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Strain Diversity of CTX-M-Producing *Enterobacteriaceae* in Individual Pigs: Insights into the Dynamics of Shedding during the Production Cycle

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The aim of this study was to evaluate the population dynamics of CTX-M-producing *Enterobacteriaceae* in individual pigs on a farm positive for CTX-M-14-producing *Escherichia coli*. Fecal samples were collected once around the farrowing time from five sows and four times along the production cycle from two of their respective offspring. Multiple colonies per sample were isolated on cefotaxime-supplemented MacConkey agar with or without prior enrichment, resulting in 98 isolates identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry and tested for $bla_{\text{CTX-M}}$. CTX-M-positive isolates ($n = 86$) were typed by pulsed-field gel electrophoresis (PFGE). Plasmids harboring $bla_{\text{CTX-M}}$ were characterized in 22 representative isolates by replicon typing and restriction fragment length polymorphism. Based on the PFGE results, all individuals shed unrelated CTX-M-14-producing *E. coli* strains during the course of life. Concomitant shedding of CTX-M-2/97-producing *Proteus mirabilis* or *Providencia rettgeri* was observed in two sows and two offspring. At least two genetically unrelated CTX-M-producing *E. coli* strains were isolated from approximately one-fourth of the samples, with remarkable differences between isolates obtained by enrichment and direct plating. A clear decrease in strain diversity was observed after weaning. Dissemination of $bla_{\text{CTX-M-14}}$ within the farm was attributed to horizontal transfer of an IncK plasmid that did not carry additional resistance genes and persisted in the absence of antimicrobial selective pressure. Assessment of strain diversity was shown to be influenced by the production stage from which samples were collected, as well as by the isolation method, providing useful information for the design and interpretation of future epidemiological studies of CTX-M-producing *Enterobacteriaceae* in pig farms.

An increasing occurrence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* has been observed worldwide among human clinical isolates over the last decade. This epidemiological trend is mainly due to the dissemination of $bla_{\text{CTX-M}}$ genes, which have replaced bla_{TEM} and bla_{SHV} genes as the predominant ESBL-encoding genes in Europe and other geographical regions (1). Concomitantly, ESBL producers, and in particular strains producing CTX-M-1, CTX-M-14, TEM-52, and/or SHV-12, have increasingly been reported among *Escherichia coli* isolates from food-producing animals and food products worldwide with geographical differences in the distribution of CTX-M variants (2). Five CTX-M groups (CTX-M-1, -2, -8, -9, and -25), each including several variants, have been recognized to date based on amino acid similarities (www.lahey.org/studies). In Denmark, CTX-M-1 and CTX-M-14 are the first (66%) and second (7%) most frequently reported ESBL variants in pigs at slaughter, respectively (3). CTX-M-14 is the only CTX-M-9 group variant associated with Danish pigs, and worldwide only a few additional variants (CTX-M-27, -65, and -98) within this CTX-M group have been described in pigs (4, 5). Among human patients in Denmark, CTX-M-15 is by far the most frequently reported ESBL variant (63%), followed by CTX-M-14 (10%) (6).

ESBL-encoding genes are generally located on conjugative plasmids of different types and sizes (7). Indistinguishable IncN plasmids carrying $bla_{\text{CTX-M-1}}$ have been observed in pigs and workers from the same farms, suggesting within-farm transmission of these plasmids between human and porcine commensal *E. coli* (8). Food-borne transmission of CTX-M-1 and TEM-52 β-lactamase-encoding genes, plasmids, and *E. coli* from poultry to humans has been inferred on the basis of associations between strains isolated from human patients and retail food in the Netherlands (9). On the basis of these and similar findings, public health authorities are concerned about possible zoonotic transmission of ESBL-producing bacteria from animals to humans (10). Elucidation of the dynamics of shedding of ESBL-producing *Enterobacteriaceae* within herds is a first step needed to develop effective control measures in livestock. We recently showed a temporal decrease in both the prevalence of pigs shedding CTX-M-producing *E. coli* and the fecal counts of CTX-resistant coliforms in individual pigs during the production cycles of two unrelated Danish farms (11). The objective of the present study was to evaluate the population dynamics of CTX-M-producing *Enterobacteriaceae* in individual pigs from birth to slaughter at one of the two farms, which was previously shown to be contaminated with CTX-M-14-producing *E. coli*.

MATERIALS AND METHODS

Sampling procedure. Fecal samples were collected at the study farm between June and November 2011. The farm had a single-sited, farrow-to-finish production system with housing of sows, piglets, weaners, and finishers on the same farm and used continuous production through all

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sections. Data on antimicrobial consumption at the farm level during the study period and for the preceding 6 months were reported by Hansen et al. (11). A total of 15 individual pigs and 45 samples were included in the study. Fecal samples were taken once from five randomly selected sows around the time of farrowing (sampling 1, between 2 days before and 3 days after delivery) and at four sampling times from two offspring per sow (sampling 2, between 5 and 10 days after birth; sampling 3, between 19 and 24 days after birth; sampling 4, ~2 months after birth; and sampling 5, ~5 months after birth). The sows were housed in individual pens in the farrowing section (samplings 1 to 3), and the offspring were housed in three separate pens at the weaning stage (sampling 4) and in six separate pens at the finishing stage (sampling 5). Fecal samples were taken manually from the rectum except for newborn piglets (sampling 2), which were sampled by using premoistened Steriswabs (MWE Medical Wire & Equipment, United Kingdom).

Isolation of cefotaxime-resistant *Enterobacteriaceae*. Each sample was processed with and without enrichment. In the enrichment procedure, 1 g of feces was dissolved in 10 ml of MacConkey broth (Merck, Denmark) supplemented with 2 mg/liter of cefotaxime (CTX), followed by overnight incubation at 37°C. Subsequently, a 10-μl volume was streaked onto MacConkey agar supplemented with 2 mg/liter of CTX. In the procedure without enrichment (direct plating), 1 g of feces was suspended in 9 ml of sterile saline, and serial 10-fold dilutions were plated on MacConkey agar supplemented with 2 mg/liter of CTX. Detailed information on the plating method and quantification results has been reported previously (11). After overnight incubation at 37°C, one and two (if available) lactose-positive colonies were randomly selected from the plates deriving from the enrichment and the direct plating procedure, respectively. Any lactose-negative colonies displaying distinct morphology in terms of size, color, or shape were also selected. All isolates were identified at the species level by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonic, Germany). McNemar's test (http://graphpad.com/quickcalcs/McNemar1.cfm) was used to compare the abilities of the two isolation protocols (with and without enrichment) to detect CTX-M-producing *E. coli*. *P* values of <0.05 were considered statistically significant.

Detection and identification of ESBL-encoding genes. Since the focus of this study was to evaluate the population dynamics of CTX-M-producing *Enterobacteriaceae*, all CTX-resistant isolates were tested for the presence of bla_{CTX-M} by PCR using universal, group-specific, and variant-specific primers (12–15). Only isolates negative for bla_{CTX-M} were further tested for bla_{TEM}, bla_{SHV}, and bla_{CMY-2} by PCR (12, 16). PCR amplicons from a selection of isolates positive for the CTX-M-9 group and from all isolates positive for the CTX-M-2 group or for TEM were sequenced (Macrogen, Inc., South Korea). The obtained sequences were assembled by CLC Main Workbench (CLC Bio, Denmark) and compared at the nucleotide and amino acid levels with sequences deposited in GenBank (www.ncbi.nlm.nih.gov) and the Lahey database (www.lahey.org/studies), respectively. Isolates negative for all *bla* genes tested were analyzed by the CT/CTL Etest (bioMérieux, France) for phenotypic confirmation of ESBL production.

PFGE analysis. All bla_{CTX-M}-positive *E. coli* strains were characterized by pulsed-field gel electrophoresis (PFGE) analysis after digestion of total chromosomal DNA with XbaI (New England BioLabs, Denmark) (17). *Salmonella enterica* serovar Braenderup H9812 was included as an internal control in all gels, and run conditions were as previously described (17). PFGE cluster analysis was carried out with Gelcompar II (Applied Maths, Belgium) using the Dice similarity coefficient and clustering by the unweighted pair group method with arithmetic averages. Band optimization and position tolerance were both set at 1%. Band patterns were visually compared to define indistinguishable and closely related subtypes differing by two or three bands in accordance with the criteria proposed by Tenover et al. (18). Each PFGE type was assigned a capital letter code, followed by a number indicating closely related subtypes (e.g., A1, A2, and A3).

Plasmid characterization. CTX-M-encoding plasmids were characterized in a subset of 22 bla_{CTX-M}-positive isolates, including one *E. coli* isolate for each PFGE subtype, three arbitrarily selected PFGE nontypeable isolates from different individuals, and all non-*E. coli* isolates. Plasmid DNA was purified with a PureLink HiPure plasmid midiprep kit (Invitrogen, Denmark) and transformed into electrocompetent Genehog *E. coli* (Invitrogen, Denmark). Transformants were selected on brain heart infusion agar (Oxoid, Denmark) supplemented with 2 mg/liter of CTX and tested by PCR to confirm the presence of bla_{CTX-M} (12). The numbers and sizes of plasmids were determined by PFGE after S1 nuclease (Thermo Scientific, Sweden) digestion of whole genomic DNA of transformants (19). XbaI-digested genomic DNA of *Salmonella enterica* serovar Braenderup H9812 was used as a molecular size marker. Plasmids extracted from transformants were characterized by PCR-based replicon typing (PBRT) (20) using a commercial kit (Diatheva, Italy) and by restriction fragment length polymorphism (RFLP) using EcoRV and SalI (Thermo Scientific, Sweden). Restriction profiles were visualized on a 0.8% agarose gel, and band patterns were compared by visual inspection. In order to detect the cotransfer of resistance not conferred by CTX-M-encoding genes, transformants were tested by disk diffusion according to Clinical Laboratory Standards Institute (CLSI) standards (21). Human CLSI breakpoints were used for compounds without established animal breakpoints (22). The following discs (Oxoid, Denmark) representing the most common antimicrobial classes used in the Danish pig production were employed: chloramphenicol (30 μg), florfenicol (30 μg), gentamicin (10 μg), sulfamethoxazole (250 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), and tetracycline (30 μg). In addition, cefoxitin (30 μg) and meropenem (10 μg) were included to detect transferable AmpC β-lactamases and carbapemenases, respectively.

In order to identify additional antimicrobial resistance genes present on the $bla_{CTX-M-14}$-positive plasmid, whole-genome sequencing (WGS) of one $bla_{CTX-M-14}$-carrying *E. coli* strain representing the most widespread PFGE type (F2, isolated from offspring 3.2 at sampling 5 [see Fig. 1]) was performed using Illumina technology. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Germany), and normalization of DNA concentrations was performed using a Qubit assay (Invitrogen, United Kingdom). Libraries were generated by using a Nextera XT DNA sample preparation kit (Illumina, USA), and sequencing was performed on an Illumina MiSeq benchtop sequencer using a MiSeq reagent kit v2 (300 cycles) and 150-bp paired-end reads. Reads from sequencing were assembled by using Velvet v1.0.11 and VelvetOptimizer. Antimicrobial resistance genes were identified using ResFinder 2.1 (http://cge.cbs.dtu.dk/services/ResFinder) and ARG-ANNOT (http://www.mediterranee-infection.com/article.php?laref=282&titer=arg-annot). BLAST at NCBI was used to infer chromosome and plasmid localization of contigs harboring resistance genes. Finally, the contig ordering tool Mauve (23, 24) was employed to order the obtained contigs, using the IncK plasmid pCT (GenBank accession no. FN868832.1) as the reference genome, thereby ensuring that the WGS data yielded an accurate representation of the IncK plasmid.

Nucleotide sequence accession numbers. The nucleotide sequences of the contigs containing antimicrobial resistance genes have been deposited in GenBank under accession numbers KJ868771 (contig 37), KJ868772 (contig 47), KJ868773 (contig 253), and KJ868774 (contig 955).

RESULTS

Occurrence of CTX-resistant bacteria. CTX-resistant colonies were cultured from all 15 animals, leading to a total of 38 (84%) positive samples and 98 CTX-resistant isolates. The seven negative samples originated from pigs at the finishing stage (sampling 5). Of the 98 CTX-resistant isolates, 93 (95%) were lactose-positive *E. coli*, while five (5%) were lactose negative and were identified as *E. coli* ($n = 1$), *Proteus mirabilis* ($n = 2$), or *Providencia rettgeri* ($n = 2$). The distribution of CTX-resistant *Enterobacteriaceae* isolated from the five sows and ten offspring at different samplings and

TABLE 1 Distribution of bacterial species and β-lactamase-encoding genes among cefotaxime-resistant *Enterobacteriaceae* isolated from five sows and ten offspring at different production stages (farrowing, weaning, and finishing)

Production stage	Animal age group/sampling no. (*n*)	Species	β-Lactamase gene	No. (%)	
				Isolates	Pigs
Farrowing	Sows[a]/sampling 1 (5)	*E. coli*	$bla_{\text{CTX-M-14}}$	11 (73)	5 (100)
			$bla_{\text{TEM-1}}$	2 (13)	1 (20)
		P. rettgeri	$bla_{\text{CTX-M-2/97}}$	2 (13)	2 (40)
	Offspring (1 wk old)/sampling 2 (10)	*E. coli*	$bla_{\text{CTX-M-14}}$	24 (77)	10 (100)
			$bla_{\text{TEM-1}}$	6 (19)	3 (30)
		P. mirabilis	$bla_{\text{CTX-M-2/97}}$	1 (3)	1 (10)
	Offspring (3 wk old)/sampling 3 (10)	*E. coli*	$bla_{\text{CTX-M-14}}$	28 (97)	10 (100)
		P. mirabilis	$bla_{\text{CTX-M-2/97}}$	1 (4)	1 (10)
Weaning	Offspring (2 mo old)/sampling 4 (10)	*E. coli*	$bla_{\text{CTX-M-14}}$	20 (100)	10 (100)
Finishing	Offspring (5 mo old)/sampling 5 (10)	*E. coli*	$bla_{\text{CTX-M-14}}$	3 (100)	3 (30)

[a] Sows were sampled within a week of delivery.

production stages (farrowing, weaning, and finishing) is shown in Table 1.

Genetic background of CTX resistance. Among the 94 CTX-resistant *E. coli* isolates, 86 (91%) were positive for the CTX-M-9 group-specific and the CTX-M-14/17 variant-specific PCRs. Sequencing of PCR amplicons from 31 isolates selected to represent each PFGE type and any PFGE-nontypeable isolates, and analysis of the WGS data from the sequenced strain confirmed the presence of $bla_{\text{CTX-M-14}}$. The remaining eight (9%) CTX-resistant *E. coli* isolates, obtained from one sow and three piglets, carried $bla_{\text{TEM-1}}$ and were negative for $bla_{\text{CTX-M}}$, bla_{SHV}, and $bla_{\text{CMY-2}}$. Further testing of these isolates by the CT/CTL Etest revealed no synergy with clavulanic acid, indicating that the cephalosporin resistance phenotype was not attributable to ESBL production. The two *P. mirabilis* and the two *P. rettgeri* isolates were positive for the CTX-M-2 group-specific PCR and yielded sequences compatible with $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-97}}$.

Comparison between isolation procedures with and without enrichment. Among the 38 samples in which CTX resistance was detected, CTX-M-producing *E. coli* were detected in 21 (55%) using both isolation procedures, in seven (18%) using the enrichment procedure only, and in 10 (26%) using direct plating only (Table 2). There was no significant difference between the two isolation procedures in relation to their ability to detect CTX-M-producing *E. coli* ($P = 0.628$). CTX-M-producing *P. mirabilis* was isolated from two samples using the procedure without enrichment only, and CTX-M-producing *P. rettgeri* was detected in one sample using the enrichment procedure and in another sample using the procedure without enrichment.

Strain diversity of CTX-M-producing *E. coli*. The 86 *E. coli* isolates positive in the CTX-M-9 group-specific PCR were subjected to PFGE; 73 isolates were typeable and belonged to 15 distinct (A to O) PFGE types, including two closely related subtypes within PFGE types F (F1 and F2), H (H1 and H2), and M (M1 and M2). The remaining 13 isolates were nontypeable by PFGE despite repeated analysis. The distribution of PFGE types among CTX-M-producing *E. coli* isolates from the five sows and ten offspring at different production stages is displayed in Fig. 1. Multiple unrelated PFGE types were detected within samples collected from one sow and seven offspring at samplings 2 and 3, leading to a total of 36% of samples harboring at least two and up to three genetically unrelated CTX-M-producing *E. coli* strains at the farrowing stage. The PFGE types detected in six of the ten newborn piglets at sampling 2 were unrelated to those displayed by the isolates from their respective dams. No within-sample strain diversity was observed in nine of ten offspring after weaning (sampling 4) and in the three positive pigs at the finishing stage (sampling 5). A total of 26% of the samples harbored genetically unrelated CTX-M-producing *E. coli* strains.

Analysis of strain diversity between samples from the same animal showed that each pig shed different strains, or at least combinations of strains, over time. Among the five PFGE types/subtypes (B, F2, H1, H2, and I) that occurred between samples from the same animal, only two (H1 and F2) were detected in two physically separated stages of the production cycle (i.e., farrowing/weaning and weaning/finishing, respectively) (Fig. 1).

In 13 (62%) of the 21 samples from which CTX-M-producing *E. coli* was isolated using both procedures, isolates obtained by enrichment displayed PFGE patterns identical or closely related to that of at least one of the isolates obtained by direct plating. In the remaining eight samples, isolates obtained by the two methods were genetically unrelated on the basis of PFGE ($n = 5$) or genetic relatedness could not be inferred since the isolates were nontypeable ($n = 3$).

Diversity of CTX-M-encoding plasmids. All transformants harbored one $bla_{\text{CTX-M-14}}$-positive IncK plasmid of a size between 78.2 and 104 kb and displaying indistinguishable RFLP patterns (see Fig. S1 and S2 in the supplemental material). The transformants were resistant to CTX, displayed intermediate resistance to cefoxitin, and were susceptible to the remaining antimicrobials tested. No antimicrobial resistance genes besides $bla_{\text{CTX-M-14}}$ were identified on the IncK plasmid by analysis of WGS data from the sequenced strain using ResFinder and ARG-ANNOT. The obtained WGS data consisted of 442,146 reads with an average length

TABLE 2 Comparison of results obtained by two selective methods for the isolation of CTX-M-producing *E. coli* with and without enrichment

Isolation without enrichment	Isolation with enrichment (no. of samples)		
	Growth	No growth	Total
Growth	21	10	31
No growth	7	7	14
Total	28	17	45

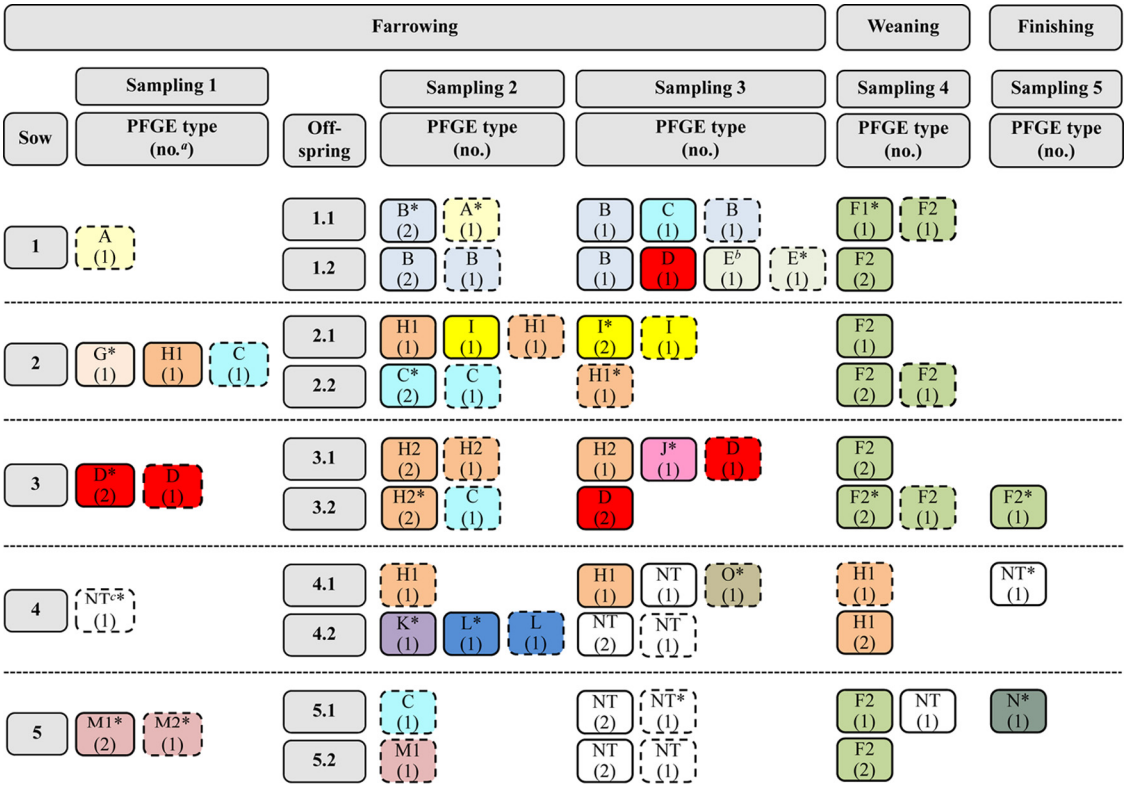

FIG 1 Distribution of PFGE types among CTX-M-producing *E. coli* isolated from five sows and two offspring per sow during the production cycle. Boxes with dashed borders indicate that the strain was isolated by the enrichment procedure. Strains displaying identical PFGE types are indicated by the same color shading. Superscript letters and symbols: *, strains selected for plasmid characterization; *a*, number of isolates representing each PFGE type; *b*, lactose-negative *E. coli*; *c*, NT indicates nontypeable by PFGE.

of 149 bp (5 Mb of data), and *de novo* genome assembly produced 998 contigs. Among these contigs, both web services identified (i) $bla_{CTX-M-14}$ on contig 955 (4,040 bp); (ii) *sul2*, *strA*, and *strB* on contig 37 (4,476 bp); and (iii) *tet*(*B*) on contig 47 (6,585 bp). In addition, ARG-ANNOT identified chromosomal *ampC* on contig 253 (8,584 bp). BLAST at NCBI revealed that contig 955 had 100% identity to the IncK reference plasmid pCT (25). In contrast, contig 37 had an identity of 99% to the *E. coli* plasmid p62 (GenBank accession no. FN822747.1), and contig 47 had an identity of 99% to the *E. coli* plasmid pTC1 (GenBank accession no. CP000913.1); p62 and pTC1 are two plasmids not related to IncK plasmids, and further indications that contigs 37 and 47 were not part of the IncK plasmid under study were given by the fact that none of the transformants displayed the phenotypes associated with the resistance genes present on these contigs. Finally, contig 253 displayed 99% identity to the *E. coli* E24377A chromosome (GenBank accession no. CP000800.1). Mapping of all contigs to the reference plasmid pCT revealed that 15 contigs (total length of 90,723 bp) created a concatenated sequence with high (97%) nucleotide identity to pCT (93,629 bp), and the IncK plasmid in our study differed from pCT mainly in the absence of three genes encoding IS*66*-family elements (data not shown). Among the four non-*E. coli* strains, CTX resistance could be transferred only by transformation into *E. coli* from one *P. rettgeri* strain. The transformant harbored a plasmid of ~104 kb, which was nontypeable by PBRT and cotransferred resistance to sulfamethoxazole and trimethoprim. The plasmid was confirmed in the donor, along with an additional plasmid of ~138 kb.

DISCUSSION

In a recent study, marked reductions in the prevalence of pigs shedding CTX-M-producing *E. coli* and in the fecal concentrations of CTX-resistant coliforms were observed during the production cycles at two Danish pig farms (11). The present study was designed to elucidate the population dynamics of CTX-M-producing *Enterobacteriaceae* associated with this observation. Using samples collected during the previous study, we analyzed retrospectively strain and plasmid diversity of CTX-M-producing *Enterobacteriaceae* in 15 individual pigs from one of the two farms. A high genetic diversity of CTX-M-14-producing *E. coli* was observed within and between samples obtained from the same individual over time. Eight of the ten offspring carried genetically unrelated CTX-M-14-producing *E. coli* at a minimum of one sampling time, as illustrated by the recovery of multiple PFGE types in the same sample (Fig. 1). A high strain diversity was also evident when analyzing each pig longitudinally, since at least three and up to four distinct PFGE types were detected in individual offspring during the course of the study. Notably, a decrease in strain diversity was observed after weaning (sampling 4), when eight of the ten offspring were found to shed a predominant CTX-M-producing *E. coli* strain (PFGE type F2). At the finishing stage (sampling 5), this strain was detected (at low numbers according to the previous study) in a single pig, and most of the offspring did not shed any CTX-M-producing *E. coli*. Altogether, these data show that the quantitative trends observed in the previous study were associated with a reduction in the diversity of the CTX-M-producing *E. coli*

after weaning. The observed selection of the dominant strain after weaning may reflect physiological changes in the pig intestinal tract, as well as specific management procedures at this production stage (e.g., changes in diet and antimicrobial treatment). Previous studies investigating the diversity of coliforms in weaned pigs by phenotypic or genotypic methods showed that weaning was associated with the appearance of dominant coliform strains (26, 27). A shift in the composition of the predominant microbiota of the pig intestine has been observed in the postweaning period using 16S rRNA gene-targeted denaturing gradient gel electrophoresis (28). It is also known that the dominant *E. coli* strains of newborn piglets may differ from those observed in the feces of their dams (27, 29). Thus, our findings are in line with the current knowledge of diversity and dynamics of *E. coli* in pigs, supporting the notion that the population dynamics of CTX-M-producing *E. coli* does not differ from that of other *E. coli*, even though these antimicrobial-resistant strains occurred at low fecal densities (approximately 10^3 to 10^7 CFU/g) compared to the total *E. coli* population, especially at later production stages (11).

In the scientific literature there is a paucity of longitudinal studies of CTX-M-producing *Enterobacteriaceae* in livestock farming. We are aware of two studies that investigated the diversity of CTX-M-producing *E. coli* at the farm level in dairy (30) and veal (31) calves. Both studies showed that changes in the diversity of CTX-M-producing strains over time were associated with the use of antimicrobials. Antimicrobial usage may also have influenced the relative abundance of different CTX-M-producing *E. coli* strains during the production cycle of the farm investigated in the present study. In the 6 months prior to the study and during the study period, pigs at the farrowing stage (sows and piglets) were treated with a wide array of antimicrobials, including extended-spectrum penicillins, tetracyclines, aminoglycosides, and sulfonamide-trimethoprim, whereas weaners and finishers were treated primarily with macrolides and tetracyclines (11). The high genetic diversity and fecal densities of CTX-M-producing strains observed at farrowing coincided with a high consumption of β-lactams at this production stage. Although it is not possible to establish a cause-effect relationship based on the results generated here, the reduction of β-lactam use after weaning (<5% of the amount used for sows and piglets) could have contributed to lower both the diversity and fecal densities of CTX-M-producing *E. coli*. A similar effect could be attributed to zinc, which is routinely used at this farm and in the vast majority of Danish pig farms for the prevention of postweaning diarrhea and has been previously shown to reduce *E. coli* strain diversity in the porcine fecal microbiota (26).

The ability to accurately characterize *E. coli* strain diversity within and between samples correlates with the underlying prevalence of each strain, which varies according to the individual host, and is influenced by the number of colonies tested (32–34). In the absence of data on the diversity of CTX-M-producing *E. coli* in pig fecal samples, we analyzed up to three isolates with typical *E. coli* morphology per sample, whenever available. It is possible that the animals harbored the sum of the strains at all sampling times with various levels of abundance that ranged from detectable to undetectable. Although our approach could underestimate strain diversity, it was a necessary compromise given the large number of positive samples and the methodology used for typing multiple isolates from the same sample. Moreover, it was sufficient to appreciate an unforeseen diversity of CTX-M-producing *E. coli* at the individual level. Diversity was even higher when considering other bacterial genera since some individuals shed concomitantly *E. coli* and non-*E. coli* strains producing distinct types of CTX-M β-lactamase.

All *E. coli* isolates examined carried $bla_{\text{CTX-M-14}}$ on indistinguishable IncK plasmids independent of strain genetic background and production stage. This finding confirms that plasmid-mediated horizontal gene transfer plays an important role in dissemination of ESBL-encoding genes within pig farms. Similar findings have been previously reported for $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ on different plasmid backbones (IncN and IncI1, respectively) (8, 35). Horizontal transmission of $bla_{\text{CTX-M-14}}$ on indistinguishable IncK plasmids among genetically unrelated *E. coli* strains of human, avian, and cattle origin has been described in the United Kingdom (30, 36). Cottell et al. (37) showed that this epidemic $bla_{\text{CTX-M-14}}$-carrying IncK plasmid conferred little or no fitness burden to *E. coli* and is likely to persist in this bacterial host in the absence of antimicrobial selective pressure. At the farm we studied, cephalosporins (the class of β-lactams that specifically selects for CTX-resistant bacteria) were not used in the study period (5 months) and in the six preceding months but were used before that time. Since the endemic IncK plasmid harboring $bla_{\text{CTX-M-14}}$ did not contain any other antimicrobial resistance genes, it is unlikely that this plasmid was selected by the usage of non-β-lactam agents. Thus, the plasmid was able to persist a long time after cephalosporins were used at the farm. A recent study of CTX-M-1-producing strains of equine origin has provided an example of how CTX-M-encoding plasmids might be coselected by nonantimicrobial factors such as the presence of sugar metabolic elements that are likely to enhance the fitness of *E. coli* in the intestinal tract of the horse (38). This finding suggests that antimicrobial use is not the only factor contributing to selection and maintenance of CTX-M-encoding plasmids in the intestinal microbiota of specific animal populations.

We showed simultaneous colonization with different CTX-M-producing *Enterobacteriaceae* species in individual sows and piglets, as previously shown among human patients colonized with *E. coli* and *Klebsiella pneumoniae* strains both producing CTX-M-3/9/15 variants (39). Interestingly, in our study there was no overlap between $bla_{\text{CTX-M}}$ gene variants and plasmid types detected in the different bacterial species within individual samples. To our knowledge, the presence of $bla_{\text{CTX-M-2/97}}$-carrying *P. mirabilis* and *P. rettgeri* in pigs has not been described before. This CTX-M variant is rare among *E. coli* isolates from pigs in Europe (2) but has been shown to be common among *E. coli* isolates from Belgian broiler farms (40). In humans, CTX-M-2 has occasionally been reported in clinical isolates of *P. mirabilis* and *Providencia stuartii* in Asia (41) and South America (42, 43). The molecular epidemiology of ESBL-encoding genes in *P. mirabilis* isolated from humans varies widely between geographical regions. TEM types, especially TEM-52 and TEM-92, dominate in France and Italy (44, 45), while CTX-M types are common in Israel (CTX-M-2), Poland (CTX-M-3), Japan (CTX-M-2), South Korea (CTX-M-14), and Argentina (CTX-M-2) (41, 42, 46–48). *Providencia* species producing ESBL types other than CTX-M-2 have only been reported on a few occasions (49).

Direct plating and plating following enrichment of feces were equally suited to detect CTX-M-producing *E. coli*. Minor differences between the two isolation procedures were observed in the prevalence of positive samples (69% versus 62%). Surprisingly, 10 samples were positive by direct plating but negative following en-

richment (Table 2). The reasons for this unexpected finding are unknown. Differences in the abilities of the two methods to detect strain diversity were also observed. The use of two methods increased the overall picture of strain diversity, and not only because more isolates were analyzed. In fact, 24% of the strains isolated following enrichment were not detected by direct plating, even if multiple colonies were analyzed by the latter method. This finding may be due to the ability of low-prevalence strains to overgrow high-prevalence strains during enrichment. Although unlikely, it is also possible that susceptible strains acquired the plasmid during enrichment and overgrew resistant strains originally present in the samples. It is worth considering these factors in future epidemiological studies since enrichment is recommended and widely used to enhance the detection of ESBL producers (50).

The inclusion of a single farm and the limited number of pigs sampled are the main limitations of the study. The numbers of farms and individuals tested were necessarily limited by the longitudinal design of the study, as well as by the use of different isolation procedures and time-consuming methods for characterization of the genetic background and plasmid content of the isolates. Due to these limitations, the results of the study must be cautiously interpreted and generalized.

In conclusion, we demonstrated that the reduction in the prevalence of pigs shedding CTX-M-producing *E. coli* during the farm production cycle was accompanied by a marked decrease in strain diversity after weaning. Newborn piglets carried multiple genetically unrelated strains that were replaced in the postweaning period by a dominant strain, which was largely lost toward the end of the production cycle. These results show that assessment of strain diversity of CTX-M-producing *Enterobacteriaceae* within pig farms is greatly influenced by the production stage from which samples are collected and by the method used for isolation. This information is relevant to the design and interpretation of future epidemiological studies in pig farming, including, for example, studies assessing transmission of CTX-M-producing *Enterobacteriaceae* between farms or between pigs and farmers within the same farm. This study also highlights the need for functional genomic research to identify the factors and mechanisms responsible for maintenance of the IncK plasmid described here and other antimicrobial resistance plasmids that are able to persist in animal populations in the absence of any evident antimicrobial selective pressure. Beyond the scientific interest of this topic, the outcome of this research may pave the way for the development of effective measures to reduce the occurrence of resistant bacteria of zoonotic interest in livestock.

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