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Changing plasmid types responsible for extended spectrum cephalosporin resistance in *Escherichia coli* O157:H7 in the United States, 1996–2009

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Abstract

Escherichia coli O157 is a major cause of foodborne illness. Plasmids are genetic elements that mobilize antimicrobial resistance determinants including bla_{CMY} β-lactamases that confer resistance to extended-spectrum cephalosporins (ESC). ESCs are important for treating a variety of infections. IncA/C plasmids are found among diverse sources, including cattle, the principal source of *E. coli* O157 infections in humans. IncI1 plasmids are common among *E. coli* and *Salmonella* from poultry and other avian sources. To broaden our understanding of reservoirs of bla_{CMY}, we determined the types of plasmids carrying bla_{CMY} among *E. coli* O157. From 1996 to 2009, 3742 *E. coli* O157 isolates were tested. Eleven (0.29%) were ceftriaxone resistant and had a bla_{CMY-2}-containing plasmid. All four isolates submitted before 2001 and a single 2001 isolate had bla_{CMY} encoded on IncA/C plasmids, while all five isolates submitted after 2001 and a single 2001 isolate had bla_{CMY} carried on IncI1 plasmids. The IncI1 plasmids were ST2, ST20, and ST23. We conclude that cephalosporin resistance among *E. coli* O157:H7 is due to plasmid-encoded bla_{CMY} genes and that plasmid types appear to have shifted from IncA/C to IncI1. This shift suggests either a change in plasmid type among animal reservoirs or that the organism has expanded into avian reservoirs. More analysis of human, retail meat, and food animal isolates is necessary to broaden our understanding of the antimicrobial resistance determinants of ESC resistance among *E. coli* O157.

Keywords

Antimicrobial resistance; *Escherichia coli* O157:H7; resistance plasmid; cephalosporin resistance

1. Introduction

Escherichia coli is commonly found in the intestinal tracks of humans and animals and can be spread to food, soil, and water by fecal contamination. While most *E. coli* is commensal,

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Shiga-toxin producing *E. coli* (STEC) has emerged as a significant public health threat. One particular STEC, O157, is a major cause of foodborne illness and can result in diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome in humans [1]. *E. coli* O157 has been the cause of many foodborne disease outbreaks and much research has focused on the prevalence of this organism in beef cattle and the resulting contamination of ground beef [2]. However, *E. coli* O157 has been identified among additional food animals including dairy cattle, swine, and poultry [3–5].

Antimicrobial treatment of STEC infections is currently not recommended due to bacterial lysis and subsequent Shiga toxin release. However, some studies have suggested that early administration of some antimicrobials may prevent disease progression [6–8]. More recently, a retrospective study of treatment outcomes from the 2011German *E. coli* O104:H4 outbreak concluded that antimicrobials may be an effective treatment [9]. Besides their use in human therapeutics and veterinary medicine, antimicrobials are also used in food animal production for growth promotion and disease prevention. This has likely led to the selection of antimicrobial resistance among STECs in food animals [10]. By identifying and characterizing the mechanisms of resistance and how they are spread, we hope to better understand the relationships between resistance attributes and possible sources of human infection among food animals and their retail meat.

Extended-spectrum cephalosporins (ESCs) are an important class of drugs used in human and veterinary medicine, and veterinary uses include treatment of food animals. Resistance to ESCs is usually mediated by the production of certain classes of β-lactamases, including AmpC β-lactamases, commonly encoded by $bla_{\text{CMY-2}}$ genes [11]. Plasmids are genetic elements that can mobilize antimicrobial resistance determinants, including bla_{CMY} β-lactamases. Plasmids can be distinguished by their incompatibility features (reflecting replication) and can therefore be grouped into several replicon (Inc) types [12]. To broaden our understanding of reservoirs of cephalosporin resistance among *E. coli* O157, we identified and characterized cephalosporin resistant isolates from humans. We identified the mechanism of resistance, determined if resistance was plasmid mediated and if so, determined the types and mobility of these plasmids. We also examined the genetic relatedness of isolates to determine if clonal spread could be responsible for the cephalosporin resistance

2. Materials and Methods

2.1 Isolate collection and testing

E. coli O157 isolates were obtained from specimens from ill persons submitted to clinical laboratories in the United States and subsequently forwarded to state public health laboratories. Participating state public health laboratories serotyped and submitted every twentieth *E. coli* O157 to the CDC National Antimicrobial Resistance Monitoring System (NARMS) laboratory for susceptibility testing. The Enteric Diseases Laboratory Branch at CDC also receives representative isolates from foodborne disease outbreaks from state and local public health laboratories, and NARMS performs susceptibility testing on those isolates as well. Broth microdilution (Sensititre®, Trek Diagnostics, Westlake, OH) was used to determine the minimum inhibitory concentrations (MIC) for 15 antimicrobial agents:

amikacin, ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole/sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. Resistance was defined by the Clinical and Laboratory Standards Institute (CLSI) interpretive standards, when available [13]. For streptomycin, where no CLSI interpretive criteria for Enterobacteriaceae isolated from humans exist, the resistance breakpoint is 64 mg/L [14]. Testing was performed according to the manufacturer's instructions and using the following quality control strains: *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853.

2.2 PCR amplification and sequencing of *bla*$_{CMY}$ and the promoter of *ampC*

For each isolate, DNA was isolated by lysing the bacteria at 95°C and collecting the supernatant following centrifugation for 10 min at 20,000 g (Sorvall RC5B Plus, SS-34 rotor, Thermo Fischer Scientific Inc., Waltham, MA). PCR reactions contained 1x Hot Start PCR Master Mix (Qiagen Inc., Valencia, CA), 0.4μM of each primer, 5μl template DNA and sterile PCR water to a final volume of 50μl. Thermal cycling was performed using the following conditions: 15 min at 95°C, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 90 s. To determine the presence of *bla*$_{CMY}$ genes, primers *ampC*1 and *ampC*2 were used [15]. These primers and internal primers CMYSEQ1 (5'-GGTTGCAGGACGCGTCTG-3') and CMYSEQ2 (5'-CCGCAATGGACTCCGGGC-3') were used to sequence the entire *bla*$_{CMY}$ gene. PCR amplification of the promoter of *ampC* was performed with primers AB1 and AmpC2 as previously described [16]. Sequencing was performed using Big Dye version 3.1 (Applied Biosystems, Foster City, CA) and sequence reactions are cleaned with Centri-sep plates (Princeton Separations, Adelphia, NJ). The reactions are electrophoresed through POP-7 polymer (Applied Biosystems) on a 3730 DNA Analyzer (Applied Biosystems) equipped with a 48-capillary, 50 cm array. Sequence analysis was performed using Lasergene 8 software (DNASTAR Inc, Madison, WI). The detection of genetic elements associated with the *bla*$_{CMY}$ gene was performed as previously described [17, 18].

2.3 Plasmid purification and characterization

Purified plasmid DNA was used to transform laboratory *E. coli* strains, separate the *bla*$_{CMY}$ plasmids from other plasmid types prior to replicon typing, and perform plasmid multi-locus sequence typing (pMLST). Plasmids were purified using the QiaFilter Midi kit (Qiagen Inc.), following a modified manufacturers protocol as previously described [19]. Electroporation of each plasmid into *E. coli* DH10B Electromax competent cells (Invitrogen, Carlsbad, CA) was performed as previously described [19]. Cells were plated on LB agar plates containing 100 mg/L of ampicillin (Sigma-Aldrich, St. Louis, MO). Plasmids were re-purified from a single *bla*$_{CMY}$ PCR-positive transformant to isolate a single plasmid from each isolate. Purification was performed as described above with the additional modification of growing the cells overnight in 25 ml of LB broth with 100 mg/L of ampicillin. Plasmid PCR-based replicon typing (PBRT) was performed as previously described [12]. Plasmid multi-locus sequence typing was performed on IncI1 plasmids as previously described [20]. Sequences were submitted to the plasmid multi locus sequence type (pMLST) web page (http://pubmlst.org/plasmid/) and the ST type was determined. Conjugation experiments

were performed as previously described using a sodium azide resistant J53 *E. coli* strain as recipient (gift from G. Jacoby) [21]. Transconjugants were selected on LB containing 200 mg/L sodium azide and 100 mg/L of ampicillin. A single colony from each transformation experiment was PCR-screened for the bla_{CMY} gene to confirm successful conjugation. Conjugation efficiency was equal to the number of recipient cells divided by the number of donor cells. Conjugation experiments were performed in duplicate. A single representative experiment is shown in Table 2. Plasmid size was determined using a pPFGE protocol [19]. Plasmids from *E. coli* strains PDK9 and V517, which contain several plasmids that range in size from 2.2 to 220 kb, were also extracted and used as size standards on the gels [22].

2.4 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed according to the CDC PulseNet protocol and all PFGE profiles generated were submitted to the PulseNet national database [23]. Gel images were captured using the GelDoc XR system (Bio-Rad Laboratories) and Quantity One 1-D analysis software (Bio-Rad Laboratories). Pattern analysis and UPGMA dendrogram generation were performed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) with the Dice coefficient and tolerance of 1.5%.

3. Results

3.1 Identification of ceftriaxone resistant isolates

From 1996 to 2009, CDC NARMS received 3742 *E. coli* O157 isolates and tested them for antimicrobial susceptibility to 15 antimicrobials. Eleven (0.29%) isolates were resistant to the third generation cephalosporin ceftriaxone (Table 1). All were isolated between 2000 and 2009 and were confirmed as serotype O157:H7. Ten of the eleven resistant isolates were submitted as part of routine surveillance; the single 2009 isolate was from an outbreak with unknown source (Table 1). The resistant isolates originated from 10 different states, with two submitted from California. All isolates were from stool samples. The patients included six females, three males, and two persons whose gender was not reported. Their ages varied from 1 to 83 years, with a median of 15 years. All cephalosporin-resistant isolates were also resistant to ampicillin, amoxicillin/clavulanic acid, cefoxitin, and ceftiofur. The four isolates obtained in 2000 and the one obtained in 2001 were also resistant to chloramphenicol, sulphamethoxazole/sulfisoxazole, streptomycin, and tetracycline. One of the two 2001 isolates and all five isolates submitted after 2001 displayed no additional resistance.

3.1 Determination of the genetic relatedness of the isolates

To determine the genetic relatedness of isolates, pulsed-field gel electrophoresis (PFGE) was performed both with *Xba*I- and *Bln*I-restricted DNA and a dendrogram was constructed (Figure 1). All isolates showed a degree of relatedness (>75%) and two sets of two isolates were indistinguishable by both enzymes. Interestingly, these 4 isolates were among the earliest identified by NARMS (year 2000–01). All of the remaining isolates had different PFGE patterns.

3.2 Characterization of the ceftriaxone resistance determinant

The observed resistance to cefoxitin, a cephamycin, and amoxicillin/clavulanic acid, a β-lactam/β-lactam inhibitor combination, suggests the presence of an AmpC-like β-lactamase. All isolates were positive for a *bla*$_{CMY}$ gene by PCR (the four isolates from year 2000 were previously shown to be *bla*$_{CMY}$ positive) [24]. Sequence analysis identified genes as *bla*$_{CMY-2}$ (Table 2). No mutations were identified within the promoter region of the chromosomal copy of *ampC* (data not shown). To determine if the *bla*$_{CMY-2}$ genes were plasmid-encoded, plasmid DNA was purified from the isolates and transferred to laboratory *E.coli* strains by electroporation. The *bla*$_{CMY-2}$ genes and corresponding resistance phenotype transferred from all 11 isolates, confirming that the *bla*$_{CMY-2}$ genes for all 11 isolates were located on plasmids. To further characterize the *bla*$_{CMY-2}$ plasmids, PCR-based incompatibility testing (PBRT) was performed on the transformants. The five isolates submitted through 2000 and a single 2001 isolate had *bla*$_{CMY-2}$ encoded on IncA/C plasmids, while all five isolates submitted after 2001 and the remaining 2001 isolate had *bla*$_{CMY-2}$ carried on IncI1 plasmids (Table 2). Interestingly, the 4 isolates which had paired PFGE patterns (2 matched sets) all had IncA/C-*bla*$_{CMY-2}$ plasmids. These IncA/C-*bla*$_{CMY-2}$ plasmids varied in size from 100–150 kb, while the IncI1-*bla*$_{CMY-2}$ plasmids varied from 90–110 kb. To determine if these plasmids were competent for conjugation, a conjugation efficiency assay was performed. Both types of plasmids were able to transfer to the recipient strain by broth conjugation. The IncA/C plasmids displayed a wide range of conjugation efficiency; three had high/moderate efficiency (10^{-2}, 10^{-3}) while two of the IncA/C plasmids had very low efficiency (10^{-8}, 10^{-9}). In contrast, all of the IncI1 plasmids displayed high conjugation efficiency (10^{-1}, 10^{-2}). The genetic elements associated with the *bla*$_{CMY}$ genes were investigated by PCR analysis. All eleven *bla*$_{CMY}$ genes were located downstream of an IS*Ecp*1 element, but 4 of these elements (found in plasmids pAM17504, pAM18099, pAM25705, and p2009EL1288) contained IS*Ecp*1 elements with 5’ deletions (data not shown). No IS*CR*1 or IS26 elements were identified.

Antimicrobial susceptibility testing performed on the plasmid transformants identified additional resistance phenotypes conferred by all of the IncA/C-*bla*$_{CMY-2}$ plasmids, including resistance to chloramphenicol, sulphamethoxazole/sulfisoxazole, streptomycin, and tetracycline. None of the IncI1-*bla*$_{CMY-2}$ plasmids conferred additional resistance. To further characterize the IncI1-*bla*$_{CMY-2}$ plasmids, plasmid multi-locus sequence typing (pMLST) was performed on the plasmid containing transformants. Of the six IncI1-*bla*$_{CMY-2}$ plasmids, three were sequence type (ST) 23, two were ST2, and one was ST20 (Table 3). There is currently no pMLST scheme for IncA/C plasmids.

4. Discussion

Extended-spectrum cephalosporins (ESC) are an important class of antimicrobials drugs used in human medicine and veterinary medicine, including in food animal production settings [25]. Although ESCs are rarely used for the treatment of STEC infections in humans, understanding the mechanisms of ESC resistance and transmission of ESC-resistant bacteria may provide us with clues into possible sources of human infection. The percentage of ESC resistance among *E. coli* O157 isolates collected from ill humans by CDC-NARMS

remained low (0.29%) over the last 13 years. The eleven ESC resistant isolates identified in this study were spread out over time (2000–2009) and location (submitted by 10 states). PFGE analysis showed a variety of subtypes and only 2 sets of 2 isolates were indistinguishable. This suggests that the observed ESC resistance among *E. coli* O157:H7 isolates in the U.S. is not being spread clonally.

All eleven isolates displaying ESC resistance were positive for the $bla_{\text{CMY-2}}$ gene, a common ESC-resistance determinant found among *Salmonella* in the United States [19]. *E. coli* also have a chromosomal *ampC* gene which can be upregulated due to promoter mutations, resulting in cephalosporin resistance. However, no mutations were identified within the promoter region of *ampC* among our isolates. Beside resistance to the ESCs ceftriaxone and ceftiofur, the $bla_{\text{CMY-2}}$ genes also conferred resistance to ampicillin, amoxicillin/clavulanic acid, and cefoxitin. All of the $bla_{\text{CMY-2}}$ genes were located downstream of IS*Ecp*1 elements which were located on plasmids, common for $bla_{\text{CMY-2}}$ genes. Depending on their origin or replication, some plasmids can be typed or grouped based on their compatibility within a host bacteria. Two plasmid types, IncA/C and IncI1, were found to carry the $bla_{\text{CMY-2}}$ gene among our isolates. Additional characteristics of the IncA/C- $bla_{\text{CMY-2}}$ and IncI1- $bla_{\text{CMY-2}}$ plasmids were similar to what was previously observed for these plasmids isolated from *Salmonella* [11, 19, 26–28]. Specifically, IncA/C plasmids are usually large (>150kb), have varying conjugation efficiencies, and carry additional resistance determinants, while IncI1- $bla_{\text{CMY-2}}$ plasmids are smaller (~100kb), highly mobile (conjugation efficiency 10^{-1}–10^{-2}), and usually do not carry additional resistance determinants. IncA/C and IncI1 are common plasmid types found to carry $bla_{\text{CMY-2}}$ among enteric bacteria in the U.S. However, we were surprised to see a shift in 2001, from IncA/C- $bla_{\text{CMY-2}}$ plasmids to IncI1- $bla_{\text{CMY-2}}$ plasmids among our ESC resistant *E. coli* O157:H7. IncA/C plasmids have been found among diverse sources, including cattle, which are believed to be the principal reservoir for *E. coli* O157:H7 and the major source of food products transmitting the organism to humans [2]. In contrast, IncI1 plasmids are most commonly found among *E. coli* and *Salmonella* isolated from poultry and other avian sources and encode a type IV pilus system which may be involved in avian pathogenicity [11, 19, 26, 29].

Although based on a very small number of isolates, this shift in plasmid type carrying the $bla_{\text{CMY-2}}$ gene from IncA/C to IncI1 suggests either a possible change in $bla_{\text{CMY-2}}$- plasmid type among cattle (from IncA/C to IncI1) or a possible expansion of the *E. coli* O157 reservoir among food animal sources (from cattle to poultry). The six IncI1- $bla_{\text{CMY-2}}$ plasmids could be further typed into sequence types (ST) and compared with additional isolates with similar plasmids in the plasmid multi-locus sequencing database [30]. ST23 plasmids have been identified previously from $bla_{\text{CMY-2}}$ positive isolates, including an *E. coli* clinical isolate obtained in Canada and *Salmonella* ser. Heidelberg clinical and a retail meat (chicken breast) isolates obtained here in the U.S. [11, 19, 31]. ST20 and ST2 plasmids have been found among $bla_{\text{CMY-2}}$ positive *E. coli* and *Salmonella* from multiple sources, including environmental and clinical samples collected in Canada and the U.S [19, 31]. These additional isolates, similar to our six isolates with IncI1 plasmids, were also collected

after 2001. This further suggests that these IncI1 plasmids may be present in different types of enteric bacteria and in different sources.

We conclude that the observed cephalosporin resistance among *E. coli* O157:H7 is due to plasmid-encoded bla_{CMY} genes and that plasmid types appear to have shifted from IncA/C to IncI1. This shift suggests a possible a change in plasmid type among animal reservoirs or the possibility that the organism has expanded into avian reservoirs. While a considerable amount of research has been performed on ESC resistance in generic *E. coli*, very little is known about resistance among STEC or serogroup O157 specifically. In particular, analysis and characterization of *E. coli* O157:H7 isolates from food animals and retail meat is necessary to better understand transit of and selection pressures for plasmid-mediated ESC resistance. Better understanding of the connection between plasmid type and animal reservoir may contribute to determination of source of human infection.

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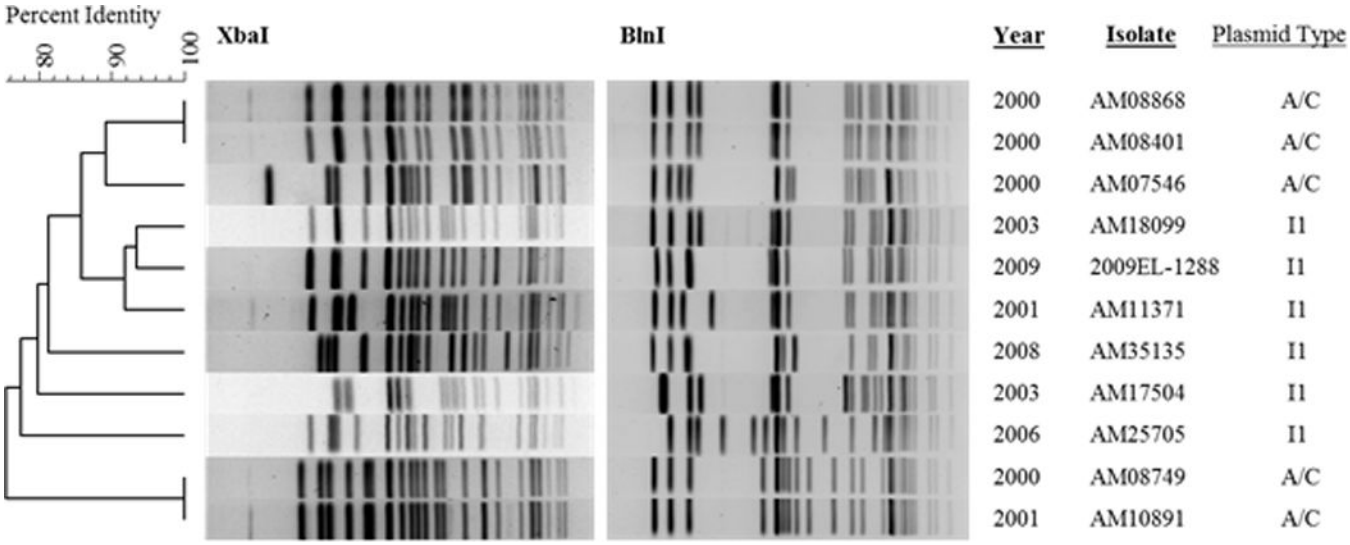

Figure 1.

PFGE patterns of ESC resistant *E. coli* O157:H7 isolated from humans in the United States. Dendrogram of percent genetic identity by PFGE was generated using BioNumerics based on *Xba*I and *Bln*I restriction digestion. Pattern analysis and UPGMA dendrogram generation were performed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) with the Dice coefficient and tolerance of 1.5%. Percent identity is located above the dendrogram. Isolate year, isolate number, and plasmid incompatibility type are listed to the right of the dendrogram.

Table 1

Demographic data on the extended spectrum cephalosporin resistant *E. coli* O157:H7.

Isolate	Year	State	Purpose	Source	Gender	Age (yrs)	Additional Resistance[a]
AM07546	2000	MN	Surveillance	Stool	F	10	CHL, FIS, STR, TET
AM08401	2000	KS	Surveillance	Stool	M	62	CHL, FIS, STR, TET
AM08749	2000	CA	Surveillance	Stool	F	1	CHL, FIS, STR, TET
AM08868	2000	CT	Surveillance	Stool	F	10	CHL, FIS, STR, TET
AM10891	2001	CO	Surveillance	Stool	M	28	CHL, FIS, STR, TET
AM11371	2001	KS	Surveillance	Stool	M	83	None
AM18099	2003	CA	Surveillance	Stool	F	1	None
AM17504	2003	GA	Surveillance	Stool	-	-	None
AM25705	2006	NV	Surveillance	Stool	-	15	None
AM35135	2008	MA	Surveillance	Stool	F	1	None
2009EL-1288	2009	WI	Outbreak	Stool	F	18	None

[a] All isolates are ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, and ceftriaxone resistant

- Not reported;

CHL, chloramphenicol; FIS, sulfamethoxazole/sulfisoxazole; STR, streptomycin; TET, tetracycline; M, male; F, female.

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Table 2

Characteristics of the bla_{CMY-2} plasmids isolated from ESC resistant *E. coli* O157:H7.

Plasmid	Year	β-Lactamase	Inc Type	Approximate Size (kb)	Conjugation Efficiency	Additional Resistance[a]
pAM07546	2000	CMY-2	A/C	150	7.5×10^{-3}	CHL FIS STR TET
pAM08401	2000	CMY-2	A/C	150	2.3×10^{-2}	CHL FIS STR TET
pAM08749	2000	CMY-2	A/C	100	6.6×10^{-8}	CHL FIS STR TET
pAM08868	2000	CMY-2	A/C	150	4.8×10^{-2}	CHL FIS STR TET
pAM10891	2001	CMY-2	A/C	100	8.3×10^{-9}	CHL FIS STR TET
pAM11371	2001	CMY-2	I1	100	1.9×10^{-1}	None
pAM17504	2003	CMY-2	I1	100	1.1×10^{-1}	None
pAM18099	2003	CMY-2	I1	90	2.7×10^{-2}	None
pAM25705	2006	CMY-2	I1	90	1.6×10^{-1}	None
pAM35135	2008	CMY-2	I1	110	8.1×10^{-2}	None
p2009EL1288	2009	CMY-2	I1	90	3.3×10^{-2}	None

[a] All transformants were resistant to ampicillin, amoxicillin/clavulanic acid, cefoxitin, and ceftiofur. Additionally, all transformants were resistant to streptomycin due to the natural resistance of DH10B cells.

CHL, chloramphenicol; FIS, sulfamethoxazole/sulfisoxazole; STR, streptomycin; TET, tetracycline

Table 3

Plasmid multi-locus sequence type of the IncI1-*bla*$_{CMY-2}$ plasmids

Plasmid	Year	Allele[a]					Sequence Type (ST)
		ardA	*pilL*	repI	*sogS*	*trbA/pndC*	
pAM11371	2001	2	1	1	2	3	2
pAM17504	2003	2	1	1	1	3	23
pAM18099	2003	2	1	1	2	3	2
pAM25705	2006	2	1	1	1	3	23
pAM35135	2008	1	1	1	9	3	20
p2009-EL1288	2009	2	1	1	1	3	23

[a] Five plasmid alleles are used to define the sequence type