic sheen (and subsequently confirmed as *E. coli* by *uidA* PCR), were subjected to antimicrobial sensitivity testing and phenotypic testing for ESBL production. The sensitivity testing was performed by disk diffusion on ISO-sensitest agar supplemented with horse blood according to the British Society of Antimicrobial Chemotherapy (BSAC) methodology.[27] *E. coli* ATCC25922 was used as control for disk diffusion susceptibility testing for every new batch of isolates using a suspension of 0.5 McFarland to obtain a semiconfluent lawn on ISO-sensitest Agar.

The antimicrobial disk sensitivity panel comprised ampicillin (25 μg), amoxicillin/clavulanic acid (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefpodoxime (30 μg), imipenem (10 μg), nalidixic acid (30 μg), ciprofloxacin (1 μg), gentamicin (30 μg), streptomycin (10 μg), tetracycline (30 μg), and trimethoprim/sulfamethoxazole (25 μg) (all disks and media from Oxoid, UK). Interpretation of results was performed according to BSAC criteria.[27] Confirmation of ESBL phenotype was performed by the double-disk synergy test (DDST).[33]

Characterization of ESBL and other resistance genes

To identify the resistance genes carried by the ESC-resistant *E. coli* isolates, cell lysates for PCR confirmation were prepared by suspending the bacterial cells in 500 μl sterile distilled water and incubating the tubes at 95°C for 10 minutes, followed by centrifugation at 10,000 *g* for 5 minutes. All cephalosporin-resistant isolates were subjected to PCR screening for the presence of bla_{SHV}, bla_{TEM}, bla_{OXA}, and $bla_{CTX\text{-}M}$ genes, with a further multiplex PCR being used for the detection of family-specific plasmid-mediated *ampC* β-lactamase genes.[17,41] In addition, the presence of the (PMQR) genes, *qnrA,B,S*, *qepA*, and the *aac(6′)-Ib-cr* gene, which confers reduced susceptibility to aminoglycosides and also fluoroquinolones, was determined as previously described.[40,43] All isolates positive for *aac(6′)-Ib* were sequenced with specific primers to confirm the presence of *aac(6′)-Ib-cr* variant.[40] In addition, PCR for the *uidA* gene was performed to confirm that the isolates were *E. coli*.[36] All human clinical isolates were screened for subgroup O25b and sequence type ST131 by allele-specific PCR.[12] To confirm gene identity and enable genotyping of isolates, DNA sequencing of both strands of PCR reaction products was done for representative isolates from groups

displaying unique gene combinations (*e.g.*, $bla_{CTX\text{-}M}$, bla_{TEM}, *aac(6′)-ib-cr*) using the same sets of primers as in the original reactions (Eurofins MWG Operon). The resulting amplicon DNA sequences were compared using BLASTn against sequences in GenBank. All chicken and human *E. coli* isolates carrying $bla_{CTX\text{-}M}$ were selected for further molecular typing.

Molecular characterization of isolates

E. coli isolates were assigned to phylogenetic groups (A, B1, B2, and D) by multiplex PCR.[11] The isolates identified as phylogenetic group B2 were screened for the O25 group by a method based on an allele-specific PCR.[13] Positive isolates were further tested to identify members of the international clone O25b-ST131 using primers for the gene *pabB* and *trpA*, as described by Clermont *et al.*[12]

Genetic relatedness between isolates carrying CTX-M-type genes was analyzed by macrorestriction pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). In total, 27 isolates, which included one representative isolate of a group with a unique resistance gene combination or having the same gene combination, but from a different phylogroup, were chosen. MLST was performed according to the protocol described by Wirth *et al.*[53] for the amplification and sequencing of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*). Allelic profiles and sequence types (ST) were assigned according to the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/).

PFGE was performed according to the PulseNet protocol of the Centers for Disease Control and Prevention (www.cdc.gov/pulsenet/pathogens/). Agarose-embedded bacterial genomic DNA was digested with restriction enzyme *Xba*I (Roche Diagnostics Ltd.), followed by separation of DNA fragments on a 1% agarose gel in a 0.5X Tris-Borate-EDTA buffer. PFGE profiles were compared using the Lambda Ladder PFG Marker (New England Biolabs) as a molecular size marker. Data were analyzed using BioNumerics software version 5.1 (Applied Maths). A position tolerance of 1.00% was selected and cluster analysis of PFGE pulsotypes was performed by the unweighted pair group method with average linkages (UPGMA) using the Dice coefficient to analyze similarities and define pulsotypes.

Resistance transfer and PCR-based replicon typing

Transfer of resistance genes by conjugation was performed by broth mating to determine whether the ESBL phenotypes were transferable. The conjugation assays were attempted for 14 isolates carrying $bla_{CTX\text{-}M}$ genes using a streptomycin-resistant *E. coli* HB101 strain as recipient as previously described.[38] Transconjugants were selected on nutrient agar (Oxoid) supplemented with streptomycin (50 μg/ml) and cefotaxime (1 μg/ml). The resistance phenotype of transconjugants was determined by disk diffusion using the same antimicrobial panel as for parental strains. Plasmid replicons were determined using a PCR-based replicon-typing scheme as previously described, on all $bla_{CTX\text{-}M}$-positive isolates and transconjugants.[8]

Results

Bacterial isolates and antimicrobial sensitivity testing

Screening of the 127 chicken cecal samples collected from both abattoirs for detection of ESC-resistant *E. coli* resulted in 90 isolates being selected for further testing (58 isolates from abattoir A1 and 32 isolates from abattoir A2). In addition, screening of the 55 *E. coli* isolates from abattoir A1 workers on the same selective media resulted in 19 ESC-resistant isolates. In total, 134 *E. coli* ESC-resistant isolates (90 cecal isolates from abattoir A1and A2, 19 isolates from A1 workers, and 25 human clinical isolates) were obtained and analyzed for antimicrobial susceptibility. Results obtained with the *E. coli* ATCC25922 control strain were within the acceptable ranges published by BSAC for ATCC control strains.

Antimicrobial susceptibility testing was performed for all 134 *E. coli* ESC-resistant isolates; in poultry isolates, both ESBL and non-ESBL producers showed high levels of resistance to ampicillin (98.9%), amoxicillin/clavulanic acid (91.1%), cefoxitin (86.6%), cefpodoxime (93.3%), cefotaxime (87.7%), ceftazidime (91.1%), ciprofloxacin (87.7%), nalidixic acid (94.4%), gentamicin (33.3%), streptomycin (80%), trimethoprim/sulfmethoxazole (77.7%), and tetracycline (81.1%). Phenotypic DDST testing of the 90 ESC-resistant chicken cecal isolates confirmed that 50 isolates (37 from abattoir A1 and 13 from A2) were ESBL producers.

None of the abattoir worker isolates were confirmed as ESBL producers by DDST. However, the sensitivity testing results for these isolates showed that they were resistant to tetracycline (78.9%), streptomycin, trimethoprim/sulfamethoxazole, and ampicillin (47.3%), as well as amoxicillin/clavulanic acid (36.84%), cefpodoxime (42.1%), cefotaxime (10.52%), ceftazidime (21.05%), ciprofloxacin (36.8%), nalidixic acid (42.1%), cefoxitin (21.05%), and gentamicin (5.26%).

All human clinical isolates were resistant to ampicillin, cefpodoxime, cefotaxime, and tetracycline, and showed resistance to amoxicillin/clavulanic acid and ceftazidime (96%), ciprofloxacin (80%), nalidixic acid (84%), gentamicin (68%), streptomycin (64%), cefoxitin (56%), and trimethoprim/sulfamethoxazole (60%).

All isolates were fully susceptible to the carbapenem, imipenem.

Characterization of ESBL and other resistance genes

One hundred thirty-four ESC-resistant *E. coli* isolates (90 cecal broiler isolates from abattoir A1and A2, 19 isolates from A1 workers, and 25 human clinical isolates) were screened for beta-lactamases, ESBL, and fluoroquinolone resistance determinants.

Among the 90 ESC-resistant *E. coli* broiler isolates analyzed, only two did not carry any plasmid-mediated AmpC beta-lactamase or ESBL resistance genes, bringing the overall ESC resistance prevalence to 69.2% (88/127) in the poultry population from the two abattoirs investigated. The $bla_{CTX\text{-}M}$ genes were identified in 35.5% (32/90) of poultry *E. coli* isolates comprising 19 (21.1%) isolates from abattoir A1 and 13 isolates (14.4%) from abattoir A2. Of the 32 $bla_{CTX\text{-}M}$ genes identified in poultry isolates, twenty-seven belonged to CTX-M group 1 (30%), and sequencing identified $bla_{CTX\text{-}M\text{-}15}$ to be the most prevalent resistance gene carried by 17 isolates (53.1%); this was followed by $bla_{CTX\text{-}M\text{-}3}$, which was carried by 7 isolates (21.9%) and $bla_{CTX\text{-}M\text{-}1}$ present in the remaining 3 isolates (9.4%). Only

five *E. coli* poultry isolates (5.6%) carried CTX-M group 9 with all five harboring $bla_{CTX\text{-}M\text{-}14}$. There were clear differences in the ESBL gene type carriage between the chicken samples from the two abattoirs, with $bla_{CTX\text{-}M\text{-}15}$ ($n=4$) and $bla_{CTX\text{-}M\text{-}1}$ ($n=1$) present in *E. coli* from A2, which had an enclosed rearing system, while all $bla_{CTX\text{-}M}$ gene variants identified in this study were present in isolates from abattoir A1, which was a slaughter unit for multiple poultry farms. With regard to the gene combinations found in the poultry *E. coli* isolates, three isolates harbored $bla_{CTX\text{-}M\text{-}15}$ only, while the remaining isolates also harbored $bla_{TEM\text{-}1}$, $bla_{SHV\text{-}12}$, $bla_{SHV\text{-}2a}$, and/or $bla_{CMY\text{-}2}$ genes (Table 1). One chicken isolate was also positive for the aminoglycoside-modifying enzyme *aac(6′)-Ib-cr*. When screened for plasmid-mediated AmpC-type beta-lactamases, 48/90 broiler isolates (53.3%) carried bla_{ampC} genes, borne singly or in various combinations with $bla_{CTX\text{-}M}$, bla_{SHV}, or bla_{TEM}. The majority of bla_{ampC} genes were identified as $bla_{CMY\text{-}2}$, except for one isolate carrying bla_{ACC} in combination with $bla_{SHV\text{-}2a}$. The prevalence of bla_{SHV} and bla_{TEM}, carried singly or in various combinations in broiler isolates, was 33.3% and 44.0%, respectively (Table 1).

Nineteen ESC-resistant *E. coli* isolates obtained from abattoir A1 workers were also screened for the presence of ESBL/AmpC resistance determinants. No $bla_{CTX\text{-}M}$ genes were identified in these isolates. However, five of the *E. coli* isolates from these workers did harbor bla_{TEM}, two isolates harbored $bla_{SHV\text{-}11}$, and one isolate coharbored a rare combination of

Table 1. Prevalence of Resistance Genes and Distribution of Resistance Gene Combinations in *Escherichia coli* Isolates from Broilers, Human Volunteers, and Human Clinical Isolates

bla *gene combinations*	*No. of isolates/group*		
	Broilers n = *90*	*Human volunteers* n = *19*	*Human clinical isolates* n = *25*
Total CTX-M (%)[a]	**32/90 (35.5%)**	—	**24 (96%)**
CTX-M-1 group	**27/90 (30%)**	—	**21/25 (84%)**
$bla_{CTX\text{-}M\text{-}1}$	1	—	—
$bla_{CTX\text{-}M\text{-}1}$, $bla_{CMY\text{-}2}$	2	—	—
$bla_{CTX\text{-}M\text{-}3}$	4	—	—
$bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$	1	—	—
$bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$, *aac(6′)-ib-cr*	1	—	—
$bla_{CTX\text{-}M\text{-}3}$, $bla_{SHV\text{-}12}$	1	—	—
$bla_{CTX\text{-}M\text{-}3/22}$, bla_{TEM}, *qnrB*	—	—	1
$bla_{CTX\text{-}M\text{-}15}$	3	—	3
$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$	2	—	1
$bla_{CTX\text{-}M\text{-}15}$, bla_{TEM}, $bla_{CMY\text{-}2}$	5	—	—
$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$	2	—	—
$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}2a}$, $bla_{CMY\text{-}2}$,	2	—	—
$bla_{CTX\text{-}M\text{-}15}$, $bla_{CMY\text{-}2}$	3	—	—
$bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, bla_{TEM}, *aac(6′)-ib-cr*	—	—	12[@]
$bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, *aac(6′)-ib-cr*	—	—	4
CTX-M-9 group (%)[a]	**5 (5.5%)**	—	**3 (12%)**
$bla_{CTX\text{-}M\text{-}14}$	1	—	3
$bla_{CTX\text{-}M\text{-}14}$, $bla_{CMY\text{-}2}$, bla_{TEM}	4	—	—
CTX-M-2 group (%)[a]	—	—	**1 (4%)**
$bla_{CTX\text{-}M\text{-}2}$, $bla_{TEM\text{-}1}$	—	—	1
Total AmpC (%)	**48**[a] **(53.3%)**	**1 (5.2%)**	—
$bla_{CMY\text{-}2}$	5	—	—
$bla_{CMY\text{-}2}$, bla_{SHV}	8	—	—
$bla_{CMY\text{-}2}$, bla_{SHV}, *qnr*S/*qnrB*	3	—	—
$bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1}$	15	—	—
bla_{ACC}, $bla_{SHV\text{-}2A}$	1	1[b]	—
Total SHV (%)[a]	**30**[a] **(33.3%)**	**3**[a] **(15.7%)**	—
bla_{SHV}	10		—
bla_{SHV}, *qnrB*	2	2	—
bla_{SHV}, *aac(6′)-ib-cr*	1	—	—
Total OXA1-like (%)[a]	—	—	**17**[a] **(68%)**
$bla_{OXA\text{-}1}$	—	—	1
Total Tem (%)[a]	**40**[a] **(44.4%)**	**5 (26.3%)**	—
bla_{TEM}	11	4	—
bla_{TEM}, *qnrB*, *qnrS*	—	1	

Bold represents beta-lactamases types.

[a]Represents total number of the screened genes present in any combination per study group (chicken, human volunteers, or human clinical isolates) @ one isolate also had *qnrS*.

[b]human isolate also had *aac(6′)-ib-cr*.

genes, bla_{ACC-1a}, bla_{SHV-12}, and *aac(6′)-Ib-cr.* Interestingly, one isolate obtained from chickens from the same abattoir (A1) also harbored bla_{ACC-1a}, and bla_{SHV-12}, but not *aac(6′)-Ib-cr.*

Screening of the 25 human clinical *E. coli* isolates originating from two large local hospitals for the presence of ESBL/AmpC resistance determinants showed that all but one harbored bla_{CTX-M} genes. Twenty-one (84%) of these belonged to CTX-M group 1 and sequencing identified that all these clinical isolates carried a $bla_{CTX-M-15}$ gene, except for one isolate, which harbored $bla_{CTX-M-3/22}$. The only isolate positive for CTX-M group 2 was identified as $bla_{CTX-M-2}$, while three remaining isolates carried $bla_{CTX-M-14}$, a gene belonging to CTX-M group 9. Regarding the combination of resistance genes identified in the human samples, of the 24 CTX-M-positive *E. coli* isolates, only six harbored the $bla_{CTX-M14/15}$ alone, while the remaining coharbored bla_{TEM-1}, bla_{OXA-1}, and *aac(6′)-Ib-cr.* None of the human isolates harbored bla_{CMY-2} or $bla_{SHV-2a/12}$ genes (Table 1). No *qepA* genes were identified in the poultry or human isolates.

Molecular typing

The PCR determination of the phylogenetic groups for the chicken isolates revealed that group D was most prevalent at 32.2% (29/90), followed by group A (28.8%, 26/90), B1 (23.3%, 21/90), and B2 (15.5%, 14/90). Isolates positive for $bla_{CTX-M-15}$ belonged to phylogenetic groups D (8/17), A (7/17), and B1 (2/17) (Table 2). All B2 poultry isolates were screened for identification of the O25b-ST131 clone, but none of them were found to belong to the pandemic *E. coli* clone or to carry $bla_{CTX-M-15}$. The majority of abattoir A1 worker isolates (18/19–94.7%) typed to phylogroup A, except for one isolate, which was identified as belonging to phylogroup D. Most of the human clinical isolates belonged to phylogroup B2 (15/25), followed by group A (6/25) and D (4/25). All but one of the human clinical *E. coli* isolates in phylogroup B2 were identified as belonging to the pandemic O25b-ST131 clone.

The clonal relationship between broiler CTX-M-producing isolates from both abattoirs and the human hospitals was assessed by MLST and PFGE. Broiler isolates showed high diversity with twelve different sequence types (STs) identified in the 13 isolates analyzed, with only one ST (ST88) identified in two isolates (Fig. 1). Conversely, human clinical isolates were less diverse with six STs identified among 12 isolates analyzed by MLST. In addition, allele-specific PCR showed that 60% (15/25) of the human clinical isolates belonged to the pandemic O25b-ST131 clone. There were no common STs found between human and poultry isolates. PFGE produced similar findings to MLST, confirming strain diversity even within strains belonging to the same ST (*i.e.*, ST131). Two clusters (defined as 70% similarity) emerged, all containing both human and broiler isolates. One cluster (Fig. 1, box A) included mostly poultry isolates from abattoir A1 (Broiler A1), but also a human clinical isolate (52H), which shared 79% similarity with a poultry isolate (36B). The second cluster (Fig. 1, box B) included isolates from abattoir A2 (Broiler A2) and isolates from one human hospital (Human H1). One human isolate from this cluster (58H), which belonged to ST73, shared 86% similarity with a poultry isolate of ST168 (100B). In addition to these two main clusters, there were four cases where human and broiler isolates shared a similarity ranging from 77% to 82%. PFGE also showed that the two *E. coli* isolates (one broiler and one human, 14B and 73H), which both carried a rare plasmid-mediated AmpC beta-lactamase, the bla_{ACC-1a}, and coharbored bla_{SHV-12}, were clonally unrelated.

Resistance transfer and PCR-based replicon typing

Conjugation experiments demonstrated the transfer of $bla_{CTX-M-15}$ as well as of $bla_{CTX-M-1}$ and $bla_{CTX-M-3}$ to an *E. coli* recipient in poultry isolates. In addition, PCR-based replicon typing (PBRT) showed that IncI1, FIA, K/B, and P were associated with transfer of ESBL genes. IncI1 was the most common replicon found in the poultry *E. coli* transconjugants, being recovered from all six transconjugants in which the CTX-M genes were successfully transferred. The remaining replicons were each found in one of the six transconjugants obtained. However, none of the $bla_{CTX-M-14}$ genes were transferred, indicating that they may be located on a nonconjugative plasmid or on the chromosome.

In all but eight of the *E. coli* isolates, multiple replicons were detected by PBRT. Nine of the 18 replicons screened were detected in our isolates and included I1, FIA, FIB, F, K, B/O, N, P, and HI2. IncI1, detected singly or in combination with other replicons, was the most prevalent replicon found in broiler ESBL isolates, followed by F ($n=22$), FIB ($n=21$), B/O ($n=11$), FIA ($n=7$), N ($n=6$), K ($n=4$), and P ($n=1$). For the human isolates, the most representative replicons were FIB ($n=20$), FIA ($n=20$), (F = 19), followed by I1 ($n=3$), HI2 ($n=1$), B/O ($n=1$), N ($n=1$), and P ($n=1$) (Table 2).

Discussion

This is the first study to investigate the prevalence of ESBL and AmpC beta-lactamase-producing *E. coli* in chicken production in Romania. Although the numbers of slaughterhouses and farms studied in this work are relatively small, the findings are quite remarkable in signaling the potential for $bla_{CTX-M-15}$ resistance determinant dissemination between chickens and humans and this will be relevant in many countries, not just the one under current investigation. The study showed a high prevalence of ESBL-producing *E. coli* isolates carrying beta-lactamases and/or ESBL resistance genes (69.2%) on the Romanian poultry farms analyzed. In addition, it revealed a high prevalence of ESC-resistant *E. coli* isolates carrying bla_{TEM} and bla_{SHV} genes, indicating poultry as a potential reservoir of these genes.

The prevalence of ESBL and AmpC production among *E. coli* isolates in broiler abattoirs has been shown to vary between European countries, ranging from 10% to 34% in Portugal, France, Denmark, and Sweden. Furthermore, the prevalence can also vary within a country; in Belgium, for example, the prevalence was shown to range from 27% to 75%.[1,16,24,34] More recently, an average within-farm prevalence of $85\% \pm 10\%$ was found in the Netherlands when a sensitive enrichment-based method was used for detection.[19]

In this study, when considering antibiotic resistance genes of bla_{CTX-M} type, CTX-M group 1 was predominant in *E. coli* isolates, while CTX-M group 9 was present in only a minority of isolates, findings which are in accordance with

Table 2. Phenotypic and Genotypic Characteristics of bla_{CTX-M}-Positive *E. coli* from Broilers and Human Clinical Specimens

Source	No of isolates (n)	PFGE isolate ID[a]	PG	CTX-M type	Associated resistance genes: TEM SHV/OXA	Plasmid-mediated AmpC	PMQR	Inc Type	Resistance profile
Chicken	4	27B	A	**CTX-M-3**				F, FIB, **I1**	AMP,CPD,CTX,CAZ,NA,CIP,TE,STX,S
Chicken	2	84B	A	**CTX-M-15**				F,FIB, **FIA, I1**	AMP, AMC, CPD, CTX, CAZ, FOX, NA
Chicken	1	110B	B1	CTX-M-3	TEM-1			B/O, F, FIA, FIB, I1	AMP,AMC, CPC, CTX,CAZ,FOX, NA, CIP, TE, STX, S
Chicken	1	88B	A	CTX-M15	TEM-1			N	AMP,AMC, CPC, CTX,CAZ,FOX, NA, CIP, TE, STX, S
Chicken	1		D	CTX-M 15	TEM-1			I1	AMP,AMC, CPC, CTX,CAZ,FOX, NA, CIP, TE, STX, S
Chicken	1		B1	CTX-M-15				F, FIA, N,I1	AMP,AMC, CPD, CTX,CAZ, NA, CIP, TE, STX, S
Chicken	1	78B	D	CTX-M-3	TEM-1		*aac(6′)-ib-cr*	FIB, HI2, I1	AMP,AMC, CTX,CAZ,FOX, NA, CIP, TE, STX, S
Chicken	5	28B	A	**CTX-M-15**	TEM-1	CMY-2		**K/B, P**, F, FIB, **I1**	AMP,AMC, CPD, CTX,CAZ,FOX, NA, CIP, TE, STX
Chicken	2	48B	B1	**CTX-M-1**		CMY-2		B/O,F,FIB.**I1**	AMP, AMC, CTX,CAZ,FOX, NA, CIP, TE, S
Chicken	3		D	**CTX-M-15**		CMY-2		B/O, F, **I1**	AMP,AMC, CPC, CTX,CAZ,FOX, NA, TE, STX,
Chicken	2	100B	D	**CTX-M-15**	SHV-12			B/O, F, FIB, N, **I1**	AMP, AMC, CPD,CTX, NA, CIP, TE,
Chicken	1	36B	D	CTX-M-3	SHV-12			B/O, F, FIB, N	AMP,AMC, CPD, CTX, NA, CIP, TE, STX, S
Chicken	1	52B	B1	CTX-M-1				N, I1	AMP, AMC, CPD, CTX, CAZ, FOX, NA, CIP, TE, STX, S, CN
Chicken	2	29B	D	CTX-M-15	SHV-2A	CMY-2		B/O, F, FIB, FIA, I1	AMP,AMC, CPD, CTX, CAZ, FOX, NA, CIP, TE, STX, S
Chicken	1	65B	D	CTX-M-14				I1	AMP, AMC, CPD, CTX,CAZ, FOX, NA, CIP, TE, STX, S
Chicken	2	23B	B1	CTX-M-14	TEM[ND]	CMY-2		K/B, P, F, FIB, I1	AMP,AMC, CPD, CTX, NA, CIP, TE, STX,
Chicken	2		B1	CTX-M-14	TEM[ND]	CMY-2		I1	AMP,AMC, CPD, CTX,CAZ,FOX, NA, CIP, TE, S
Chicken	1	73B-AW	D		SHV-2A	ACC-1a		B/O, FIA,FIB,F	AMP, AMC,CPD,CTX,CAZ,FOX,TE,

(*continued*)

Table 2. (Continued)

Source	No of isolates (n)	PFGE isolate ID[a]	PG	CTX-M type	Associated resistance genes: TEM SHV/OXA	Associated resistance genes: Plasmid-mediated AmpC	Associated resistance genes: PMQR	Inc Type	Resistance profile
Human	1	14B	A		SHV-2A	ACC-1a	*aac(6′)-ib-cr*	B/O, FII_A	AMP, AMC, CPD, CTX,CAZ, FOX, NA, CIP, TE, STX, S
Human	1	52H	D	CTX-M-15	TEM[ND]			B/O, FIA	AMP, AMC, CPD, CTX,CAZ, FOX, NA, CIP, TE, STX, S, CN
Human	1	2H	A	CTX-M-3/22	TEM-1		*qnr b*	F, FIB, I1	AMP, AMC, CPD, CTX,CAZ, TE, STX, S
Human	1	17H	A	CTX-M-15	TEM[ND] OXA-1		*aac(6′)-ib-cr*	F, FIB, FIA	AMP, AMC, CPD, CTX,CAZ, TE, S, CN
Human	2	79H	D	CTX-M 15	OXA-1		*aac(6′)-ib-cr*	NT	AMP, AMC, CPD, CTX,CAZ, FOX, NA, CIP, TE, STX, S
Human	1	6H	B2	CTX-M-15	TEM-1 OXA-1		*aac(6′)-ib-cr, qnrS*	F, FIB, FIA,N	AMP, AMC, CPD, CTX,CAZ, NA, CIP, TE, S
Human	1	12H	A	CTX-M-15	OXA-1		*aac(6′)-ib-cr*	F, FIB, FIA, I1	AMP, AMC, CPD, CTX,CAZ, FOX, NA, CIP, TE, CN
Human	1		A	CTX-M-15	OXA-1		*aac(6′)-ib-cr*	I1	AMP, AMC, CPD, CTX,CAZ, TE, S, CN
Human	2		B2	CTX-M-15	OXA-1		*aac(6′)-ib-cr*	F, FIB, FIA	AMP, AMC, CPD, CTX, CAZ, FOX, NA, CIP, TE, STX, S, CN
Human	1	5H	B2	CTX-M-15				F, FIB, FIA	AMP, AMC, CPD, CTX, CAZ, NA, CIP, TE, STX, S,
Human	8	24H	B2	CTX-M-15	TEM[ND] OXA-1		*aac(6′)-ib-cr*	F, FIB, FIA	AMP, AMC, CPD, CTX, CAZ, NA, CIP,
Human	1	56H	B2	CTX-M-15	TEM[ND] OXA-1		*aac(6′)-ib-cr*	HI2	AMP, AMC, CPD, CTX, CAZ, NA, CIP,
Human	1	58H	D	CTX-M-15				I1	AMP, AMC, CPD, CTX, CAZ, FOX, TE, STX, S,
Human	3	68H	B2	CTX-M-14				F, FIB, FIA	AMP, AMC, CPD, CTX, CAZ, FOX, NA, CIP, TE, STX, S,
Human	1	41H	A	CTX-M − 2	TEM-1			P, HI2	AMP, AMC, CPD, CTX, CAZ, FOX, NA, CIP, TE, STX, S,

Two isolates (one human-73H and one from an abttoir worker 14B-AW) carrying the bla_{ACC-1a} beta-lactamase are also included. Underlining and bold indicate *ESBL/amp*C genes and plasmids transferred by conjugation.

[a]Isolate identification given in Fig. 1; NT-not typable by PBRT; B, broiler isolates; H, human clinical isolates; B-AW, broiler abattoir worker isolate; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPD, cefpodoxime; NA, nalidixic acid; CIP, ciprofloxacin, CN, gentamicin, S, streptomycin; TE, tetracycline, STX, trimethoprim/sulfamethoxazole.

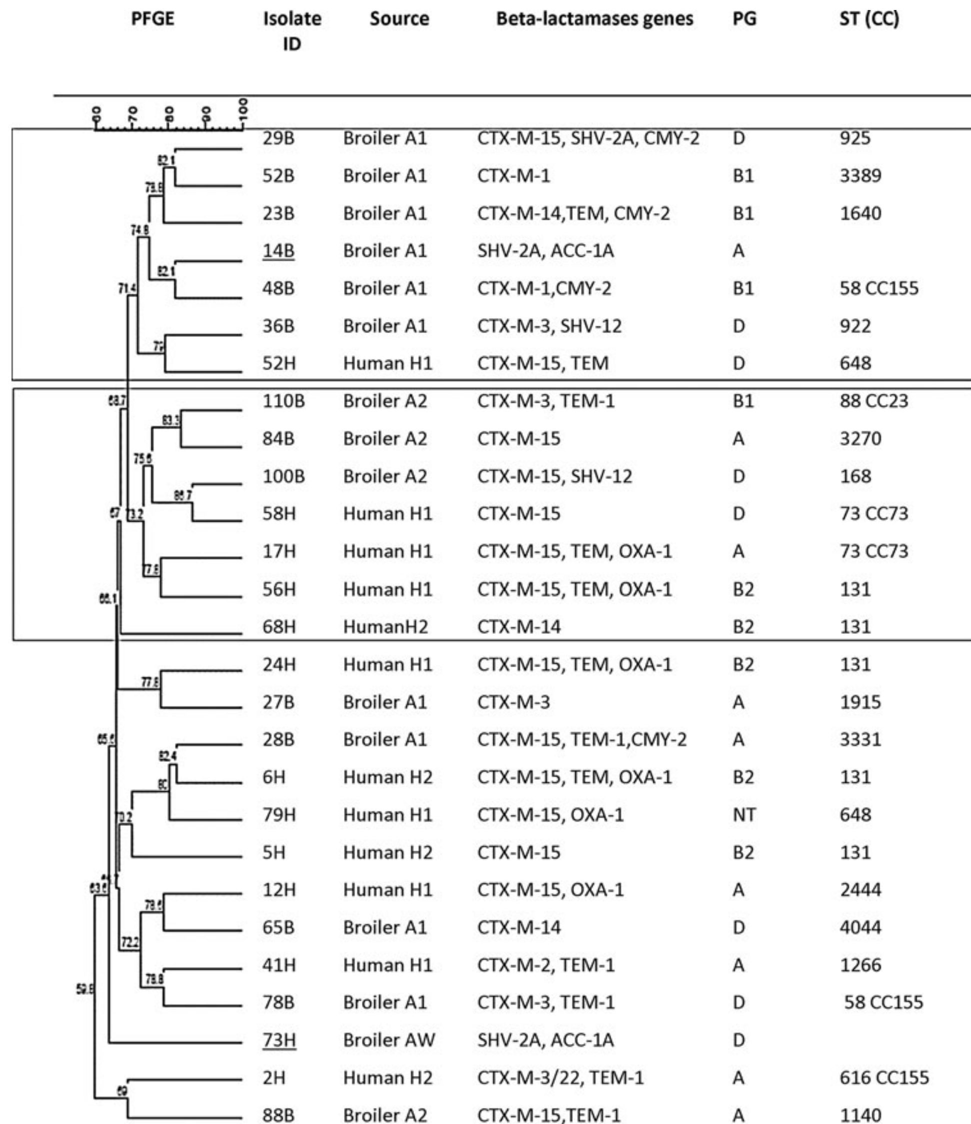

FIG. 1. Dendogram showing cluster analysis of *Xba*I PFGE patterns of 25 CTX-M producing *E. coli* broiler isolates from abattoirs A1 and A2 and human clinical isolates from two hospitals (H1 and H2). Two isolates (both underlined, one human-73H and one from abattoir workers 14B) carrying the $bla_{\text{ACC-1a}}$ beta-lactamase are also included. The columns to the right of the PFGE pattern indicate the isolates ID, their origin, beta-lactamase genes carried, phylogroup (PG), sequence type (ST) and clonal complex (CC) where known. Boxes (A & B) indicate main clusters defined as 70% similarity. NT, not typeable; PFGE, pulsed-field gel electrophoresis.

other studies from European countries.[3,24,31,46] However, within the CTX-M group 1 family, $bla_{\text{CTX-M-1}}$ was the most prevalent gene found in poultry or poultry retail meat in France, Sweden, the Netherlands, and Belgium.[10,22,29,39] Interestingly, in our study, $bla_{\text{CTX-M-1}}$ was present in only three isolates, while $bla_{\text{CTX-M-15}}$, which is the most common type of ESBL gene found in human clinical isolates worldwide, was found in 53% of poultry isolates exhibiting ESC resistance. This finding in chickens in Romania is surprising given the general low prevalence of $bla_{\text{CTX-M-15}}$ in poultry isolates reported elsewhere. For instance, CTX-M-15 enzymes were found in 11.9% and 2% of poultry samples from Great Britain and Belgium and were found in only one isolate from 600 cloacal samples from broilers analyzed in Switzerland.[22,42,46] Moreover, $bla_{\text{CTX-M-15}}$ was not found in chicken retail meat isolates from the Netherlands, Sweden,[29,31,39] or in *E. coli* from samples from poultry in the Netherlands, Italy, and Denmark,[5,20] as well as in Japan or China.[28,55] To the best of our knowledge, this is the highest prevalence of $bla_{\text{CTX-M-15}}$ reported in *E. coli* isolates from poultry. This is a worrying finding, considering the evidence from other studies suggesting that the transfer of resistant isolates or their mobile genetic elements from broilers to humans is highly likely.[29,31,39]

Romania contributes to the overall data collected by the European Antimicrobial Resistance Surveillance Network (EARS-Net) and its data show a high prevalence (41%) of ESBL-producing *E. coli* invasive isolates. However, data on the molecular characterization of *E. coli* clinical isolates from Romanian hospitals are largely lacking. The human clinical isolates used in this study were previously phenotypically confirmed as ESBL producers,[48] but additional sequencing carried out in this study showed that 80% of these isolates carried the $bla_{\text{CTX-M-15}}$ gene alone or in combination with other genes. Moreover, many of the human clinical isolates (48%) carrying the $bla_{\text{CTX-M-15}}$ gene also coharbored $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, and *aac(6′)-Ib-cr*, and this is the first report, which demonstrates the prevalence of this genotype in human clinical isolates in this part of the country.

Although clonal dissemination has been implicated in the worldwide spread of $bla_{\text{CTX-M-15}}$,[14] both MLST and PFGE approaches used in our study indicated diversity of human

and poultry strains. Apart from ST131, which was the most prevalent sequence type found in our human isolates, we also identified other sequence types, which are associated with human disease and which have the potential to be human–animal shared. For instance, in our study, we only identified ST73 in human isolates, but the MLST database shows that this ST is common in human and animal species and has also been described in companion animals, ducks, and chickens in Germany.[49] Similarly, ST648, which we identified in two human isolates from the same hospital, was previously reported as one of the most frequent STs found in extraintestinal pathogenic *E. coli* (ExPEC) from Brazil.[35] In addition, we have identified ST616 among the Romanian *E. coli* human isolates, a sequence type, which was previously reported in avian pathogenic *E. coli* (APEC) from Brazil.[35] Moreover, some STs, which we found in the chicken isolates analyzed, were previously associated with human pathogenic strains in other studies. For instance, ST88, which we identified in one poultry isolate, was also found in Dutch chicken meat and was reported to be the most common ST of the uropathogenic *E. coli* isolates analyzed from the northeast of England.[23,30,54] In addition, in poultry isolates, we have identified three STs (ST168, ST4044, and ST1915), which were only previously identified in human isolates according to the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), indicating their zoonotic potential.

Although on the basis of MLST and PFGE results no common sequence types/clones were identified, the high prevalence of $bla_{CTX\text{-}M\text{-}15}$ in both human and poultry groups may suggest that other vectors, such us mobile genetic elements, may have played a role in the spread of genetic determinants in the poultry population. Plasmid replicon typing showed that most CTX-M genes were linked to IncI1, F, and FIB in the poultry isolates and to F, FIA, FIB, and also to IncI1 in the human isolates. Moreover, our conjugation experiments showed that IncI1 and FIA were closely associated with the transfer of $bla_{CTX\text{-}M\text{-}15}$ as well as of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}3}$ to a recipient *E. coli* strain. IncI1 was identified in all poultry *E. coli* transconjugants, indicating its likely association with the transfer of $bla_{CTX\text{-}M}$ genes. The association of $bla_{CTX\text{-}M\text{-}15}$ with IncI1, FIB, and FIA plasmids in both poultry and human clinical isolates suggests a role in horizontal ESBL gene transmission. In addition, although PFGE showed that the two *E. coli* isolates, one broiler and one human (14B and 73H), which both carried a rare plasmid-mediated AmpC beta-lactamase, $bla_{ACC\text{-}1a}$, and also coharbored $bla_{SHV\text{-}12}$, were clonally unrelated, both isolates carried B/O Inc-type replicons, suggesting involvement in gene transmission. These findings are in agreement with Borjesson *et al*.[3] who showed that spread of $bla_{CTX\text{-}M\text{-}1}$ in Swedish broilers is driven by the spread of an IncI plasmid and not by a specific clone.[3] Furthermore, Leverstein-van Hall *et al.*[31] showed that the same ESBL genes may be found in Dutch patients, retail chicken meat, and poultry and are located on IncI1 plasmids from human samples that were genetically indistinguishable from those obtained from poultry meat. More recently, Wang *et al.*[50] highlighted the role that Inc-type plasmids have in the spread of bla_{ESBL} determinants between the human and food-producing animal isolates.

Our study has some limitations as it also aimed to study the relatedness of poultry *E. coli* isolates and those recovered from the abattoir workers and human clinical isolates from two large hospitals in the same geographical area. In this study, making comparisons of ESBL carriage between chickens and humans has to be tempered by the relatively low number of human clinical isolates available and human volunteer isolates provided by the National Institute of Public Health, Iasi, for this study. However, the data, particularly with regard to high prevalence of $bla_{CTX\text{-}M\text{-}15}$ in chicken and human samples are solid and worrying.

In addition to the high prevalence of $bla_{CTX\text{-}M\text{-}15}$ found in Romanian poultry isolates, the current study indicates a high rate of AmpC-producing *E. coli* isolates (53%), which carried plasmid-mediated *ampC* genes, mostly of the CMY-2 type. The high prevalence of CMY-2 *E. coli* in food-producing animals and in humans has been reported in both Europe and the United States.[52] Within European countries, the CMY-2 prevalence in Romanian chickens was higher than that found in Holland, Switzerland, and Spain, but lower than in Poland (78%).[15,19,22,51] Interestingly, in the present study, $bla_{CMY\text{-}2}$ was not identified in any human *E. coli* isolates either from abattoir workers or from human clinical isolates in Romania. In addition to a high prevalence of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$, we found that bla_{TEM} and bla_{SHV} (mainly $bla_{SHV\text{-}12}$) genes were widespread in the ESC-resistant *E. coli* isolates from poultry (44% and 33%, respectively). In Europe, the prevalence of $bla_{SHV\text{-}12}$ was high in chickens in Italy (64%) and was 14–16% in poultry meat samples in the Netherlands, while the narrow-spectrum beta-lactamase TEM-1 was present in 41% of Belgian broilers.[4,29,31,39,46]

Although poultry samples from only two abattoirs were sampled in the present study, abattoir A1 processed poultry from four farms spread throughout the northeast of Romania. From this abattoir, a multitude of beta-lactamases and ESBLs genes were identified, indicating a diversity of resistance genes in *E. coli* strains in Romanian poultry farms.

This study shows a high prevalence of $bla_{CTX\text{-}M\text{-}15}$ in both the poultry *E. coli* isolates and also the human clinical isolates. However, further comparison of the *E. coli* genotypes from these two groups shows some degree of segregation of AmpC/ESBL gene distribution. For instance, $bla_{CMY\text{-}2}$ was found exclusively in poultry and $bla_{OXA\text{-}1}$ was found exclusively in human clinical isolates, while $bla_{SHV\text{-}12}$ and bla_{TEM} were mainly found in poultry, but also in a number of isolates (two and four, respectively) from human abattoir workers. This is also reflected in the susceptibility profile of isolates, which showed high levels of penicillin and cephalosporin resistance in both human and poultry isolates, while cefoxitin resistance, a marker of AmpC production, was more prevalent in the poultry *E. coli* isolates. The high prevalence of cefoxitin resistance and of $bla_{CMY\text{-}2}$ in poultry isolates may reflect the antimicrobial selective pressure exerted by the prophylactic use of antimicrobials (most likely cephalosporins) on farms in the Romanian poultry industry.

We could not obtain specific data regarding use of antimicrobials at the local farm level either for treating infections or for prophylaxis. The off-label use of ceftiofur is restricted in poultry in the European Union, but it is sometimes used to prevent mortality in 1-day-old chickens.[45] Antimicrobial use in Romania is not as strictly monitored as in other European countries and the high prevalence of AmpC/ESBL in the poultry isolates may be a reflection of this situation.

In addition to the high numbers of Romanian poultry *E. coli* isolates carrying AmpC/ESBL resistance genes, the most remarkable finding of this study is the high prevalence of $bla_{CTX\text{-}M\text{-}15}$ in these poultry isolates. These findings warrant investigation of $bla_{CTX\text{-}M\text{-}15}$ origin in the broiler production chain, of the role that chickens may play as a potential reservoir of plasmids and resistance genes for humans through the food chain, and its implication for public health. Previous studies have pointed to potential infection sources, such as river water, contaminated feed, or vectors (*i.e.*, flies or rodents), which may play a major role in the transmission epidemiology of ESBL-producing isolates.[2,18,25,26] Identification of the factors, which may have been involved in the emergence of $bla_{CTX\text{-}M\text{-}15}$ or of the plasmids carrying them in the Romanian broiler *E. coli* isolates, requires further investigation. Recognition of $bla_{CTX\text{-}M\text{-}15}$ sources in the broiler production chain and their subsequent control is critical for reducing the burden of resistance genes circulating through the food chain and the associated risks for local or European (through export) food industries and markets.

Conclusion

This study identified that chickens in Romania have the highest prevalence of $bla_{CTX\text{-}M\text{-}15}$-positive *E. coli*, the most prevalent ESBL type in human clinical isolates worldwide, as well as a high prevalence of isolates carrying plasmid-mediated *ampC* genes. Mobile genetic elements, especially IncI1 replicons, rather than bacterial clones, may be associated with the transfer of these resistance determinants. Identification of $bla_{CTX\text{-}M\text{-}15}$ sources in the broiler production chain is critical for reducing the zoonotic risks through the food chain.

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Author Disclosure Statement

All authors declare that they have no conflicts of interest or any other competing financial interests to disclose.

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