Evaluación del uso de antimicrobianos como factor de riesgo relacionado con la aparición de resistencia a cefalosporinas en *Escherichia coli* y *Salmonella* en cerdos

Antimicrobial use as a risk factor associated with the emergence of cephalosporin resistance in *Escherichia coli* and *Salmonella* spp in pigs

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“Si tan solo amas la vida, tu bienestar aumentara, tus valores serán más fuertes, tu proyección se consolidara. Solo amate, disfruta tu vida y observa cuanta gente vendrá a ti” Y.B

ANEXOS

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Impact of the Use of β-Lactam Antimicrobials on the Emergence of *Escherichia coli* Isolates Resistant to Cephalosporins under Standard Pig-Rearing Conditions

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The aim of this study was to evaluate if the treatments with ceftiofur and amoxicillin are risk factors for the emergence of cephalosporin resistant (CR) *E. coli* in a pig farm during the rearing period. One hundred 7-day-old piglets were divided into two groups, a control ($n = 50$) group and a group parenterally treated with ceftiofur ($n = 50$). During the fattening period, both groups were subdivided in two. A second treatment with amoxicillin was administered in feed to two of the four groups, as follows: group 1 (untreated, $n = 20$), group 2 (treated with amoxicillin, $n = 26$), group 3 (treated with ceftiofur, $n = 20$), and group 4 (treated with ceftiofur and amoxicillin, $n = 26$). During treatment with ceftiofur, fecal samples were collected before treatment (day 0) and at days 2, 7, 14, 21, and 42 posttreatment, whereas with amoxicillin, the sampling was extended 73 days posttreatment. CR *E. coli* bacteria were selected on MacConkey agar with ceftriaxone (1 mg/liter). Pulsed-field gel electrophoresis (PFGE), MICs of 14 antimicrobials, the presence of cephalosporin resistance genes, and replicon typing of plasmids were analyzed. Both treatments generated an increase in the prevalence of CR *E. coli*, which was statistically significant in the treated groups. Resistance diminished after treatment. A total of 47 CR *E. coli* isolates were recovered during the study period; of these, 15 contained $bla_{\text{CTX-M-1}}$, 10 contained $bla_{\text{CTX-M-14}}$, 4 contained $bla_{\text{CTX-M-9}}$, 2 contained $bla_{\text{CTX-M-15}}$, and 5 contained $bla_{\text{SHV-12}}$. The treatment with ceftiofur and amoxicillin was associated with the emergence of CR *E. coli* during the course of the treatment. However, by the time of finishing, CR *E. coli* bacteria were not recovered from the animals.

During the last decade, resistance to extended-spectrum beta-lactams (ESBLs), especially third- and fourth-generation cephalosporins and penems, has raised the concern of the scientific community. The World Health Organization has defined third- and fourth-generation cephalosporins as being "critically important" for use in humans (1) since the increased presence of resistance to these antimicrobials could seriously compromise the treatment of some life-threatening infections, including bacteremia and meningitis.

A third-generation cephalosporin, ceftiofur, and a fourth-generation cephalosporin, cefquinome, have been developed strictly for veterinary use (2). Ceftiofur is widely used in many different food animals to treat respiratory diseases. Cefquinome can also be used for the treatment of mastitis metritis agalaxia syndrome in sows, exudative epidermitis, and meningitis (3). The systemic use in food animals of cephalosporins that could potentially select for resistant organisms is worrisome due to the role that food-producing animals may play in the spread of extended-spectrum cephalosporinases into the community.

Previous studies have demonstrated a statistically significant association between the use of ceftiofur and reduced susceptibility to third-generation cephalosporins in *Escherichia coli* (4, 5). However, these studies did not find an association between ceftiofur usage and the presence of ESBL genes ($bla_{\text{CTX-M}}$), and, more importantly, none of these studies has examined other drug use practices that can cross- or coselect for cephalosporin resistance. To our understanding, there is a lack of comprehensive studies performed under standard pig-rearing conditions analyzing the presence and factors that can contribute to both the emergence and the increase in occurrence of cephalosporin-resistant (CR) *E. coli* in pig farms.

For this reason, this study intends to evaluate if the treatments with two different beta-lactams, ceftiofur and amoxicillin, are risk factors associated with the emergence of CR *E. coli* during two stages (preweaning-growing and finishing) of the rearing period and assess if there is enough selective pressure to maintain resistant strains during the lifetime of the animals.

MATERIALS AND METHODS

Study design. This study was conducted on a conventional commercial pig farm in the northeast of Spain. During the 6 months previous to the study, the site remained depopulated, and it was cleaned and disinfected using standard operation procedures under field conditions. Sixty-eight sows were housed in a climate-controlled house, and fecal samples were

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TABLE 1 Results obtained during the visits after treatment with ceftiofur

Sampling day[a]	Age (days)	No. of positive animals by group (%)	
		Control ($n = 50$)[b]	Treated ($n = 50$)
0	6–8	5 (10)	7 (12)
2	8–10	5 (10)	11 (26)
7	13–15	1 (2)	0
14	20–22	1 (2)	5 (8)
21	27–29	0	0
41	47–49	0	0

[a] Sampling on day 0 was performed prior to injecting the animals with ceftiofur.
[b] Three animals from the control group died after 7 days of treatment, decreasing the size of the group to 47 animals.

TABLE 2 Results obtained during the course of the study after treatment with amoxicillin

Sampling day[a]	Age (days)	No. of positive animals (%) in[b]:			
		Group 1 ($n = 20$)	Group 2 ($n = 26$)	Group 3 ($n = 20$)	Group 4 ($n = 26$)
0	70	0	0	0	0
2	72	0	2 (8)	0	0
7	77	0	7 (27)	0	0
14	84	0	1 (4)	0	0
21	115	0	0	0	1 (4)
45	138	0	1 (4)	0	0
73	155	0	0	0	0

[a] Sampling in day 0 was performed just before the beginning of the treatment.
[b] Group 1, untreated with antimicrobials; group 2, untreated with ceftiofur and treated with amoxicillin; group 3, treated with ceftiofur and not treated with amoxicillin; group 4, treated with ceftiofur and with amoxicillin.

collected to examine the presence of CR *E. coli*. After farrowing, a total of 100 7-day-old piglets from 10 different sows were spatially divided into two groups: untreated controls ($n = 50$) and animals parenterally treated ($n = 50$) with ceftiofur (5 mg/kg of body weight in one shot) according to the product characteristics of a commercial preparation (Naxcel; Zoetis SLU). Three animals from the control group died of noninfectious causes during the course of the study. Fecal samples were taken manually from the rectum of piglets on six occasions: before treatment (day 0) and at days 2, 7, 14, 21, and 42 posttreatment (Table 1).

During the fattening period (day 70), each of the original two groups was subdivided into two (Table 2). A treatment with amoxicillin (Maymoxi; Laboratorios Maymó) was administered in feed for 14 days to two of the four resulting groups (10 mg/kg of body weight/day), consisting of the following: group 1, untreated control group, or animals that did not receive any treatment with beta-lactams ($n = 20$); group 2, animals orally treated with amoxicillin during finishing ($n = 26$); group 3, animals parenterally treated with ceftiofur during preweaning ($n = 20$); and group 4, animals treated with ceftiofur and amoxicillin ($n = 26$). The four groups remained spatially separated until their departure to the abattoir. Fecal samples were taken from all animals before administration of amoxicillin (day 0) and on days 2, 7, 14, 21, 45, and 73 posttreatment. A final sampling was performed at the time of slaughter. During the course of the study, farm biosecurity was extreme. Animals of different groups were spatially separated in designated pens to avoid contact. Overshoes were used by investigators and replaced at the entrance of each pen. Sampling was always initiated from the control group to the treated group to minimize transmission of resistant bacteria from pen to pen.

The study was performed in a commercial farm where the treatments, housing, and husbandry conditions conformed to the European Union (EU) Guidelines. In particular, the medicinal product used in this study (Naxcel) is EU registered (EU/2/05/053/001), and it was used according to veterinary rules without any additional requirement. Thus, it was not necessary to comply with additional ethical standards or approvals to carry out this experimental work since it did not require any invasive procedures (only collection of fecal samples) or management other than the field standard protocols set by the company.

***E. coli* isolation and identification.** Fecal samples were transported to the laboratory at 4°C on the same day of sampling. During the first two visits to the farm, a total of 268 fecal samples were collected from the sows ($n = 68$) and the piglets ($n = 200$), and a comparative study of isolation methods was performed. For each sample, direct plating of a loopful of homogenized feces onto MacConkey agar with ceftriaxone (1 mg/liter) was carried out in parallel to the following enrichment method. One gram of feces was suspended in 10 ml of MacConkey broth supplemented with ceftriaxone (1 mg/liter). After overnight enrichment at 37°C, 10 μl was plated onto MacConkey agar with ceftriaxone (1 mg/liter). Three colonies for each plate were stored, and one was confirmed as *E. coli* by Vitek-2 (bioMérieux) and further characterized.

PFGE and phylotyping. To assess the clonality of the isolates and their epidemiological relatedness, all isolates were analyzed for genetic relatedness by pulsed-field gel electrophoresis (PFGE) using XbaI according to the CDC PulseNet protocol (6). The *Salmonella* Braenderup H9812 strain was used as molecular standard. PFGE profiles were compared using Fingerprinting II Informatixe software (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to have a unique pattern when at least one band difference was detected. The analysis of the bands generated was performed using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.5% and position tolerance of 1.5%).

The isolates were discriminated in phylogenetic groups (A, B1, B2, C, D, and E) according to the method previously described by Clermont et al. (7, 8).

Antimicrobial susceptibility testing. Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, United Kingdom): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime-clavulanic acid, 30 plus 10 mg (30/10 mg), respectively; and ceftazidime-clavulanic acid, 30/10 mg, respectively. The disc combinations of cefotaxime and cefotaxime-clavulanic acid and of ceftazidime and ceftazidime-clavulanic acid were used for the identification of ESBLs; cefoxitin was used for the detection of *ampC*-type beta-lactamase (9). MICs of ampicillin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, tetracycline, florfenicol, colistin sulfate, sulfamethoxazole, trimethoprim, chloramphenicol, kanamycin, cefotaxime, and ceftazidime were determined by microdilution methods (VetMIC GN-mo; National Veterinary Institute, Uppsala, Sweden). Results were interpreted as epidemiological cutoff values following EUCAST recommendations (http://www.eucast.org/).

Detection of resistance genes. Resistance to third-generation cephalosporins was analyzed by PCR for the presence of the bla_{TEM}, bla_{CTX}, bla_{CMY-1}, bla_{CMY-2}, and bla_{SHV} genes as described previously (10). Detection of plasmid-mediated AmpC beta-lactamase genes was assessed by multiplex PCR (11). Sequence analysis was performed using Vector NTI Advance, version 11 (InforMax, Inc., Bethesda, MD). The amplified nucleotide sequences were compared to previously described sequences obtained from public databases (www.ncbi.nlm.nih.gov and http://www.lahey.org/Studies/).

Mating experiments and plasmid characterization. Filter mating experiments were performed to assess the capacity of the plasmids to conjugate. For this analysis, 14 isolates containing ESBL genes were selected. They comprised representative isolates from five PFGE clusters and nine PFGE types. Mating assays were performed as described elsewhere (12), using the isolates as donors and rifampin-resistant *E. coli* HB101 as a recipient. Transconjugants were selected on LB agar plates containing rifampin (50 mg/liter) and ceftriaxone (1 mg/liter) and were confirmed by PFGE.

Plasmidic DNA was purified from these 14 wild-type (WT) isolates and later from transformants using a Qiagen Plasmid Midi kit (Qiagen,

Hilden, Germany) according to the manufacturer's recommendations. Plasmids were introduced to electrocompetent plasmid-free *E. coli* cells by electroporation. Transformants were selected in brain heart infusion agar supplemented with ceftriaxone (1 mg/liter), and PCR for confirmation of the cephalosporin-resistant genes was performed. The presence of a unique plasmid in the transformants and their sizes were determined using S1 nuclease digestion followed by PFGE (S1-PFGE) (13). Finally, plasmids were classified by PCR-based replicon typing (14). Additionally, susceptibility testing was performed in all transformants to assess transferability of resistance genes unrelated to cephalosporins.

RESULTS

Emergence of cephalosporin resistance during treatment. In the first visit 168 samples were obtained (100 from piglets and 68 from sows). None of the samples was positive for CR *E. coli* by direct plating, in contrast with 11 positive piglets obtained with enrichment methods. Similar results (8 positive piglets versus 16, respectively) were obtained in the second visit ($n = 100$); furthermore, the 8 positive samples obtained by direct plating were also detected by the enrichment method. These results convinced the authors to continue the study using only the enrichment methodology.

All 68 sows were negative for CR *E. coli*. However, before administration of ceftiofur, five and seven of the 7-day-old piglets among the control and the treated groups, respectively, yielded CR *E. coli* (Table 1). During this first treatment, a total of 12 (4.1%) and 23 (8%) CR *E. coli* strains were isolated from the control ($n = 288$ samples) and the treated ($n = 300$ samples) groups, respectively. The difference in the proportion of CR *E. coli* recovered in the two groups was statistically significant ($P = 0.04$). The highest percentage of samples positive for CR *E. coli* was obtained within the treated group (22%) at 48 h posttreatment, showing a statistical tendency ($P = 0.1$) compared to the corresponding figure (10%) of the control group.

A total of 552 fecal swabs were collected during the second part of the study when animals were treated with amoxicillin in feed (Table 2). Previous to the treatment, all animals were negative for CR *E. coli*. CR *E. coli* isolates were recovered from group 2 (treated only with amoxicillin) after 2 (two isolates), 7 (seven isolates), 14 (one isolate), and 45 (one isolate) days posttreatment. One extra isolate was obtained from group 4 (treated with ceftiofur and amoxicillin) after 21 days posttreatment. No other positive samples were obtained in the rest of the groups during the study period. The highest percentage of samples positive for CR *E. coli* (27%) was obtained after 7 days of amoxicillin treatment within the group treated with amoxicillin and with no history of ceftiofur use. Significant differences were observed (Fisher test, $P = 0.02$) between the proportion of CR *E. coli* isolated from animals treated with amoxicillin and the rest of the groups after 7 days of treatment. By the finishing stage, all animals were negative for CR *E. coli*.

PFGE and phylogenetic analysis. Electrophoresis of XbaI-digested genomic DNA from the 47 CR *E. coli* isolates revealed 22 different profiles (Fig. 1). XbaI profiles typically had 14 to 21 restriction fragments between 20 and 1,135 kb (Fig. 1). Indistinguishable fingerprints were present in isolates from different animals and also in isolates obtained from the same animal at different sampling times (see Table S1 in the supplemental material), indicating the persistence of clones during the course of the treatment. None of the clones obtained during the treatment with ceftiofur was recovered during treatment with amoxicillin. Additionally, 10 of 12 isolates recovered during amoxicillin treatment presented identical PFGE patterns. A total of 66%, 25%, 4%, and 4% belonged to phylogroups A, B1, C, and E, respectively.

MIC determination. All 47 CR *E. coli* isolates (Fig. 1) were resistant to ampicillin (for the WT, MIC $\leq$ 8 mg/liter) and cefotaxime (for the WT, MIC $\leq$ 0.25 mg/liter), and all but four (belonging to the ceftiofur study) were resistant to ceftazidime (for the WT, MIC $\leq$ 0.5 mg/liter). Regarding the remaining antimicrobial families tested (tetracyclines, sulfamides, trimethoprim, aminoglycosides, quinolones, phenicols, and polymyxins), all isolates but two were multiresistant (15), ranging from resistance to three families of antimicrobials to resistance to six. MIC differences were detected among isolates according to treatment and sow. Higher levels of resistance were found during the ceftiofur treatment against phenicols (both chloramphenicol [for the WT, MIC $\leq$ 16 mg/liter] and florfenicol [for the WT, MIC $\leq$ 16 mg/liter]) and gentamicin (for the WT, MIC $\leq$ 2 mg/liter) than during the amoxicillin treatment, whereas levels of resistance were lower against ciprofloxacin (for the WT, MIC $\leq$ 0.064 mg/liter), nalidixic acid (for the WT, MIC $\leq$ 16 mg/liter), trimethoprim (for the WT, MIC $\leq$ 2 mg/liter), and kanamycin (for the WT, MIC $\leq$ 8 mg/liter). In the litter from sow number 25, all 10 positive CR isolates except 1 had the same resistance phenotype (beta-lactams, quinolones, and trimethoprim), whereas the remaining isolates obtained from the rest of the sows exhibited higher diversity of resistance traits. One isolate was resistant to colistin (for the WT, MIC $\leq$ 2 mg/liter).

Detection of genes responsible for ESBL resistance. ESBL genes were detected in 36 of these 47 CR *E. coli* isolates and in most cases were combined with the $bla_{\text{TEM-1}}$ gene. Fifteen isolates were confirmed to contained $bla_{\text{CTX-M-1}}$ (GenBank accession number X92506), 10 contained $bla_{\text{CTX-M-14}}$ (GenBank accession number AF252622), 4 contained $bla_{\text{CTX-M-9}}$ (GenBank accession number AF174129), 2 contained $bla_{\text{CTX-M-15}}$ (GenBank accession number AY044436), and 5 contained $bla_{\text{SHV-12}}$ (GenBank accession number AJ920369). Four isolates were resistant to cefoxitin, and the genotype could not be determined. Seven isolates with MICs of 0.5 mg/liter and 2 mg/liter for cefotaxime and ceftazidime, respectively, were negative for all PCRs tested, suggesting low susceptibility to cephalosporins, probably by upregulation of the AmpC promoter.

Conjugation and transformation. Eight of the 14 selected isolates were able to transfer the cephalosporin-resistant genes by conjugation. Additionally, 11 out of 14 isolates transferred cephalosporin-resistant genes to the electrocompetent strain. The 11 transformants together with the three transconjugants resulting from the wild-type strains were subjected to S1 nuclease, and the presence of one unique plasmid was confirmed. Sizes of plasmids varied between approximately 33.4 kb and 173.4 kb (Table 3). IncI1 was the most common replicon, followed by IncN. Four of the isolates presented two different replicons on the same plasmid, and no replicons were detected in one of the transformants.

The transformants/transconjugants were also resistant to streptomycin ($n = 10$), tetracycline ($n = 9$), sulfamethoxazole ($n = 8$), trimethoprim ($n = 4$), ciprofloxacin ($n = 2$), and kanamycin ($n = 1$).

DISCUSSION

Cephalosporin-resistant *E. coli* isolates were found in samples from 7-day-old piglets prior to the administration of any medica-

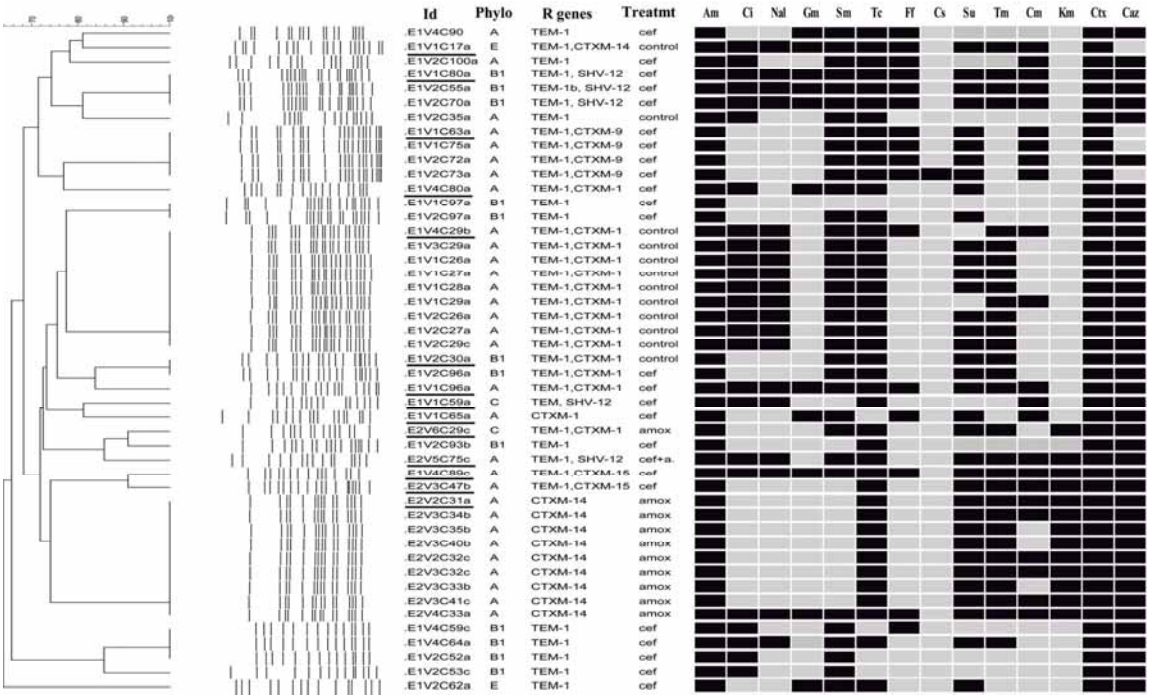

FIG 1 Dendrogram showing the genotypic relatedness of the CR *E. coli* bacteria isolated during the course of the study, the phylogeny, cephalosporin resistance genes, treatment, and phenotypic diversity. Am, ampicillin; Ci, ciprofloxacin; Nal, nalidixic acid; Gm, gentamicin; Sm, streptomycin; Tc, tetracycline; Ff, florfenicol; Cs, colistin; Su, sulfamethoxazole; Tm, trimethoprim; Cm, chloramphenicol; Km, kanamycin; Ctx, cefotaxime; Caz, ceftazidime. Strains selected for transformation and conjugation experiments are underlined. Phylo, phylogroup; Id, identification number; cef, ceftiofur; amox, amoxicillin; cef+a, ceftiofur and amoxicillin.

tion. Moreover, we could not detect the isolates from the sows even though we used an enrichment step for isolation of the specific resistance trait. The high clonality of the isolates demonstrated by PFGE does not argue for vertical transmission but, rather, for multiple acquisitions of isolates with limited colonization properties, perhaps from an external origin (personnel working at the farm, the food source, or the presence of rodents or other vectors). Other studies have also detected high diversity of CR isolates in newborn piglets, especially when enrichment methods were used for isolation due to the ability of low-prevalence strains

TABLE 3 Results of the conjugation and transformation experiments together with plasmid replicons and plasmid sizes obtained

Strain	Resistance gene	Conjugation result[a]	Transformation result[a]	Inc family member found[b]					Mol size (kb)
				I1	N	FIA	FIB	A/C	
E1V1C17a	CTXM-14	TC1b	**TF1a**	+					120
E1V1C80a	SHV-12	**TC2a**		+					138.9
E1V1C63a	CTXM-9	**TC3b**		+					138.9
E1V4C80a	CTXM-1	TC4a	**TF4a**		+				40
E1V4C29b	CTXM-1		**TF5a**		+		+		140
E1V2C30a	CTXM-1		**TF6a**	+					138
E1V1C96a	CTXM-1	TC7a	**TF7a**		+				40
E1V1C59a	SHV-12	TC8c	**TF8**	+	+				180
E1V1C65a	CTXM-1		**TF9a**		+				50
E2V6C29c	CTXM-1	TC10a	**TF10a**					+	180
E2V5C75c	SHV-12		**TF11a**						140
E1V4C89c	CTXM-15		**TF12a**			+	+		150
E2V3C47b	CTXM-15		**TF13a**			+	+		150
E2V2C31a	CTXM-14	**TC14a**		+					120

[a] Transconjugants and transformants used for replicon typing are in boldface.
[b] Inc family members are those present in the wild type and in the corresponding transformant or transconjugant.

to overgrow high-prevalence strains during enrichment (16). Additionally, weaning poses enough stress that it may contribute to *E. coli* overgrowth in pigs (17, 18). On the other hand, in some cases, PFGE results suggest that some of the clones were shared among piglets of the same pen (for example, the litters from sows numbers 25 and 11) (see Table S1 in the supplemental material), indicating a common source within the pen. Several studies have demonstrated a short-lived increase in the *E. coli* population after antimicrobial treatment or a stressful event. Since the sows were far from these events, they may carry undetectable amounts of CR *E. coli* (17, 18), and the limitations of the bacteriological techniques did not allow their detection. Hence, the farm was cleaned and depopulated during the 6 months prior to the study; incorrect cleaning and disinfection of the premises may play a role in the persistence of these organisms. Since environmental samples of the barn were not taken prior to the study, this option cannot be ruled out. Thus, a further visit to this farm, after 1 year of finishing this trial and applying a cleaning and disinfection protocol, demonstrated the presence of CTX-M-producing *E. coli* in the environment with a PFGE profile different from the profiles isolated from feces (data not shown).

After 48 h of the parenteral treatment with ceftiofur, an increase in the prevalence of CR *E. coli* was detected. These levels decreased after the first week of treatment. In the case of in-feed amoxicillin treatment, a similar increase was observed after 7 days of treatment. In the last visit, prior to departure to the abattoir, all the animals were negative for CR *E. coli*. Results from this study are in agreement with those of other studies performed in calves (19, 20), in which CR *E. coli* emerged for a short time while in the course of treatment and diminished shortly after treatment. Perhaps the resistant population could not compete well with the sensitive population after withdrawal of the antibiotic (19). However, during treatment with beta-lactam antimicrobials, animal feces could become a source of resistant bacteria. Biosecurity measures should be undertaken during treatment, such as feces removal or isolation of animals under medication, to avoid transfer of resistance. Additionally, farmers are at potential risk of contamination during exposure to animals shedding CR bacteria. Studies have demonstrated that ESBL genes and plasmids obtained from *E. coli* of farmers exhibited genetic similarity to those obtained from *E. coli* bacteria isolated from animals belonging to their farms (21).

It appears that both treatments with beta-lactams have selected for a wide range of cephalosporin resistance genes from different families, and these genes were recovered during both treatments. Previous studies analyzing the presence of cephalosporin resistance genes in pig farms in Spain described the presence of different *bla* genes, with SHV-12 being the most frequent (22), which is a completely different picture from what is found in other European countries, where SHV-12 is associated with human infections (23). Results from this study have shown the coexistence of many different resistance genes within one farm. The most frequent CTX-M variants in ESBL producers in animals and food of animal origin are currently CTX-M-1 and CTX-M-14, while CTX-M-15 ESBL-producing *E. coli* isolates have only exceptionally been observed in the veterinary context (24). However, this study has demonstrated in healthy pigs the presence of CTX-M-15 genes harbored in high-molecular-weight plasmids of approximately 150 kb containing two replicons, FIA and FIB. Are we seeing a change in the evolution of resistance similar to that we have perceived on the human side (25), where it was a shift in occurrence from CTX-M-14 and CTX-M1 toward CTX-M15?

Transformation experiments and replicon typing revealed the presence of a great variety of plasmids of many different sizes harboring the same resistance genes, with the most common replicons being IncI1 and IncN. However, further studies should be performed at the animal level and at the farm level to assess both the occurrence and spread of plasmids within the pig bacterial population in a particular farm and the persistence and transmission of these plasmids from herd to herd.

Additionally, CR *E. coli* isolates recovered during the course of the study were phenotypically resistant to different families of antimicrobials, and half of them were resistant to ciprofloxacin even though fluoroquinolones were never used to treat these animals. These results are in line with a high background of antibiotic resistance genes in the gut bacteria of livestock after over 60 years of antibiotic use (26). Although fluoroquinolone resistance is mostly conferred via a *gyrA* or *parC* mutation in the bacterial chromosome, two of the transformants exhibited resistance to fluoroquinolones. Plasmid-mediated quinolone resistance in some cases has been associated to the same plasmids as those harboring cephalosporin resistance genes (27). Furthermore, as demonstrated by the phenotype of the transformants exhibiting resistance to several antimicrobial families, coselection by plasmids bearing resistance genes for different antimicrobial families probably plays an important role in the maintenance of resistance mechanisms, as demonstrated via metagenomics in the gut bacteria of swine (28). In-depth studies should be performed to avoid the transmission of these resistance genes from farm to fork since several studies have demonstrated the presence of resistant *E. coli* and, in particular, CR *E. coli* of pig origin in the abattoir (29–31). Although animals from this study departed to the abattoir free of CR *E. coli*, it should be noted that this study was conducted under control conditions, and no extra medication apart from ceftiofur and amoxicillin was applied during the course of the study. However, conventional farming could also require the administration of macrolides, polymyxins, and tetracyclines during the fattening period, which could coselect for CR *E. coli* (32, 33). Currently, there is a scarcity of data linking antimicrobial consumption in veterinary medicine and the generation of antimicrobial-resistant bacteria; hence, it seems clear that the use of different families of antimicrobials in the same population could be a risk factor for the development of antimicrobial resistance in several microorganisms under field conditions (26, 34).

Taken together, these results suggest that the use of ceftiofur and amoxicillin at different stages of the rearing cycle are independent risk factors for the selection of CR *E. coli*. Both beta-lactam antimicrobials select for resistant *E. coli* during the course of treatment. However, CR *E. coli* bacteria were not detected in the absence of the selective pressure or when the animals departed to the abattoir. Further studies should be designed to identify other risk factors associated with the persistence of resistance determinants to minimize the recirculation of isolates and/or plasmids within farms.

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