Carriage and Fecal Counts of Cefotaxime M-Producing *Escherichia coli* in Pigs: a Longitudinal Study

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Current knowledge on extended-spectrum beta-lactamases (ESBLs) in animals is based largely on cross-sectional studies and qualitative data. The aim of this longitudinal study was to elucidate carriage proportions and fecal counts of ESBL-producing *Escherichia coli* in pigs during the production cycle. At each of three ESBL-positive single-sited farrow-to-finisher pig farms (farms A, B, and C) included in the study, individual fecal samples were taken from 17 to 20 sows 1 week before farrowing and from 2 piglets of each sow’s litter four times from birth to slaughter (as piglets, weaners, and finishers). Cefotaxime (CTX)-resistant coliforms in feces were counted on MacConkey agar containing 2 μg/ml CTX and characterized for the presence of ESBL-encoding genes by PCR and sequencing. CTX-M-positive pigs were detected in all age groups at farms A (bla<sub>CTX-M-1</sub> group, compatible with bla<sub>CTX-M-1/61</sub>), B (bla<sub>CTX-M-1</sub> group, compatible with bla<sub>CTX-M-1/61</sub>), whereas only three weaners were positive at farm C (bla<sub>CTX-M-1</sub> group, compatible with bla<sub>CTX-M-1/61</sub>). A significant decrease in carriage was detected during the production cycle, with an average 50% carriage immediately after birth, 58% just before weaning, 29% during weaning, and 12% during finishing. The observed reduction in numbers of CTX-M-positive pigs was accompanied by a significant reduction in mean fecal counts of CTX-resistant coliforms from ~10<sup>7</sup> CFU/g in piglets to ~10<sup>5</sup> CFU/g in finishers (P < 0.001). These findings provide novel information about the epidemiology of ESBLs at the farm level and have important implications for assessments of risks of meat contamination during slaughter.

Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* represent one of the fastest-emerging resistance problems worldwide. At the gene level, bla<sub>CTX-M</sub> has spread successfully in recent years, replacing bla<sub>TEM</sub> and bla<sub>SHV</sub> as the predominant ESBL-encoding gene in Europe (1, 2). Cefotaxime (CTX-M) enzymes comprise a genetically diverse and rapidly growing family, and so far, five groups, named groups 1, 2, 8, 9, and 25, have been described (1).

The occurrence of ESBL-producing bacteria in different food-producing animals has been increasingly reported in recent years (3, 4). CTX-M-1 is the most frequently reported ESBL type in food animals in Europe, and this was also the first type discovered in Danish pigs in 2005 (5). The same type was also predominant in a recent study of pigs at slaughter in Denmark, followed by CTX-M-14, CTX-M-2, and CTX-M-15 (6). In Europe, CTX-M-1 and CTX-M-14 have also been detected among pigs in Spain, France, the United Kingdom, and Switzerland (7–11). An association between the presence of CTX-M-producing *Escherichia coli* and the usage of cephalosporins in pigs has been demonstrated (12, 13), leading to a self-ban of these antibiotics by the Danish pig industry in 2010. The occurrence of ESBLs in production animals has important implications for public health, because these clinically relevant resistance determinants can be transmitted from animals to humans through either acquisition via the food chain (14) or direct transmission to farm workers (15).

Current knowledge of ESBLs in animals is based largely on cross-sectional studies and qualitative data. The objective of the present study was to characterize longitudinal shedding patterns and fecal counts of ESBL-producing *E. coli* in individual pigs monitored from birth to slaughter. For this purpose, a longitudinal study was conducted at three Danish pig farms known to be ESBL positive and having a history of cephalosporin use. Quantitative data on ESBL carriage during the production cycle were generated for 54 sows and 108 pigs randomly selected as the offspring of these sows.

**MATERIALS AND METHODS**

**Sampling sites and methods.** Three ESBL-positive farms (farms A, B, and C) were selected based on the following inclusion criteria: (i) positive ESBL status based on previous screening, (ii) single-sited farrow-to-finish production system (housing of sows, piglets, weaners, and finishers on the same farm), and (iii) high-level consumption of cephalosporins (in the upper 10th percentile of usage of cephalosporins in Denmark from April 2009 through March 2010). Sampling was carried out from June through December 2011. Data on antimicrobial consumption at the farm level during the study and 6 months previously were obtained from the national veterinary prescription database, VetStat, at the Danish Veterinary and Food Administration. These data and the size of each farm are summarized in Table 1. Farms A and B used continuous production in the farrowing, weaning, and finishing sections, while farm C used an all-in/all-out management in the farrowing and finishing sections and continuous production in the weaning section.

At each farm, 20 to 25 sows in their last week of gestation or up to 4 days after farrowing were randomly selected for the study (69 sows in total). Upon enrollment, some sows had to be excluded due to either (i) cross-fostering (n = 4), (ii) not having farrowed (n = 8), or (iii) movement to other sections of the farm (n = 3), resulting in a final study population of 54 sows. In the first week after farrowing, two healthy piglets...
were randomly selected from each sow and ear tagged in both ears for identification throughout the study period. During the course of the study, offspring were excluded due to either (i) cross-fostering (*n* = 4), (ii) movement to other pens (*n* = 4), (iii) loss of ear tag (*n* = 4), or (iv) death (*n* = 5). The sampling strategy and the total numbers of fecal samples obtained from each farm and age group are summarized in Table 2.

Fecal samples were taken from the rectum of all animals except for newborn piglets, which were sampled by using rectal swabs (premoistened Steriswab; MWE Medical Wire & Equipment, Corsham, Wiltshire, United Kingdom). Samples were collected from the sows immediately before or after farrowing (sampling 1) and from the offspring four times during the production cycle: in the first week after birth (sampling 2), just before or after farrowing (sampling 1), and from the offspring four times during the production cycle: in the first week after birth (sampling 2), just before or after farrowing (sampling 1), and around 3 weeks before slaughter (sampling 3), as shown in Table 2. All samples were kept cool during transportation to the laboratory and processed within 24 h after sampling.

**Quantification and isolation of cefotaxime-resistant *E. coli*.** Fecal counts (CFU/g or CFU/swab) of cefotaxime (CTX)-resistant coliforms were quantified in the following way: 1 g of feces was suspended in 9 ml 0.9% sterile saline followed by the spotting of 20 μl of serial 10-fold dilutions (10⁻¹ to 10⁻⁵ dilutions) onto MacConkey agar (Merck, Darmstadt, Germany) supplemented with 2 μg/ml CTX (Sigma-Aldrich, St. Louis, MO). Plates were incubated at 37°C for 24 h. Lactose-positive colonies (coliforms) were counted according to recommendations by the Nordic Committee on Food Analysis (16). This method had a minimum detection limit of 500 CFU/g of feces or CFU/swab. From each sample resulting in growth, one lactose-positive colony was randomly selected for further characterization. Lactose-positive isolates were confirmed as *E. coli* by the indole and citrate tests.

**Identification of CTX-M-producing *E. coli*.** All selected isolates were screened for the presence of *bla*<sub>CTX-M</sub> by PCR using CTX-M universal primers (17). From each farm and sampling time, three *bla*<sub>CTX-M</sub>-positive isolates from randomly selected individuals were further characterized by PCR using CTX-M group-specific primers (18). *bla*<sub>CTX-M</sub>-negative isolates were screened for the presence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub> by PCR (17, 19). Amplicons were purified and sequenced by Macrogen Inc. (Seoul, South Korea). Nucleotide sequences were analyzed by using the Basic Local Alignment Search Tool (BLAST) available on the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Isolates negative for all tested genes were subjected to the CT/CTL Etest (bioMérieux, France) for the phenotypic identification of ESBL.

**Statistical analysis.** Pigs were classified as CTX-M positive if they had at least one isolate confirmed to be *E. coli* and were positive for *bla*<sub>CTX-M</sub> by PCR. Swabs from newborn piglets (sampling 2) were used for the detection of CTX-resistant isolates but could not be used for the calculation of mean CFU per gram of feces. Carriage proportions of CTX-M-positive pigs were estimated for each sampling time and each farm, and the longitudinal shedding patterns were characterized by estimating the proportion of pigs changing carriage status between different sampling points.

Mean CFU counts per gram of feces within age groups (piglet, weaner, and finisher) and farms (farms A, B, and C) were compared by using a zero-inflated negative binomial regression model with the Genmod procedure in SAS, version 9.3 (SAS Institute, Cary, NC). An interaction term between age group and farm was included to assess if counts differed between age groups in different farms.

Shifts in carriage status were examined statistically by comparing the status as CTX-M-positive piglets with the status as finishers using exact logistic regression. The carriage status of the dam as a predictor of the status of the piglet was also assessed while taking into account the herd-level carriage proportion and that each sow had multiple piglets. This

<table>
<thead>
<tr>
<th>TABLE 1 Usage of antimicrobials from January through December 2011 in each farm and stratified by age group in ADD kg&lt;sup&gt;a&lt;/sup&gt; per pig</th>
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</thead>
<tbody>
<tr>
<td><strong>Antimicrobial agents</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Farm</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>Weaner</td>
</tr>
<tr>
<td>Finisher</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>Weaner</td>
</tr>
<tr>
<td>Finisher</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>Weaner</td>
</tr>
<tr>
<td>Finisher</td>
</tr>
</tbody>
</table>

<sup>a</sup> ADD kg is the defined animal daily dose, the assumed average maintenance dose per day for treatment of 1 kg animal for the main indication in a specified species (26).

<sup>b</sup> Aminoglycosides include neomycin, gentamicin, and amikacin; lincosamides include lincomycin; macrolides include tylosin, tilmicosin, and tulathromycin; penicillins with an extended spectrum include amoxicillin and ampicillin; pleuromutilins include tiamulin; polypeptides include colistin; and tetracyclines include chlorotetracycline, doxycycline, and oxytetracycline.

<sup>c</sup> The average weight of sows plus piglets is 200 kg. The average weight of weaners is 15 kg. The average weight of finishers is 50 kg.

<table>
<thead>
<tr>
<th>TABLE 2 Sampling strategy and total numbers of fecal samples in all three farms</th>
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<tbody>
<tr>
<td>Sampling</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

<sup>d</sup> Samples were taken from sows in their last week of gestation, but for a few sows, samples had to be taken up to 4 days after farrowing.

<sup>e</sup> Day zero is farrowing.

<sup>f</sup> Rectal swabs were taken from newborn piglets.
analysis was carried out as a logistic regression analysis using the Genmod procedure in SAS. Statistical significance in all analyses was deemed at $P$ values of $<0.05$.

**RESULTS**

**Longitudinal carriage of $bla_{CTX-M}$-positive $E. coli$.** CTX-M-positive animals were detected in all age groups at farms A and B, whereas only three weaners tested positive at farm C. Among 176 CTX-resistant $E. coli$ isolates tested, 168 (95%) carried $bla_{CTX-M}$. Based on sequence analysis, all $bla_{CTX-M}$-positive isolates from farm A ($n = 15$) carried the $bla_{CTX-M-9}$ group (compatible with $bla_{CTX-M-14/17}$), whereas all isolates from farms B ($n = 15$) and C ($n = 3$) carried the $bla_{CTX-M-1}$ group (compatible with $bla_{CTX-M-1/61}$). Eight CTX-resistant isolates without $bla_{CTX-M}$ were isolated from two sows and three piglets at farm A and from three sows at farm C. All these isolates were positive for $bla_{TEM-1}$ and negative for $bla_{SHV}$ and $bla_{CMY-2}$. Further testing of these eight isolates by the Etest for ESBL detection revealed cefotaxime MICs of 3 to 8 g/ml and no synergy with clavulanic acid. The data for each sampling time stratified by farm are shown in Table 3. In farms A and B, the CTX-M status of the piglets was not influenced by the CTX-M status of the dam ($P = 0.80$) but varied significantly along the production cycle. The carriage prevalence increased until weaning, while a decrease was observed in the weaning and finishing sections, with the lowest carriage prevalence being found just before slaughter. In farm C, all sows, piglets, and finishers were CTX-M negative, while only 10% of sampled weaners were CTX-M positive. Overall, finishers were more likely to be CTX-M positive if they had been positive as piglets, with an odds ratio of 12 (95% confidence interval [CI], 2.3 to infinity; $P = 0.0068$).

**Fecal concentrations of CTX-resistant coliforms.** Mean fecal counts of CTX-resistant coliforms in different age groups are displayed in Fig. 1. The counts decreased significantly from piglets to weaners and from weaners to finishers at both farms A and B ($P < 0.001$). Among carriers, the average counts in the three age groups were $10^7$, $10^5$, and $10^4$ CFU/g, respectively.

**DISCUSSION**

This study provides useful insights into the epidemiology of ESBLs in pig farms. Irrespective of the CTX-M type, an overall decrease in the carriage prevalence of CTX-M-producing $E. coli$ was seen during the production cycle, with individual pigs being more frequently positive before weaning than after weaning and during finishing. Counts of CTX-M-producing coliforms also decreased throughout the production cycle and correlated with carriage prevalence. The higher carriage prevalence and counts found during preweaning than during postweaning and finishing could be due to age-related changes in the gut microbiome or dietary changes occurring after weaning, which may result in an alteration of the intestinal flora and the replacement of CTX-M-producing $E. coli$ strains by other strains. A previous study showed that the composition of the intestinal $E. coli$ floras of pigs changes over time (20). Another explanation for the gradual reduction in the carriage of CTX-M-producing $E. coli$ could be the use of all-in/all-out management systems with thorough cleaning and disinfection between batches. Such a system was used at farm C in the farrow-

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**TABLE 3** $bla_{CTX-M}$ carriage prevalences$^a$ in different age groups at the three farms under study

<table>
<thead>
<tr>
<th>Farm</th>
<th>Sampling 1, sows</th>
<th>Sampling 2, piglets</th>
<th>Sampling 3, piglets</th>
<th>Sampling 4, weaners</th>
<th>Sampling 5, finishers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>% positive isolates</td>
<td>No. of isolates</td>
<td>% positive isolates</td>
<td>No. of isolates</td>
</tr>
<tr>
<td>A</td>
<td>17</td>
<td>71</td>
<td>34</td>
<td>91</td>
<td>34</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>35</td>
<td>39</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>C</td>
<td>17</td>
<td>0</td>
<td>34</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>35</td>
<td>107</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ Pigs were classified as positive if they had at least one isolate confirmed to be a $bla_{CTX-M}$-positive $E. coli$ isolate.

**FIG 1** Mean fecal counts (log CFU/g) of cefotaxime-resistant coliforms in different age groups (piglets, weaners, and finishers) at farms A, B, and C. Error bars indicate the 95% confidence intervals for each age group.
ing and finishing sections but not in the weaning section, which might explain the finding of CTX-M-positive pigs only among weaners at this farm.

The data from the farrowing sections apparently suggest a link between the bla\textsubscript{CTX-M} status of the sows and that of their offspring, since both sows and piglets were bla\textsubscript{CTX-M} negative in farm C, whereas high percentages of positive sows and piglets were found in farms A and B. However, statistical analysis revealed no significant association between the bla\textsubscript{CTX-M} status of the dams and that of their offspring. It appears that the longitudinal carriage of CTX-M-producing \textit{E. coli} in pig farms may differ from that of livestock-associated methicillin-resistant \textit{Staphylococcus aureus} (LA-MRSA) (21). While our findings indicate that CTX-M carriage decreases significantly after weaning, a similar longitudinal study (22) in pig farms in Denmark and the Netherlands showed a significant increase in the prevalence of LA-MRSA-positive animals after weaning. A similar trend was observed in another study from Canada (23). It remains unclear how the three pigs in farm C suddenly became CTX-M-positive carriers in the weaning section and negative again as finishers, while all other pigs included in the farm remained negative throughout the study.

Previous studies have shown that the use of antibiotics, especially cephapirinos, is a risk factor for the selection of ESBL-producing bacteria in both humans (24) and animals (12, 13, 25). The use of broad-spectrum veterinary cephapirinos (especially “third-generation” and “fourth-generation” cephapirinos, such as ceftiofur and cefquinome) has been proposed as an important reason for the occurrence of these bacteria among food-producing animals and meat products (6). In Denmark, the consumption of cephapirinos in pigs increased from 2001 through July 2010, when the pig industry decided to ban their use (26). The farms included in this study were selected for having a high level of consumption of cephapirinos before the ban, but the pigs were sampled approximately 1 year after the ban, indicating that CTX-M-producing \textit{E. coli} can persist in pigs for a long time in the absence of any direct selective pressure. The reasons for this persistence are unknown and may include different factors such as coselection by the use of other antimicrobials, strain fitness, and plasmid stability. Antimicrobial usage patterns varied between farms and age groups. Although the data are too limited to infer any significant association between antibiotic use and the occurrence of CTX-resistant \textit{E. coli}, the farm sections where pigs had the highest fecal densities of CTX-resistant \textit{E. coli} (sows/piglets from farms A and B and weaners from farm C) were treated with extended-spectrum penicillins during the study period (Table 1), suggesting a possible association between CTX resistance and use of amoxicillin or ampicillin. This apparent association needs to be verified by future studies specifically designed to assess the influence of antibiotic use on the shedding of CTX-resistant \textit{E. coli}.

It was shown previously that \textit{E. coli} originating from the pigs’ own feces or cross-contamination between pigs slaughtered on the same day is an important source of carcass contamination (27). For cattle, it was suggested that a high concentration of \textit{E. coli} O157 in feces might pose an increased risk of meat contamination and that a few infected animals shedding high concentrations of \textit{E. coli} O157 might be of greater importance than an overall high prevalence among animals when assessing the risk of contamination (28). Similarly, several studies suggested that “high-density shedders of CTX-M-producing \textit{E. coli}” (≥1 × 10\textsuperscript{8} CFU/g) pose an increased risk for contamination of the food chain (10, 12). In our study, the lowest counts of CTX-resistant coliforms were detected in finishers sampled just before slaughter (average fecal counts in finishers of <1.2 × 10\textsuperscript{5} CFU/g), indicating a presumably lower risk of meat contamination than with high-density shedders. As the quantitative data generated by this study are not accompanied by any data on carcass contamination, quantitative studies conducted during the slaughtering process are needed to assess the possible correlation between fecal counts and meat contamination with CTX-M-producing \textit{E. coli}.

The finding of bla\textsubscript{CTX-M-1} and bla\textsubscript{CTX-M-14}-Positive \textit{E. coli} isolates in the study farms is not surprising, as these are the two predominant CTX-M types among \textit{E. coli} strains isolated from slaughter pigs in Denmark (66% and 7%, respectively) (6). In Denmark and many other European Union countries, the most prevalent CTX-M variant among human isolates is CTX-M-15 (60%), followed by CTX-M-14 (12%) and CTX-M-1 (11%) (29). CTX-M-15 is rarely detected in animals (2%) (6), thus indicating that the majority of human infections caused by CTX-M-producing \textit{E. coli} are not attributable to zoonotic transmission. However, the epidemiology of ESBLs may undergo rapid changes, and the possible flow of CTX-M-1 and CTX-M-14 from livestock to humans and that of CTX-M-15 from humans to livestock require careful monitoring and prevention over the next years.

The presence of the \textit{bla}\textsubscript{TEM-1} gene in CTX-M-negative isolates was not surprising due to the ubiquitous spread of this gene (1) but does not explain resistance to CTX. We did not investigate the possible presence of mutations in the promoter of the chromosomal \textit{ampC} gene in these isolates because the distribution of resistance determinants was beyond the scope of this study. Nevertheless, the results of the Etest indicated that \textit{ampC} upregulation could likely explain the observed phenotype. AmpC upregulation was previously shown to be the second most common mechanism of cephapirin resistance in Danish pigs (15%) (6).

The longitudinal design of this study provided original data on the shedding of bla\textsubscript{CTX-M}-Positive \textit{E. coli} over time. Such a study design is resource demanding and time-consuming (i.e., over 400 fecal samples were collected over a period of 7 months for the testing of three farms) and therefore limits the number of farms that can be tested. This is the main limitation of the study.

In conclusion, significant decreases in the carriage prevalence of CTX-M-producing \textit{E. coli} and fecal counts of CTX-resistant coliforms were detected during the pig production cycle. These findings provide a new insight into the epidemiology of these bacteria in pig production and have important implications for assessments of the risks of meat contamination during slaughter. As such, the epidemiological data generated by this study may be extremely informative for the design of future surveillance studies at farms and slaughterhouses.

ACKNOWLEDGMENTS

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ERRATUM

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Page 794, column 1, paragraph 1, lines 5 and 6: “Cefotaxime M (CTX-M) enzymes” should read “CTX-M enzymes.”

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