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Host-Specific Patterns of Genetic Diversity among IncI1-Iγ and IncK Plasmids Encoding CMY-2 β-Lactamase in *Escherichia coli* Isolates from Humans, Poultry Meat, Poultry, and Dogs in Denmark

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ABSTRACT

CMY-2 is the most common plasmid-mediated AmpC β-lactamase in *Escherichia coli* isolates of human and animal origin. The aim of this study was to elucidate the epidemiology of CMY-2-producing *E. coli* in Denmark. Strain and plasmid relatedness was studied in 93 CMY-2-producing clinical and commensal *E. coli* isolates collected from 2006 to 2012 from humans, retail poultry meat, broilers, and dogs. Multilocus sequence typing (MLST), antimicrobial susceptibility testing, and conjugation were performed in conjunction with plasmid replicon typing, plasmid multilocus sequence typing (pMLST), restriction fragment length polymorphism (RFLP), and sequencing of selected bla_{CMY-2}-harboring plasmids. MLST revealed high strain diversity, with few *E. coli* lineages occurring in multiple host species and sample types. bla_{CMY-2} was detected on plasmids in 83 (89%) isolates. Most (75%) of the plasmids were conjugative and did not (96%) cotransfer resistance to antimicrobials other than cephalosporins. The main replicon types identified were IncI1-Iγ (55%) and IncK (39%). Isolates from different host species mainly carried distinct plasmid subtypes. Seven of the 18 human isolates harbored IncI1-Iγ/sequence type 2 (ST2), IncI1-Iγ/ST12, or IncK plasmids highly similar to those found among animal isolates, even though highly related human and animal plasmids differed by nonsynonymous single nucleotide polymorphisms (SNPs) or insertion sequence elements. This study clearly demonstrates that the epidemiology of CMY-2 can be understood only by thorough plasmid characterization. To date, the spread of this β-lactam resistance determinant in Denmark is mainly associated with IncK and IncI1-Iγ plasmids that are generally distributed according to host-specific patterns. These baseline data will be useful to assess the consequences of the increasing human exposure to CMY-2-producing *E. coli* via animal sources.

IMPORTANCE

CMY-2 is the most common plasmid-mediated AmpC β-lactamase in *Escherichia coli*. This β-lactamase is poorly inhibited by clavulanic acid and confers resistance to cephamycins, third-generation cephalosporins, and aztreonam. Furthermore, resistance to carbapenems has been reported in *E. coli* as a result of production of plasmid-encoded CMY-2 β-lactamase in combination with decreased outer membrane permeability. The gene encoding CMY-2 generally resides on transferable plasmids belonging to different incompatibility groups. The prevalence of CMY-2-mediated cephalosporin resistance in *E. coli* varies significantly depending on the geographical region and host. This study demonstrates that the epidemiology of CMY-2 can be understood only by thorough plasmid characterization. To date, the spread of this β-lactam resistance determinant in Denmark is mainly associated with IncK and IncI1-Iγ plasmids, which are generally distributed according to host-specific patterns. These data will be useful to assess the consequences of the increasing human exposure to CMY-2-producing *E. coli* via animal sources.

CMY-2 is the most common plasmid-mediated AmpC β-lactamase in *Escherichia coli* (1). This class C β-lactamase is poorly inhibited by clavulanic acid and confers resistance to cephamycins, third-generation cephalosporins, and aztreonam (2). Furthermore, resistance to carbapenems has been reported in *E. coli* as a result of production of plasmid-encoded CMY-2 β-lactamase in combination with decreased outer membrane permeability (3). The gene encoding CMY-2 (bla_{CMY-2}) generally resides on transferable plasmids belonging to different incompatibility groups, including IncI1-Iγ, IncA/C, IncF, and IncK (4, 5).

The prevalence of CMY-2-mediated cephalosporin resistance in *E. coli* varies significantly depending on the geographical region and host (6, 7). In Denmark, the burden of resistance to third-generation cephalosporins in human infections is only marginally influenced by CMY-2, with an estimated prevalence lower than

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1% (8). Among *E. coli* isolates of animal origin, CMY-2 was the most commonly detected cephalosporin resistance determinant in Danish (83%) and imported (42%) broiler meat until 2013 (9). However, in 2014, a reduction in the occurrence of CMY-2-producing *E. coli* was observed in both Danish (23%) and imported (33%) broiler meat, most likely as a result of discontinued use of third-generation cephalosporins in hatcheries at the top of the broiler production pyramid (10). On the other hand, bla_{CMY-2} is rarely detected in cephalosporin-resistant *E. coli* from Danish pigs and cattle (11). Poultry has been allegedly indicated as a possible source of bla_{CMY-2} in humans. In Sweden, similar bla_{CMY-2}-carrying IncK plasmids were detected in genetically unrelated *E. coli* isolates from broilers, broiler meat, and human clinical isolates, suggesting possible plasmid-mediated zoonotic transmission of bla_{CMY-2} (12, 13). In the Netherlands, highly related IncI1-Iγ and IncK plasmids harboring bla_{CMY-2} were found in human, broiler, and broiler meat isolates (14, 15). Additionally, this resistance determinant occurs at high frequencies among *E. coli* isolates from dogs with an AmpC phenotype (16).

The objective of this study was to investigate possible epidemiological pathways of transmission of CMY-2 β-lactamase across humans, dogs, and poultry in Denmark.

MATERIALS AND METHODS

Strain collection. The bacterial isolates characterized in this study were 93 bla_{CMY-2}-positive *E. coli* samples collected in Denmark in the period from 2006 to 2012. The collection included one isolate from each of 18 humans (16 patients and 2 healthy individuals), 33 poultry meat products (25 broiler meat and 8 turkey meat), 25 chicken flocks (11 healthy broiler flocks and 14 healthy parent flocks), and 17 dogs (7 patients and 10 healthy individuals) (Table 1). The human clinical isolates were collected at the Department of Clinical Microbiology at Hvidovre Hospital (HH) as part of point prevalence studies carried out in October 2006, 2009, and 2011 (17). Isolates from healthy humans were collected in 2008 and kindly provided by the Statens Serum Institut (18). The isolates of poultry origin were kindly provided by the National Food Institute, Technical University of Denmark (DTU). The poultry meat isolates included (i) all available isolates ($n = 13$) from Danish broiler meat and a subset ($n = 12$) of isolates from imported broiler meat (representing different countries and, when applicable, different slaughterhouses within a country) obtained in the national surveillance program (DANMAP) in 2009 and 2010 (19–21) and (ii) isolates ($n = 8$) from turkey meat imported from Germany (the main source of turkey meat consumed in Denmark) representing all five batches positive for CMY-2-producing *E. coli* out of 69 batches randomly collected at Danish retail outlets in 2011. Isolates from Danish broiler flocks and parent flocks were collected in 2010 and 2011, respectively, and encompassed the majority (29 out of 35) of *E. coli* pulsed-field gel electrophoresis (PFGE) types previously identified (22). The clinical canine isolates were collected at the veterinary diagnostic laboratory of the University of Copenhagen between 2008 and 2012, whereas the isolates from healthy dogs originated from fecal samples collected in 2007 and 2008 (23). All the isolates originated from different individuals and were confirmed to carry bla_{CMY-2} by PCR and sequencing (Macrogen Inc.) using primers and under conditions previously described (24).

Strain typing. Multilocus sequence typing (MLST) analysis of four Danish broiler meat isolates and all but one isolate from broiler chickens was performed as part of a previous study (22). The remaining isolates were genotyped by MLST in this study according to published methods (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

***bla*$_{CMY-2}$ transferability and plasmid typing.** Transferability of bla_{CMY-2}-harboring plasmids was analyzed by filter-mating experiments using a rifampin-resistant, lactose-negative *E. coli* K-12 J62-2 strain as the recipient (25). Transconjugants were selected on MacConkey agar (Merck, Denmark) supplemented with 2 mg/liter cefotaxime (CTX) and 25 mg/liter rifampin and were tested by colony PCR to confirm the presence of bla_{CMY-2}. Plasmids carrying bla_{CMY-2} were typed following transfer of plasmid DNA purified either by the PureLink HiPure Plasmid Midiprep kit (Invitrogen, Denmark) or by alkaline lysis into electrocompetent Genehog *E. coli* (Invitrogen, Denmark) by electroporation. Transformants were selected on brain heart infusion agar (Oxoid, Denmark) supplemented with 2 mg/liter CTX and tested by colony PCR to confirm the presence of bla_{CMY-2}. The number and sizes of plasmids were determined by PFGE after S1 nuclease (Thermo Scientific, Sweden) digestion of whole genomic DNA of transformants (26). Transformants positive for bla_{CMY-2} and carrying a single plasmid were further characterized by PCR-based replicon typing (PBRT) (27) using a commercially available kit (Diatheva, Italy). Plasmid multilocus sequence typing (pMLST) was performed for plasmids belonging to typeable groups (28). The sequences obtained were assembled with CLC Main Workbench 6.8.4 (CLC bio, Denmark) and compared to sequences deposited in the pMLST database (http://pubmlst.org/plasmid/). Plasmid types shared by different host species were further characterized by restriction fragment length polymorphism (RFLP) using the following FastDigest enzymes (Thermo Scientific, Sweden): (i) BglII and PstI for IncI1-Iγ plasmids; (ii) EcoRV and SalI for IncK plasmids; and (iii) BamHI, HindIII, and SalI for IncA/C plasmids. Restriction profiles were visualized on a 0.8% agarose gel, and band patterns were visually compared to define indistinguishable and closely related subtypes differing by two or three bands. Each RFLP type was assigned a capital letter code, followed by a number indicating closely related subtypes (e.g., A1, A2, and A3).

In order to detect cotransfer of resistance to antimicrobial agents other than cephalosporins, transformants were phenotypically tested by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) standards (29). The following discs (Oxoid, Denmark) were used: amoxicillin-clavulanic acid (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), trimethoprim-sulfamethoxazole (25 μg), and tetracycline (30 μg). *E. coli* ATCC 25922 was used as a quality control strain.

Selected indistinguishable and closely related plasmids found in both human and animal isolates were sequenced using either Illumina MiSeq (Illumina, USA) or the 454-Genome Sequencer FLX procedure (Roche Diagnostic, Monza, Milan). Plasmid DNA was extracted from transformants with the PureLink HiPure Plasmid Midiprep kit (Invitrogen, Denmark). For Illumina MiSeq sequencing, libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina, USA) and sequenced using 150-bp paired-end reads. Contigs were assembled using Velvet (v1.0.11) and Velvet Optimizer (v2.1.7). For 454-generated sequences, contigs were obtained using the GS De Novo Assembler software and assembled *in silico* by the 454 ReadStatus output file identifying reads overlapping adjacent contigs. Open reading frames (ORFs) were predicted and annotated using Artemis software version 8 (Sanger Institute; http://www.sanger.ac.uk/resources/software/artemis/ngs/), and each predicted protein was compared to the all-protein database at the National Center for Biotechnology Information (NCBI) using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). IncI1 plasmid R64 (GenBank accession no. NC_005014.1) and IncK plasmid pCT (GenBank accession no. FN868832.1) were used as references for annotating the plasmid sequences. Plasmid genome comparisons were performed using CLC Genomics Workbench v.7. Nucleotide BLAST at NCBI was used to determine the level of identity with publicly available plasmid sequences. ISfinder was used for identification of insertion sequences (ISs) (30).

Nucleotide sequence accession numbers. Sequence data for pR7AC and pC-6 are available at GenBank under accession numbers KF434766 and KT186369, respectively. Sequence data for the remaining plasmids were deposited in the European Nucleotide Archives under the study accession number PRJEB9625.

TABLE 1 Characteristics of *bla*$_{CMY-2}$-harboring plasmids among *E. coli* isolates from humans, animals, and meat in Denmark

Isolate[a]	Host species	Isolation sample	Yr of isolation	Country of origin[p]	Plasmid characterization						Multilocus sequence type
					PBRT[q]	Plasmid size (kb)[b]	pMLST[c]	pRFLP[d]	Transferability of *bla*$_{CMY-2}$	Cotransferred resistance[r]	
C-6[k]	Human	Urine	2009	DK	I1-Iγ	86	2	A1	Positive	None	657
R1AC	Dog	Feces	2008	DK	I1-Iγ	86	2	A1	Positive	None	2196
R2AC	Dog	Feces	2008	DK	I1-Iγ	86	2	A1	Positive	None	2168
R3AC	Dog	Feces	2008	DK	I1-Iγ	86	2	A1	Positive	None	746
R7AC	Dog	Feces	2008	DK	I1-Iγ	86	2	A1	Positive	None	297
R13AC	Dog	Feces	2008	DK	I1-Iγ	86	2	A1	Positive	None	457
C-28397[k]	Dog	Urine	2011	DK	I1-Iγ	86	2	A1	Negative	None	68
C-29199[k]	Dog	Urine	2012	DK	I1-Iγ	86	2	A1	Positive	None	2558
C-30029[k]	Dog	Abdominal fluid	2012	DK	I1-Iγ	86	2	A1	Positive	None	3574
C-30104[k]	Dog	Ear	2012	DK	I1-Iγ	86	2	A1	Positive	None	372
R6AC	Dog	Feces	2008	DK	I1-Iγ	86	23[e]	A1	Positive	None	963
A1	Dog	Feces	2008	DK	I1-Iγ	86	2	A2	Positive	None	68
R5AC	Dog	Feces	2008	DK	I1-Iγ	86	2	NT	Positive	None	546
J20	Dog	Feces	2007	DK	I1-Iγ	86	2	NT	Positive	None	2144
C-9[k]	Human	Urine	2009	DK	I1-Iγ	86	116[f]	D1	Negative	None	963
C-4[k]	Human	Urine	2009	DK	I1-Iγ	82	55	C1	Positive	None	1193
C-21[k]	Human	Urine	2011	DK	I1-Iγ	82	55	C2	Positive	None	1193
C-22[k]	Human	Urine	2011	DK	I1-Iγ	100	12	B1	Negative	None	484
1061-1	Broiler	Meat	2010	DK	I1-Iγ	100	12	B1	Positive	None	10[m]
5499-28	BF[n]	Feces	2010	DK	I1-Iγ	100	12	B1	Positive	None	1056[m]
5498-25	BF	Feces	2010	DK	I1-Iγ	100	12	B1	Positive	None	219[m]
7077-63	BF	Feces	2010	DK	I1-Iγ	100	12	B1	Positive	None	115[m]
5498-4	BF	Feces	2010	DK	I1-Iγ	100	12	B1	Positive	None	212[m]
2028-8	PF	Feces	2011	DK	I1-Iγ	100	12	B1	Positive	None	10[m]
2067-2	PF[o]	Feces	2011	DK	I1-Iγ	100	12	B1	Positive	None	131[m]
2054-7	PF	Feces	2011	DK	I1-Iγ	100	12	B1	Positive	None	48[m]
2028-4	PF	Feces	2011	DK	I1-Iγ	100	12	B1	Positive	None	4048
2028-7	PF	Feces	2011	DK	I1-Iγ	100	12	B1	Positive	None	745[m]
C-24716[k]	Dog	Skin	2008	DK	I1-Iγ	100	12	B1	Positive	None	405
C-20[k]	Human	Urine	2011	DK	I1-Iγ	100	12	B2	Positive	None	155
5499-25	BF	Feces	2010	DK	I1-Iγ	100	12	B3	Positive	None	1775[m]
7077-60	BF	Feces	2010	DK	I1-Iγ	100	12	B3	Positive	None	410[m]
2123-1	PF	Feces	2011	DK	I1-Iγ	100	12	B3	Positive	None	350[m]
2115-5	PF	Feces	2011	DK	I1-Iγ	100	12	B4	Positive	None	616[m]
5499-19	BF	Feces	2010	DK	I1-Iγ	100	12	B5	Positive	None	746[m]
2028-14	PF	Feces	2011	DK	I1-Iγ	100	12	B6	Positive	None	3272[m]
2067-1	PF	Feces	2011	DK	I1-Iγ	100	12	B7	Negative	None	88[m]
2054-6	PF	Feces	2011	DK	I1-Iγ	100	12	B8	Positive	None	206[m]
2028-10	PF	Feces	2011	DK	I1-Iγ	100	12	B9	Positive	None	1303[m]
7075-31	Broiler	Meat	2010	DK	I1-Iγ	86	12	B10	Positive	None	10[m]
2054-3	PF	Feces	2011	DK	I1-Iγ	100	66[g]	B11	Positive	None	1585[m]
C-24[k]	Human	Urine	2011	DK	I1-Iγ	65	NT[h]	E1	Negative	None	155
C-28464[k]	Dog	Biopsy	2011	DK	I1-Iγ	65	NT[i]	F1	Negative	None	361
C-29870[k]	Dog	Skin	2012	DK	I1-Iγ	55	NT[j]	G1	Negative	None	448
C-8[k]	Human	Skin	2006	DK	K	78		A1	Positive	None	46
3786Z08	Human	Feces	2008	DK	K	78		A1	Positive	None	1822
3823Z08	Human	Feces	2008	DK	K	78		A1	Negative	None	1800
C-12[k]	Human	Urine	2009	DK	K	78		A1	Negative	None	117
637	Turkey	Meat	2011	DE	K	78		A1	Negative	None	117
671	Turkey	Meat	2011	DE	K	78		A1	Negative	None	117
885-01	Broiler	Meat	2009	DK	K	78		A1	Positive	None	69[m]
1472-03	Broiler	Meat	2009	DK	K	78		A1	Positive	None	4243[l]
2285-1	Broiler	Meat	2009	DE	K	78		A1	Positive	None	115
99-1	Broiler	Meat	2010	DE	K	78		A1	Positive	None	115
131-1	Broiler	Meat	2010	DE	K	78		A1	Positive	None	1640
2510-6	Broiler	Meat	2010	DE	K	78		A1	Positive	None	1594
1967-1	Broiler	Meat	2009	FR	K	86		A1	Positive	None	1594
821-1	Broiler	Meat	2009	DE	K	78		A2	Positive	None	4124[l]

(Continued on following page)

TABLE 1 (Continued)

Isolate[a]	Host species	Isolation sample	Yr of isolation	Country of origin[p]	Plasmid characterization						Multilocus sequence type
					PBRT[q]	Plasmid size (kb)[b]	pMLST[c]	pRFLP[d]	Transferability of bla_{CMY-2}	Cotransferred resistance[r]	
648-1	Broiler	Meat	2009	BR	K	78		A3	Positive	None	38
1239-2	Broiler	Meat	2010	DK	K	74		B1	Negative	None	38
6870-55	Broiler	Meat	2010	DK	K	74		B1	Positive	None	38
7075-4	Broiler	Meat	2010	DK	K	74		B1	Positive	None	38
7622-28	Broiler	Meat	2010	DK	K	74		B1	Negative	None	38
7625-26	Broiler	Meat	2010	DK	K	74		B1	Positive	None	38
9832-4	Broiler	Meat	2010	DK	K	74		B1	Positive	None	38
131-4	Broiler	Meat	2010	DK	K	74		B1	Positive	None	38
7077-57	BF	Feces	2010	DK	K	74		B1	Positive	None	38[m]
2120-1	PF	Feces	2011	DK	K	74		B1	Positive	None	38[m]
7075-19	Broiler	Meat	2010	DK	K	74		B1	Positive	None	115[m]
7077-11	BF	Feces	2010	DK	K	74		B1	Positive	None	23[m]
5499-9	BF	Feces	2010	DK	K	74		B1	Positive	None	69[m]
7077-7	BF	Feces	2010	DK	K	74		B1	Negative	None	1594[m]
2054-5	PF	Feces	2011	DK	K	74		B1	Positive	None	1518[m]
1184-1	Broiler	Meat	2009	FR	K	78		D1	Negative	None	93
2075-10	Broiler	Meat	2010	PL	K	78		C1	Positive	None	4125[l]
C-1[k]	Human	Urine	2006	DK	A/C	204		A	Positive	CHL, GEN, TET	48
1428-1	Broiler	Meat	2009	DE	A/C	86		B	Negative	TET	117
458	Turkey	Meat	2011	DE	FII	64	29		Positive	None	4240[l]
456	Turkey	Meat	2011	DE	FII, R	104			Positive	CHL, SXT, TET	1196
C-23[k]	Human	Urine	2011	DK	NT	82			Negative	None	131
7075-13	Broiler	Meat	2010	DK	ND1	68, 91, 104			Positive		23
243-1	Broiler	Meat	2010	DE	ND1	78, 138			Positive		115
R4AC	Dog	Feces	2008	DK	ND1	24, 110, 128			Positive		38
C-2[k]	Human	Urine	2006	DK	ND2	ND2			Positive		652
C-3[k]	Human	Urine	2006	DK	ND2	ND2			Negative		448
C-10[k]	Human	Urine	2009	DK	ND2	ND2			Negative		448
C-11[k]	Human	Urine	2009	DK	ND2	ND2			Negative		117
C-13[k]	Human	Urine	2009	DK	ND2	ND2			Negative		410
6870-58	Broiler	Meat	2010	DK	ND2	ND2			Positive		2040[l]
457	Turkey	Meat	2011	DE	ND2	ND2			Negative		1196
459	Turkey	Meat	2011	DE	ND2	ND2			Negative		1196
444	Turkey	Meat	2011	DE	ND2	ND2			Positive		428
683	Turkey	Meat	2011	DE	ND2	ND2			Positive		919

[a] Isolates whose bla_{CMY-2}-positive plasmids were sequenced are in boldface.
[b] Plasmid size was deduced by comparing the migration of the transformant's bands with that of the closest corresponding marker's bands.
[c] pMLST was performed on IncI1-Iγ and IncFII plasmids.
[d] pRFLP was performed on plasmid types shared by different host species.
[e] SLV of ST2.
[f] Novel sequence type, SLV of ST2.
[g] SLV of ST12.
[h] No detection of the *sogS* allele; SLV of ST55.
[i] Novel *ardA* allele (*ardA*, 20) and no detection of the *sogS* allele. The remaining allele variants were *repI1*, 4; *trbA*, 15; *pilL*, 3.
[j] No detection of both the *sogS* and the *pilL* alleles. The remaining allele variants were *repI1*, 4; *ardA*, 5; *trbA*, 15.
[k] Clinical isolate.
[l] New ST identified in this study.
[m] MLST data previously reported (22).
[n] BF, broiler flock.
[o] PF, parent flock.
[p] DK, Denmark; DE, Germany; FR, France; BR, Brazil; PL, Poland.
[q] NT, nontypeable; ND1, not determined, as more than one plasmid was present in the transformant; ND2, not determined, as the plasmid could not be transformed.
[r] CHL, chloramphenicol; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

RESULTS

Strain diversity. The 93 *E. coli* isolates belonged to 57 sequence types (STs), with five isolates (5%) displaying new STs (ST2040, ST4124, ST4125, ST4240, and ST4243) (Fig. 1). Overall, high strain diversity was observed across the different host species, with few exceptions. ST117 was detected among isolates from human patients ($n = 2$) and imported broiler ($n = 1$) and turkey ($n = 2$) meat. ST48, ST131, and ST410 occurred in single isolates from humans and broilers. ST448 and ST963 were detected in isolates of human and canine origin. ST38 strains were identified in broiler parents ($n = 1$), broilers ($n = 1$), Danish ($n = 7$) and imported ($n = 1$) broiler meat, and healthy dogs ($n = 1$) (Table 1). ST10

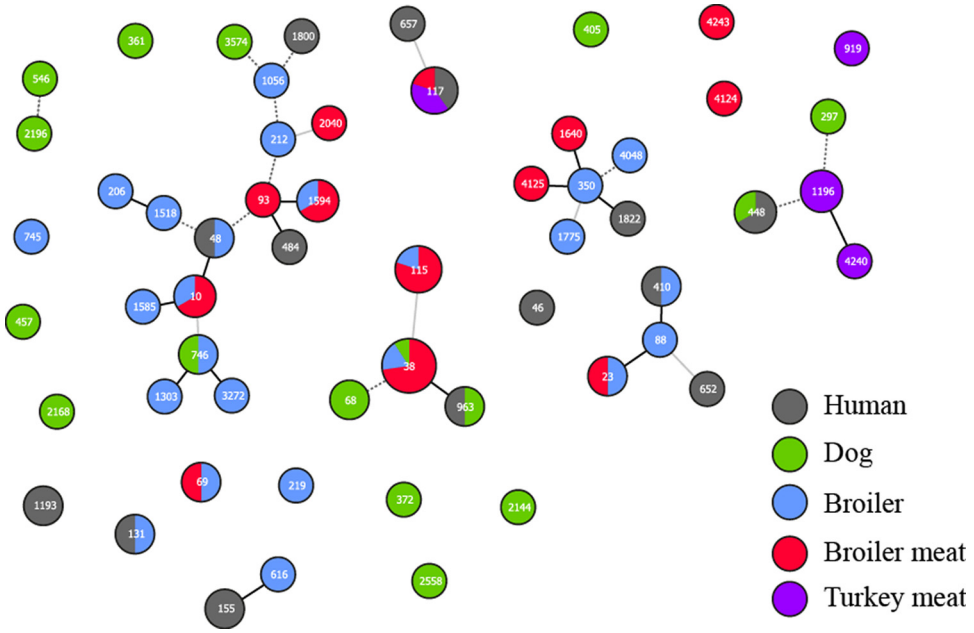

FIG 1 Multilocus sequence typing patterns of *bla*$_{CMY-2}$-carrying *E. coli* isolates from humans, dogs, broilers, and poultry meat in Denmark. Sequence types are shown as numbers. Black connecting lines indicate single-locus variants, gray connecting lines indicate double-locus variants, and dashed gray connecting lines indicate triple-locus variants. The figure was generated using PHYLOViZ software (46).

strains were identified in broiler parents (n = 1) and Danish broiler meat (n = 2). Finally, ST115 was identified in broilers (n = 1) and in Danish (n = 1) and imported (n = 2) broiler meat.

Plasmid transferability and diversity. Conjugative transfer of *bla*$_{CMY-2}$ was observed for 70 (75%) out of 93 isolates (Table 1). Transformation experiments indicated that *bla*$_{CMY-2}$ was plasmid borne in at least 83 (89%) isolates. Three transformants harbored multiple plasmids and were not further analyzed. The remaining 80 plasmids ranged in size from approximately 55 kb to 204 kb and displayed the following incompatibility groups: IncI1-Iγ (n = 44; 55%); IncK (n = 31; 39%), IncA/C (n = 2; 3%), and IncFII (n = 1; 1%). Apparently one (1%) transformant harbored a plasmid positive for both IncFII and IncR, as suggested by the results of S1 PFGE (one band detected) and PBRT (two replicons detected), and one (1%) transformant was nontypeable by PBRT (Table 1). IncI1-Iγ and IncK plasmids were detected in seven (54%) and four (31%) of the 13 (72%) human isolates for which transfer of *bla*$_{CMY-2}$ succeeded. IncI1-Iγ was the dominant replicon type among isolates from the parent flocks (86%), the broiler flocks (64%), and dogs (100%), while IncK was prevalent among isolates from Danish (83%) and imported (90%) broiler meat (Fig. 2).

Cotransfer of resistance to non-β-lactam antimicrobials was

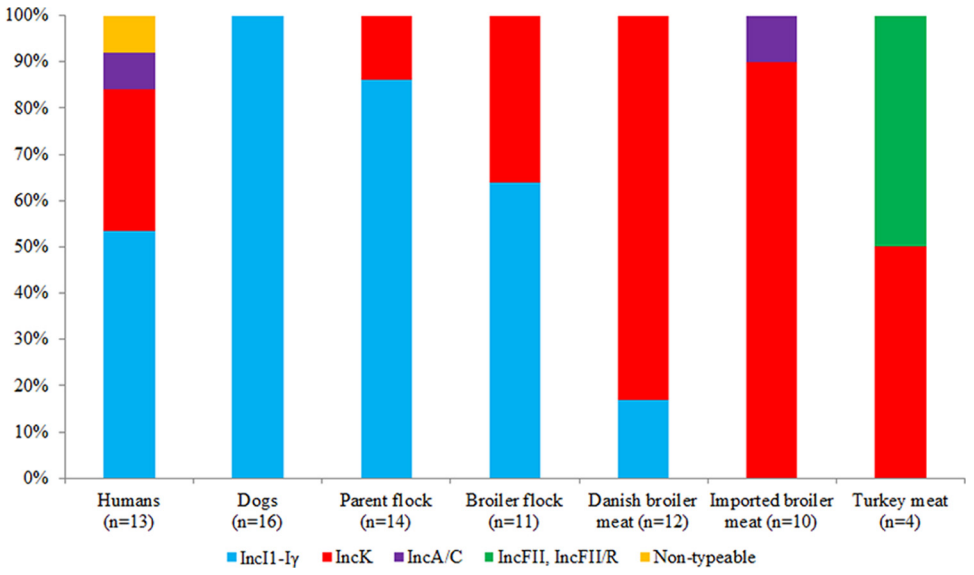

FIG 2 Replicons detected by PBRT in *bla*$_{CMY-2}$-harboring plasmids among *E. coli* isolates from different host species for which transfer of *bla*$_{CMY-2}$ succeeded.

limited to three (4%) plasmids (Table 1). Both IncA/C plasmids cotransferred resistance to tetracycline, and one of them also cotransferred resistance to chloramphenicol and gentamicin. The IncFII/R multireplicon plasmid originating from imported turkey meat cotransferred resistance to chloramphenicol, trimethoprim-sulfamethoxazole, and tetracycline (Table 1).

IncI1-Iγ plasmids. Subtyping of IncI1-Iγ plasmids by pMLST revealed two dominant sequence types: ST12 ($n = 23$; 52%) was identified in *E. coli* isolates from all sources except healthy humans and healthy dogs, and ST2 ($n = 13$; 30%) was found in *E. coli* isolates from a human patient and dogs (Table 1). The remaining STs were one single-locus variant (SLV) of ST12 (ST66) in a broiler parent isolate and different SLVs of ST2 (ST23, ST55, and the newly identified ST116) in human and canine isolates. Three IncI1-Iγ plasmids were not typeable by pMLST, as some target genes were not amplified (Table 1). Among the 23 IncI1-Iγ/ST12 plasmids examined by RFLP (see Fig. S1 and S2 in the supplemental material), 12 yielded indistinguishable RFLP profiles, designated subtype B1 (human patient, $n = 1$; Danish broiler meat, $n = 1$; broilers, $n = 4$; broiler parents, $n = 5$; and canine patient, $n = 1$) (Table 1). These plasmids measured approximately 100 kb by S1 PFGE. The remaining 11 IncI1-Iγ/ST12 plasmids mainly exhibited unique RFLP profiles (Table 1).

Sequences were obtained for 11 IncI1-Iγ/ST12 plasmids (Table 1). Sequence assembly generated contigs ranging from 99,835 bp (p2054-3) to 105,364 bp (p2028-14). These plasmids closely resembled the reference IncI1 plasmid, R64, in the organization of the backbone structure comprised of approximately 76 kbp, including the IncI1 plasmid replicon, conjugative-transfer regions (*tra*, *trb*, and *pil* operons), genes encoding plasmid partitioning and stability proteins (*parAB*, *stbAB*, and *psiAB*), and proteins involved in DNA modification and processing (*nikAB* and *impAB*). Comparative analysis of the plasmid sequences in this study showed high similarity, not only in the backbone structure, but also in the accessory region. Nine plasmids from human ($n = 1$), broiler parents ($n = 6$), broilers ($n = 1$), and broiler meat ($n = 1$) had a maximum of nine single nucleotide polymorphisms (SNPs) across 101 kbp and differed mainly in the integration of different ISs at different sites of the plasmid backbone (Fig. 3a). The remaining plasmids from broilers (p7077-60) and broiler parents (p2054-3) differed from the previous group by numerous SNPs accumulated in specific regions. The genetic context of bla_{CMY-2} consisted of an intact IS*Ecp1* upstream and *blc* followed by *sugE* downstream. This structure was inserted between a *repZ-yafB* region upstream and a colicin Ib-synthesizing gene downstream. These I1-Iγ/ST12 plasmids shared at least 94% of their nucleotide sequences (with 99% identity) with pCVM29188_101 (NC_011077.1) from a *Salmonella enterica* serovar Kentucky isolate from poultry.

Among the 13 IncI1-Iγ/ST2 plasmids examined by RFLP (see Fig. S1 and S2 in the supplemental material), plasmids from one human patient and nine dogs (canine patients, $n = 4$; healthy individuals, $n = 5$) yielded indistinguishable RFLP profiles (subtype A1). Additionally, an IncI1-Iγ/ST23 plasmid from a healthy dog also displayed RFLP subtype A1. The RFLP profiles among the remaining 10 plasmids belonging to IncI1-Iγ were unique, or the plasmids were nontypeable by RFLP despite repeated analysis (Table 1). Sequencing of a human (pC-6) and a canine (pR7AC) IncI1-Iγ/ST2/A1 plasmid yielded partial sequences of 90,486 and 90,449 bp, respectively, closely resembling (86% coverage; 99% nucleotide sequence identity) the reference IncI1 plasmid, R64, in the organization of the backbone structure. Such sequences were identical except for (i) 36 additional nucleotides in pC-6 representing part of the shufflon region (not included in the pR7AC sequence) and (ii) a nonsynonymous SNP in both *ychA* and *repA4*. bla_{CMY-2} was inserted within the *repZ-yafB* region and had an IS*SboI*-ΔIS*Ecp1* upstream and a *blc-sugE-orf1* segment downstream (Fig. 3b). These plasmids exhibited 99% nucleotide sequence identity (96% coverage) to pCVM22462 (GenBank accession no. CP009566.1) from an *S. enterica* serovar Newport isolate from a dog in the United States.

IncK plasmids. Two major clusters of IncK plasmids were identified by RFLP (see Fig. S3 and S4 in the supplemental material): subtypes A1 (42%) and B1 (45%). Subtype A1 was identified in four human (patients, $n = 2$; healthy individuals, $n = 2$) and nine poultry meat (Danish broiler meat, $n = 2$; imported broiler meat, $n = 5$; turkey meat, $n = 2$) plasmids. These plasmids measured approximately 78 kb, with the exception of an 86-kb plasmid from imported broiler meat (1967-1) (Table 1). RFLP subtype B1 was identified in plasmids from Danish broiler meat ($n = 9$), broilers ($n = 4$), and broiler parents ($n = 2$). The remaining four IncK plasmids yielded unique restriction profiles. Sequences were obtained for 12 IncK plasmids (Table 1) and ranged from 79,431 bp (p2120-1) to 90,439 bp (p1967-1). These plasmids closely resembled the reference pCT plasmid in the organization of the backbone structure comprised of approximately 68 kbp, including conjugative-transfer regions (*tra*, *trb*, and *pil* operons), genes encoding an endonuclease (*parB*), proteins involved in DNA modification and processing (*nikAB* and *impABC*), and proteins involved in inhibition of the SOS response (*psiAB*). The target of the PCR for IncK replicon typing differed from pCT by three SNPs (5G→A, 10T→C, and 135C→G) identified in all sequenced plasmids and one additional SNP identified in a subset of plasmids only (13G→A in p2120-1, p5499-9, p131-4, and p1967-1; 138G→T in p637). Comparative analysis showed high homology across the plasmid sequences in this study. Within 76,863 bp identified in all sequenced plasmids, between 1 and 46 SNPs were identified, and this difference was reduced to a maximum of 14 SNPs by excluding the most divergent plasmid (p648-1) from the comparison. In p648-1, SNPs were mainly accumulated in three specific regions (conceptually translated as hypothetical proteins). These plasmids mainly differed in various insertions/deletions represented in Fig. 3c. The genetic context of bla_{CMY-2} consisted of an intact IS*Ecp1* upstream and *blc-sugE* elements downstream. Interestingly, in p1184-1, deletion of the last 253 bp of *blc* and the last 132 bp of *sugE* was observed. Further downstream, two open reading frames coding for hypothetical proteins not previously associated with bla_{CMY-2} were detected. These elements were embedded between *traT* and *traU*. The plasmids exhibited 99% nucleotide sequence identity (96% to 99% coverage) to p53C_3 (BioProject accession no. PRJNA260957) from an *E. coli* isolate from chicken meat in the Netherlands in 2010 and 99% nucleotide sequence identity (88% to 100% coverage) to pNVI1292 (GenBank accession no. KU312044) from an *E. coli* isolate from chicken meat in Norway in 2012.

IncA/C plasmids. Subtyping of IncA/C plasmids by RFLP showed distinct plasmid subtypes in *E. coli* isolates from a human patient and imported broiler meat (Table 1).

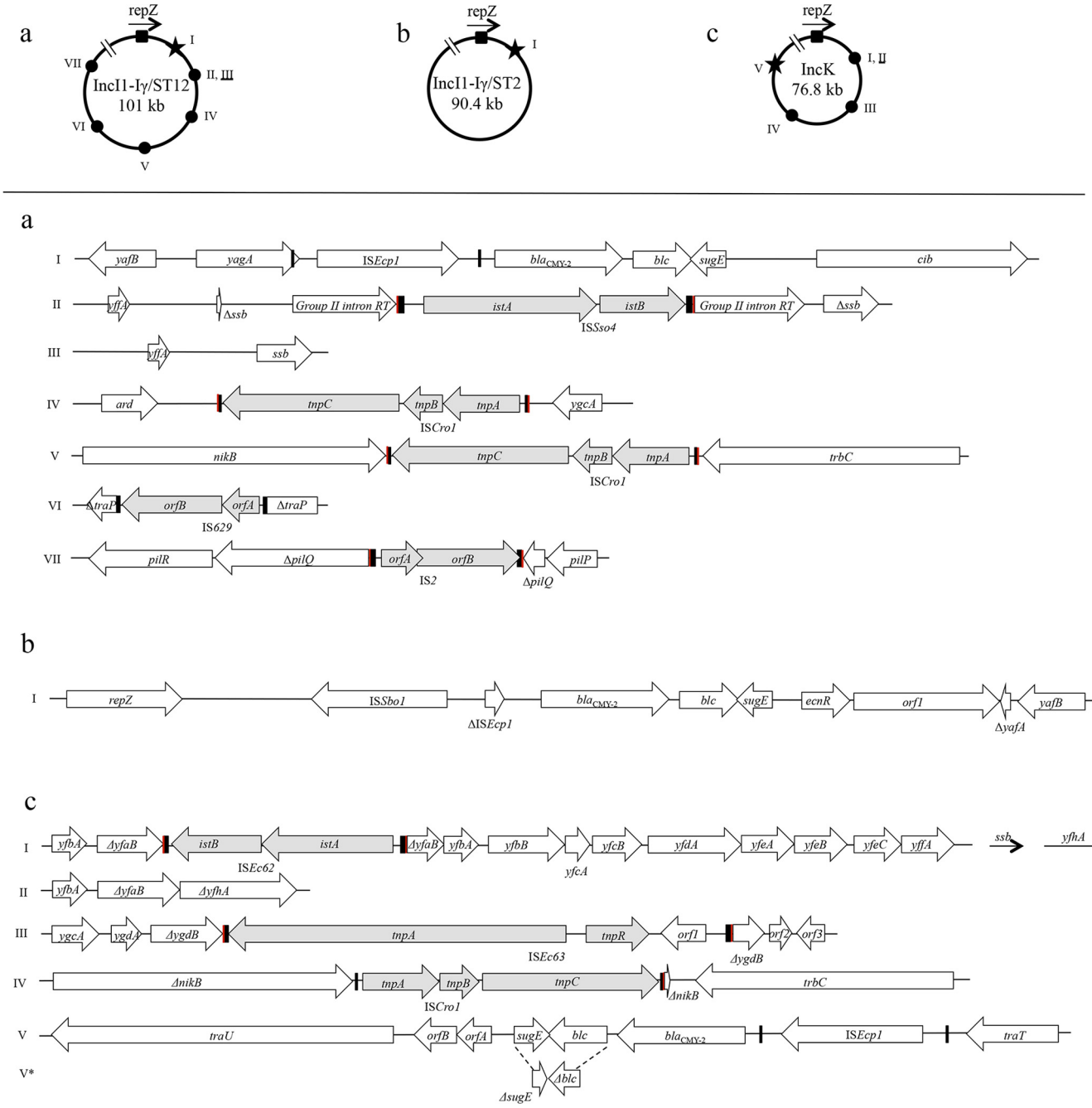

FIG 3 Representation of the genetic context of *bla*$_{CMY-2}$ and insertions/deletions within otherwise identical plasmid sequences in *E. coli* across different host species. The diagrams at the top illustrate the positions of *bla*$_{CMY-2}$ (indicated by a star) and of insertions/deletions (indicated by circles) in the different plasmids compared to *repZ* (indicated by a square, with arrowheads showing the direction of transcription). Distances from *repZ* are not drawn to scale. Each specific genetic context is identified by a roman numeral and is illustrated in the panels below. Underlined roman numerals indicate deletions. In the bottom panels, red and black rectangles represent direct repeats and inverted repeats, respectively, whereas gray shading indicates inserted genes. (a) IncI1-Iγ/ST12 plasmids. I, *bla*$_{CMY-2}$ genetic context conserved across all sequenced plasmids; II, p2028-14 (parent flock origin); III, pC20 (human origin); IV, p2028-10 (parent flock origin); V, pC20 (human origin); VI, p2067-1 (parent flock origin); VII, p2054-6 (parent flock origin). (b) IncI1-Iγ/ST2 plasmids. I, *bla*$_{CMY-2}$ genetic context conserved across all sequenced plasmids. (c) IncK plasmids. I, p2075-10 (Polish broiler meat origin) (IS*Ec62* is a new IS belonging to the IS*21* family); II, p131-4 (Danish broiler meat origin), p2120-1 (parent flock origin), p5499-9 (broiler flock origin); III, p1967-1 (French broiler meat origin) (IS*Ec63* is a novel element belonging to the Tn*3* transposon family); IV, p1184-1 (French broiler meat origin); V, *bla*$_{CMY-2}$ genetic context conserved across all sequenced plasmids except V*. *orfA* and *orfB* code for hypothetical proteins and have not been associated with *bla*$_{CMY-2}$ previously. V*, p1184-1 (broiler meat origin), in which deletions of 253 bp at the 3′ end of *blc* and 132 bp at the 3′ end of *sugE* were observed.

DISCUSSION

This study investigated the epidemiology of CMY-2-producing *E. coli* in Denmark in a set ($n = 93$) of isolates collected between 2006 and 2012 from humans and from the main animal reservoirs of CMY-2 known to date, namely, chickens, chicken meat, and dogs (7). The study provides molecular epidemiology data supporting the results of Carmo et al. (31), who concluded that the relative contribution of meat to infections by extended-spectrum β-lactamase (ESBL)/AmpC-producing *E. coli* in humans in Denmark was limited and indicated broiler meat as the main food source contributing to human exposure to this type of bacteria, especially CMY-2-producing *E. coli*. Indeed, only 4 of the 16 human clinical isolates analyzed in our study harbored the specific IncI1-Iγ/ST12 and IncK plasmid types linked to broiler meat (Table 1). Altogether, the results show that the occurrence of CMY-2-producing *E. coli* in different host species is mostly shaped by independent processes, as indicated by the presence of bla_{CMY-2} on distinct plasmid vectors shared to a minor extent across different host species.

CMY-2-encoding plasmids in human isolates showed a relatively high level of diversity, with at least four plasmid types and five plasmid subtypes detected in 13 isolates, which may indicate plasmid acquisition from multiple sources and various selective pressures favoring different *E. coli* strains and plasmids in individual hosts. Similar findings were reported in studies from the United States, Canada, and Norway (5, 16, 32). On the other hand, CMY-2-encoding plasmids identified in Danish dogs and Danish poultry showed limited diversity within the respective host species. The majority ($n = 12$; 71%) of canine isolates harbored bla_{CMY-2} on IncI1-Iγ/ST2 plasmids, also a predominant plasmid type in *E. coli* isolates from healthy dogs in France (33). This finding differs from reports of bla_{CMY-2} on a broad variety of plasmid types in canine isolates from the United States, Italy, and South Korea (16, 28, 34), highlighting geographical differences in regard to the plasmid vectors carrying bla_{CMY-2} in *E. coli* of canine origin. The reasons for these geographical differences remain unexplained, though they might be linked to different practices in antimicrobial use. In Danish broiler and broiler meat isolates, bla_{CMY-2} was nearly exclusively associated with IncI1-Iγ/ST12 and IncK plasmids. Notably, the majority (83%) of Danish broiler meat isolates harbored bla_{CMY-2} on IncK plasmids, while IncI1-Iγ seemed to be the prevalent plasmid among Danish broilers. However, this observation is biased by the criteria used for strain selection, as each isolate from Danish broilers represented a different *E. coli* PFGE type but the distribution of isolates within PFGE types was uneven. Indeed, a single PFGE type associated with IncK plasmids encompassed approximately 40% of bla_{CMY-2}-producing *E. coli* isolates from Danish broilers in the previous study (22).

Plasmid sequencing allowed us to discover that human and animal plasmids indistinguishable by RFLP presented a limited number of nonsynonymous SNPs and the presence/absence of ISs, which may have biological significance. One example is a nonsynonymous SNP in *repA4* found in human and canine IncI1-Iγ/ST2 plasmids. Such a difference may affect vertical plasmid transfer, as it has been previously shown that *repA4* mutations cause unstable inheritance of IncF plasmids. However, no such studies have been performed on IncI1-Iγ plasmids to date (35). A further example is p2067-1, an IncI1-Iγ/ST12 plasmid isolated from the broiler parent flock that was unique for the presence of IS*629* elements disrupting *traP*. Insertion mutations in *traP* have been shown to completely abolish transfer activity of R64, the IncI1 prototype plasmid (36), and indeed, p2067-1 did not transfer by conjugation (Table 1). Similarly, the IncK plasmid p1184-1 isolated from imported broiler meat had IS*66* elements causing partial deletion of NikB, which is indispensable for transfer of R64 (37), and accordingly, it did not transfer by conjugation. Identification of different genetic environments of bla_{CMY-2} among IncI1-Iγ/ST2, IncI1-Iγ/ST12, and IncK plasmids suggested that at least IncI1-Iγ/ST2 and IncI1-Iγ/ST12 plasmids acquired the resistance gene independently, while the possibility that IncK plasmids acquired the IS*Ecp1-sugE* structure from I1-Iγ/ST12 plasmids or vice versa cannot be excluded. The genetic context of bla_{CMY-2} in IncI1-Iγ/ST2 plasmids was nearly identical to previous descriptions (38–40), except for three gaps and four SNPs that were either synonymous or in intergenic regions. The genetic context of bla_{CMY-2} in IncI1-Iγ/ST12 was highly similar to that of pNF1358 (GenBank accession no. DQ017661) (38), while the genetic context of bla_{CMY-2} in IncK was unique for the presence of two genes coding for hypothetical proteins downstream of *sugE*. An IncK plasmid (p1184-1) had an additional unique feature consisting of partial deletion of *blc* and *sugE*.

The sequenced IncI1-Iγ/ST2, IncI1-Iγ/ST12, and IncK plasmids were highly similar (99% identity; 88% to 100% coverage) to plasmids detected in *Enterobacteriaceae* isolated from dogs (I1-Iγ/ST2) and poultry (IncI1-Iγ/ST12 and IncK) in the United States, the Netherlands, and Norway (14, 41–43). The recovery of highly similar plasmids from different *E. coli* lineages and from unrelated individuals of the same host species suggests that these plasmids may possess traits enhancing adaptation of *E. coli* to the intestinal tracts of specific animal hosts.

We detected a high diversity of STs within and between different sources and no linkage of bla_{CMY-2}-positive plasmids to specific *E. coli* lineages. The criteria for the selection of poultry strains may have influenced this finding. Nevertheless, the finding was expected based on a previous study (44). Further characterization of STs shared by human and animal sources by plasmid typing demonstrated that such strains were diverse (Table 1), supporting the current notion that there is no evidence of whole-bacterium transmission of cephalosporin-resistant *E. coli* from poultry meat to humans (45). ST10, ST38, and ST115 strains were detected at different levels of Danish broiler meat production. Taking into consideration previous PFGE typing data (22) and our extensive plasmid characterization, it appears that clonal transmission of CMY-2-producing *E. coli* within the Danish poultry production system is mainly attributable to an *E. coli* ST38 lineage susceptible to non-β-lactam antimicrobials and carrying bla_{CMY-2} on IncK plasmids. CMY-2-producing *E. coli* ST38 harboring bla_{CMY-2} on IncK plasmids has also been reported in Swedish and Norwegian broiler meat production (13, 43), even though plasmid-subtyping data were not available in the Swedish study. Vertical transmission of this clone between Scandinavian countries is likely, since 1-day-old chickens for Danish parent flock production are acquired from Sweden. None of the human isolates belonged to this clone, possibly due to fact that isolates from broilers and broiler meat were collected in 2010 while most human isolates were collected in previous years.

The main limitations of the study were the fact that human isolates were collected before the sharp increase in the prevalence of CMY-2-producing *E. coli* in Danish poultry meat products in

2011 (11) and the relatively low numbers of isolates from humans and dogs compared to the number of isolates from poultry. CMY-2-producing *E. coli* isolates are not commonly isolated from human patients in Denmark and are not collected in national surveillance of ESBLs in *E. coli* bacteremia and urinary tract infections (11). Thus, these isolates are not easily available in Denmark. Continuous monitoring is warranted in consideration of the occurrence of these bacteria as contaminants in poultry products and as commensals in dogs (11, 33). In this respect, our study provides useful baseline data that will enable future studies to assess whether the exposure of humans to *bla*$_{CMY-2}$ of poultry origin will result in an increase in the frequency of this β-lactam resistance determinant in human clinical isolates.

A further limitation of the study is the fact that plasmid sequences were not fully assembled. However, only very short parts of the sequences were missing, because the contigs obtained covered the length expected on the basis of the S1 PFGE results and all conserved elements expected in the respective plasmid types were identified.

In conclusion, the spread of *bla*$_{CMY-2}$ in Denmark is mainly associated with IncK and IncI1-Iγ plasmids, with limited similarity of plasmid subtypes across different host species. These plasmids were not linked to a specific *E. coli* genetic background, and no apparent clonal transfer of CMY-2-producing *E. coli* across host species was observed. The absence of linkage between plasmids and the *E. coli* genetic background confirms that plasmid horizontal transfer is more important than clonal dissemination for transmission of CMY-2-mediated cephalosporin resistance between animals and humans. The most common plasmids detected in Danish isolates were highly similar to plasmids detected in distant geographical regions, indicating that plasmid diversity is remarkably limited compared to *E. coli* strain diversity in the epidemiology of this β-lactamase.

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