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Within-farm dynamics of ESBL/AmpC-producing *Escherichia coli* in veal calves: a longitudinal approach

Joost Hordijk[1,2]*, Dik J. Mevius[1,2], Arie Kant[2], Marian E. H. Bos[3], Haitske Graveland[3], Angela B. Bosman[1], Cedric M. Hartskeerl[1], Dick J. J. Heederik[3] and Jaap A. Wagenaar[1,2]

[1]*Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.165, 3508 TD Utrecht, The Netherlands;* [2]*Central Veterinary Institute of Wageningen UR, PO Box 65, 8200 AB Lelystad, The Netherlands;* [3]*Division of Environmental Epidemiology, Institute for Risk Assessment Sciences, Utrecht University, PO Box 80.178, 3508 TD Utrecht, The Netherlands*

*Corresponding author. Tel: +31-30-2532459; E-mail: j.hordijk@uu.nl

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Objectives: To assess the within-farm dynamics of extended-spectrum β-lactamase (ESBL)/AmpC-producing *Escherichia coli* in veal calves.

Methods: Three veal-calf fattening farms were screened. Faecal samples from all calves within a compartment (109–150 per farm) were taken upon arrival on the farm (T0) and after 3, 6, 8 and 10 weeks (T3–T10). ESBL/AmpC genes were characterized by PCR and sequencing. Plasmids were characterized by transformation, PCR-based replicon typing and plasmid multilocus sequence typing (MLST). *E. coli* genotypes were analysed by MLST.

Results: At T0 the prevalence of ESBL/AmpC-producing *E. coli* ranged from 18% to 26%. These were predominantly isolates carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ genes, located on various plasmids and *E. coli* sequence types (STs). Farm 1 was negative for ESBL/AmpC-producing *E. coli* after T0. Farm 2 showed an increase up to 37% at T3, which subsequently decreased gradually to 0% at T10. The presence from T3 to T10 on farm 2 was mainly caused by the clonal spread of a multiresistant *E. coli* ST57 harbouring $bla_{\text{CTX-M-14}}$ on an IncF F2:A-:B- plasmid. Farm 3 showed a gradual decrease in prevalence to 1.4% at T10, with a relative increase of the identical clonal variant as shown for farm 2. A second clonal variant found in farm 3 was a multiresistant *E. coli* ST10 harbouring $bla_{\text{CTX-M-14}}$ on an IncK plasmid.

Conclusions: The prevalence of ESBL/AmpC-producing *E. coli* decreased over time. A clonal spread was observed on farm 2 and farm 3, illustrative of the complex dynamics probably associated with the use of antimicrobials.

Keywords: cattle, faecal carriage, antimicrobial resistance

Introduction

The increasing prevalence of resistance to extended-spectrum cephalosporins (ESCs) is of growing concern and is reported worldwide, not only in both human and animal clinical settings but also in the community and in food-producing animals.[1,2] Resistance to ESCs in Enterobacteriaceae is caused mainly by extended-spectrum β-lactamases (ESBLs) or AmpC-type β-lactamases, which enzymatically degrade these antibiotics.[3] Genes encoding these enzymes are often located on plasmids,[4] which can be transferred horizontally within and between different bacterial species. So far, many different ESBL/AmpC genes have been reported, and the number of novel gene variants is still expanding (http://www.lahey.org/studies).

Food-producing animals have been indicated as an important reservoir for ESBL/AmpC-producing bacteria.[5] Faecal carriage of ESBL/AmpC-producing *Escherichia coli* in food-producing animals has been reported previously,[6–10] and a partial genetic relatedness of genes, plasmids or host bacteria from food-producing animals and humans has been described.[6,11,12] Evidence of direct transfer was lacking. Faecal carriage in cattle has been reported previously.[9,13–18] However, cattle production is very diverse and includes dairy cattle and veal calves, which are kept in farms under different circumstances. In the Netherlands, dairy farms are generally closed production systems, whereas veal-calf farms often maintain an 'all-in all-out' system, in which animals originate from different farms and are imported from several European countries. Furthermore, dairy cattle and veal calves have a different lifespan, a different diet and are kept in different housing facilities. Finally, dairy cattle are generally exposed to considerably lower amounts of antimicrobials than veal calves.[19] Several studies on dairy farms have shown that young calves rapidly acquire antibiotic-resistant *E. coli*,[20] which are often multiresistant.[21] These studies also showed an age-related decline in the resistance levels of various antimicrobial-resistant

E. coli.[20,22] Recent studies have shown that from 2005 to 2010, resistance to ESC in veal calves was mainly caused by various CTX-M enzymes.[23] Furthermore, 66% of animal herds screened at slaughter were positive for ESBL/AmpC-producing *E. coli* [J. Hordijk, J. A. Wagenaar, A. Kant, A. Van Essen-Zandbergen, C. Dierikx, K. Veldman, B. Wit and D. Mevius (2013), unpublished results]. These studies provide a clear picture of how the prevalence of ESBL/AmpC genes developed over the years, and the genetic diversity that was present in animals at slaughter. Nonetheless, little is known about the short-term dynamics within a farm or a possible relationship between the prevalence of ESBL/AmpC-producing bacteria and antimicrobial use. In veal-calf farming, young animals are more at risk of acquiring an infectious disease due to the assembly of new herds. Furthermore, young calves generally receive more antimicrobials than older animals.

The purpose of this study was to determine the within-farm dynamics in the prevalence and molecular characteristics of ESBL/AmpC-producing *E. coli* isolated from individual veal calves during the first 10 weeks upon arrival at the fattening farm. The animals were ∼2 weeks of age when they arrived at the farm. Antimicrobial treatments were recorded and could therefore be associated with the prevalence of ESBL/AmpC-producing *E. coli*.

Methods

Study design

Three Dutch white veal-calf farms were included in the study and sampled over a 10 week time period, starting in October 2009. Farms were selected according to the following requirements: (i) the farm maintained an all-in all-out system; (ii) no professional activities concerning other food-producing animals were carried out; (iii) all calves originated from Dutch dairy farms to exclude differences between countries; and (iv) concerning the original design for methicillin-resistant *Staphylococcus aureus* (MRSA), the farm was positive for the presence of MRSA in the previous round (based on dust samples). All calves included in the study were housed in separated closed compartments within the stables. On farm 1, two compartments containing a total of 109 calves were included. On farm 2, one compartment containing 150 calves was included, and on farm 3, two compartments containing a total of 140 calves were included. Each compartment contained multiple pens.

During the first 6 weeks, all animals were housed in individual boxes within a pen. One day after T6, all calves were released from their individual boxes and relocated in pens containing an average of six animals. The calves were regrouped in the pens within the compartments based on their weight and/or feed intake. All animals in these compartments were sampled individually five times, starting at the moment the calves arrived at the farm (T0), and subsequently after 3 weeks (T3), 6 weeks (T6), 8 weeks (T8) and 10 weeks (T10). The study population did not change during this 10 week period. Calves that were relocated to stables outside the study compartments were excluded from the study. Calves from outside a study compartment were not allowed in. However, on farm 1, three calves were introduced to a study compartment at a later stage (two at T6, one at T10). These were not found positive for MRSA upon arrival (based on nose and rectal swabs), but were not tested for ESBL/AmpC carriage upon arrival. Antimicrobial use during the period of study was recorded.

E. coli isolation

At each sampling moment rectal swabs were taken from each individual calf using a sterile cotton-wool swab (Cultiplast®). The swabs were processed the same day, by suspending the faeces in 0.5 mL of buffered peptone water with 30% glycerol, and stored at −80°C. ESBL/AmpC-producing *E. coli* were isolated by inoculating 10 μL of the faecal suspension on a MacConkey agar plate (product no. 212123, Becton Dickinson) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (MC+; direct plating), as well as in 1 mL of Luria–Bertani selective enrichment broth (Becton Dickinson) supplemented with 1 mg/L cefotaxime (LB+; enrichment). After overnight aerobic incubation at 37°C, three typical red colonies were selected from MC+ plates showing growth, and each isolate was pure-cultured on sheep blood agar plates (Biotrading, the Netherlands). After overnight enrichment all LB+ samples were also inoculated on MC+ plates and, after overnight incubation, one typical red colony was selected from plates showing growth and pure-cultured on sheep blood agar plates. After *E. coli* confirmation by checking for tryptophan hydrolysis, the isolates were stored at −80°C as suspensions in buffered peptone water with 30% glycerol, pending molecular analysis.

Gene characterization

All *E. coli* isolates that were selected after direct plating or after enrichment were screened by PCR for $bla_{\text{CTX-M}}$ (including group-specific PCRs for $bla_{\text{CTX-M}}$ groups 1, 2, 8 and 9), bla_{TEM} and bla_{SHV} as described previously.[24] The presence of plasmid-mediated AmpC β-lactamase genes (including bla_{CMY}, bla_{ACC} and bla_{FOX}) was screened for by PCR as described by Pérez-Pérez and Hanson.[25] The presence of bla_{OXA} groups 1 and 2 was screened for as described by Voets *et al.*[26] For the promoter region of chromosomal *ampC*, primers were used as described by Caroff *et al.*[27] The designation of chromosomal *ampC* subtypes was conducted as described by Mulvey *et al.*[28] Since various $bla_{\text{CTX-M}}$ genes that belong to group 1 are described as occurring frequently in cattle, in all isolates where $bla_{\text{CTX-M}}$ group 1 genes were detected, these genes were sequenced using Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems, USA).

Subsequently, a selection of isolates was made for further molecular characterization by calculating the square root of the number of isolates harbouring a specific $bla_{\text{CTX-M}}$ group 1 gene or gene family groups (e.g. $bla_{\text{CTX-M}}$ group 2, bla_{CMY}, etc.) at each sampling moment. To calculate the number of bla_{TEM} genes to be sequenced, only the isolates positive solely for bla_{TEM} and no other ESBL/AmpC genes were taken into account. This was based on previous results that multiple ESBL/AmpC genes in one cell are rare in veal calves, and that the majority of bla_{TEM} genes that occur in veal-calf isolates in addition to any ESBL or AmpC genes are the narrow-spectrum $bla_{\text{TEM-1}}$ genes.[23] The calculations were based on one isolate per calf, unless in a sample from one animal different genes were found. All ESBL/AmpC genes in isolates selected for molecular characterization were sequenced using the kit described earlier and primers described previously[24] with additional primers for bla_{TEM} (TEM-Fseq: 5′-GCCAACTTA CTTCTGACAACG) and bla_{CMY} (CMY-F-838: 5′-TGGCGTATTGGCGATATGTA and CMY-R-857: 5′-TACATATCGCCAATACGCCA). Sequences were analysed using Sequencher v4.9 software (Gene Codes Corporation, USA).

Plasmid typing and E. coli multilocus sequence typing (MLST)

From the *E. coli* isolates selected for molecular characterization, plasmids carrying ESBL/AmpC were isolated and electroporated to competent Electromax DH10B cells (Invitrogen, USA). Isolates with ESBL/AmpC-carrying plasmids were pure-cultured on MC+ agar. In these transformants, the plasmids were identified by PCR-based replicon typing (PBRT) as described previously.[7,29,30] Plasmids showing a multiple replicon type were checked by PFGE, Southern blot and hybridization experiments, as described previously.[7] Isolates harbouring $bla_{\text{CTX-M-14}}$ on IncF plasmids were further analysed by IncF replicon sequence typing.[31] *E. coli* was characterized by MLST, as described by Wirth *et al.*,[32] and analysed using BioNumerics v6.6

software (Applied-Maths, Belgium). The sequences obtained were compared with sequences on the MLST reference web site.[33]

Susceptibility testing

All isolates included in the molecular characterization, and the corresponding transformants, were tested for antimicrobial susceptibility by broth microdilution according to ISO20776-1:2006, using microtitre trays with a custom-made dehydrated panel of antibiotics (Sensititre, Trek Diagnostic Systems, Basingstoke, UK). The following antibiotics were included: ampicillin, cefotaxime, ceftazidime, tetracycline, sulfamethoxazole, trimethoprim, ciprofloxacin, nalidixic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin and colistin. All results were interpreted using epidemiological cut-off values defined by EUCAST.[34] For sulfamethoxazole, the MIC breakpoint used was defined by the CLSI.[35]

Statistical analysis

A comparison of prevalence between farms was made by means of the GLIMMIX procedure in SAS (v9.2), accounting for clustering of data on pen and compartment level.

Results

Animal characteristics and antimicrobial usage

At T0, all calves were ∼2 weeks of age. The included calves on farms 1 and 3 were bulls, while those on farm 2 were heifers. On all farms the stables were cleaned but not disinfected before the calves entered the stable. Multiple antimicrobial group treatments (of all animals within the herd) were administered on all farms (Figure 1). Within the study period, most treatments were administered within the first 30 days after arrival on the farm. On all farms, oxytetracycline and colistin were orally administered upon arrival on the farm for the prophylaxis of bacterial diseases. On farms 1 and 3 these were administered for 7 days, and on farm 2 for 10 days. All further treatments differed in the type of antimicrobial and/or timing for each farm (Figure 1). Possible exposure to antibiotics before the study period was not known.

ESBL/AmpC carriage

The prevalence of animals positive for ESBL/AmpC-producing *E. coli* after arrival on the farms (T0) ranged from 18% to 26% (Figure 2). After T0, each farm showed a different pattern of prevalence. At farm 1, no animals harbouring ESBL/AmpC-producing *E. coli* were detected from weeks 3 to 10 (T3–T10). At farm 2 the prevalence first increased to 37% at T3 and then gradually decreased to 0% at T10. At farm 3, the prevalence gradually decreased after arrival on the farm to 1.4% at T10. In general, statistical analysis did not show significant differences in prevalence between farms.

Molecular characterization

The presence of different gene families varied greatly with time (Figure 2). At T0, all farms showed more or less the same distribution of mainly $bla_{\text{CTX-M}}$ genes, of which $bla_{\text{CTX-M}}$ group 1 was present in 70%–81% of all ESBL/AmpC-producing *E. coli*. At T3, by contrast, 96% of the *E. coli* isolates from farm 2 and 72% from farm 3 harboured $bla_{\text{CTX-M}}$ group 9 genes (Figure 2). These genes remained predominant until T10. On all farms, β-lactamase genes other than $bla_{\text{CTX-M}}$ were found only incidentally.

From all isolates, a selection was made for gene sequence analysis, plasmid identification and *E. coli* typing. From farm 1, 13 of 27 isolates were selected for gene sequence analysis, plasmid identification and *E. coli* typing. From farm 2, 36 of 107 were selected, and from farm 3, 31 of 69 were selected. At T0 at all farms, a variety of ESBL/ampC genes were detected in combination with plasmid replicon types and *E. coli* sequence types (STs). At farm 1, the $bla_{\text{CTX-M}}$ genes belonging to group 1 were $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$. $bla_{\text{CTX-M-1}}$ was found on IncI1, IncF and IncN plasmids, all in *E. coli* with different STs (Table 1). By contrast, $bla_{\text{CTX-M-15}}$ was found only on IncF plasmids. Two isolates harbouring $bla_{\text{CTX-M-15}}$ on IncF plasmids belonged to ST361, implicating a clonal relatedness. Furthermore, both parent strains and transformants of these two isolates showed non-wild-type susceptibility to the same antibiotics (Table 1). $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CMY-2}}$ were all located on IncI1-type plasmids in *E. coli* with different STs. $bla_{\text{CMY-2}}$ was detected in one isolate on farm 1 only.

At farm 2, the genes that belong to $bla_{\text{CTX-M}}$ group 1 found at T0 were $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-32}}$ (Table 2). Furthermore, at T0, $bla_{\text{CTX-M-2}}$ was found on various plasmids in *E. coli* with different STs. The predominant plasmid types were IncB/O (harbouring $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$) and IncF (harbouring $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-15}}$). IncI1 (carrying $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$) and IncHI2 (carrying $bla_{\text{CTX-M-2}}$) were also detected. One plasmid carrying $bla_{\text{CTX-M-32}}$ was non-typeable. Two Inc B/O+P multireplicon-type plasmids were found (Table 2). All genes were present in *E. coli* with different STs, except for two $bla_{\text{CTX-M-15}}$ isolates. These two genes were located on IncF plasmids in an *E. coli* ST410. Both transformants containing the $bla_{\text{CTX-M-15}}$ gene showed similar non-wild-type susceptibilities, while the donor isolates showed some differences (Table 2).

In farm 3, all isolates showed different gene, plasmid and *E. coli* combinations (Table 3). $bla_{\text{CTX-M-1}}$ was found on IncN, IncB/O, IncF and one non-typeable plasmid. $bla_{\text{CTX-M-2}}$ was located on an IncN plasmid and $bla_{\text{CTX-M-15}}$ was found on IncI1 and IncF plasmids. *E. coli* isolates that harboured mutations in the chromosomal *ampC* promoter region, with no plasmid-mediated ESBL/AmpC genes, were only observed at T0 (Tables 1 and 2). These isolates harbouring *ampC* promoter mutations were also positive for the narrow-spectrum β-lactamase $bla_{\text{TEM-1}}$.

In contrast to the diversity that was observed at T0 on all farms, farm 1 was negative for ESBL/AmpC-producing *E. coli* after T0, and farm 2 and farm 3 showed only little diversity at all sampling moments after T0. Despite the low diversity, a relatively high prevalence of $bla_{\text{CTX-M-14}}$ was found in both farm 2 and farm 3. This $bla_{\text{CTX-M-14}}$ gene belongs to the $bla_{\text{CTX-M}}$ group 9 shown in Figure 2, and was not detected at T0 on farm 2 and farm 3. It was the only group 9 gene variant detected in this study. On farm 2, all $bla_{\text{CTX-M-14}}$ genes were located on IncF plasmids. They were all identified in *E. coli* ST57 and located on indistinguishable F2:A-:B- plasmids. Furthermore, all isolates harbouring $bla_{\text{CTX-M-14}}$ on this IncF replicon ST showed non-wild-type susceptibility to seven classes of antimicrobials other than β-lactam antibiotics: quinolones (ciprofloxacin and nalidixic acid), phenicols (florfenicol and chloramphenicol), trimethoprim, sulphonamides (sulfamethoxazole), tetracycline, aminoglycosides (gentamicin, kanamycin and streptomycin) and polymyxins (colistin) (Table 2). However, the transformants of these isolates, harbouring only the

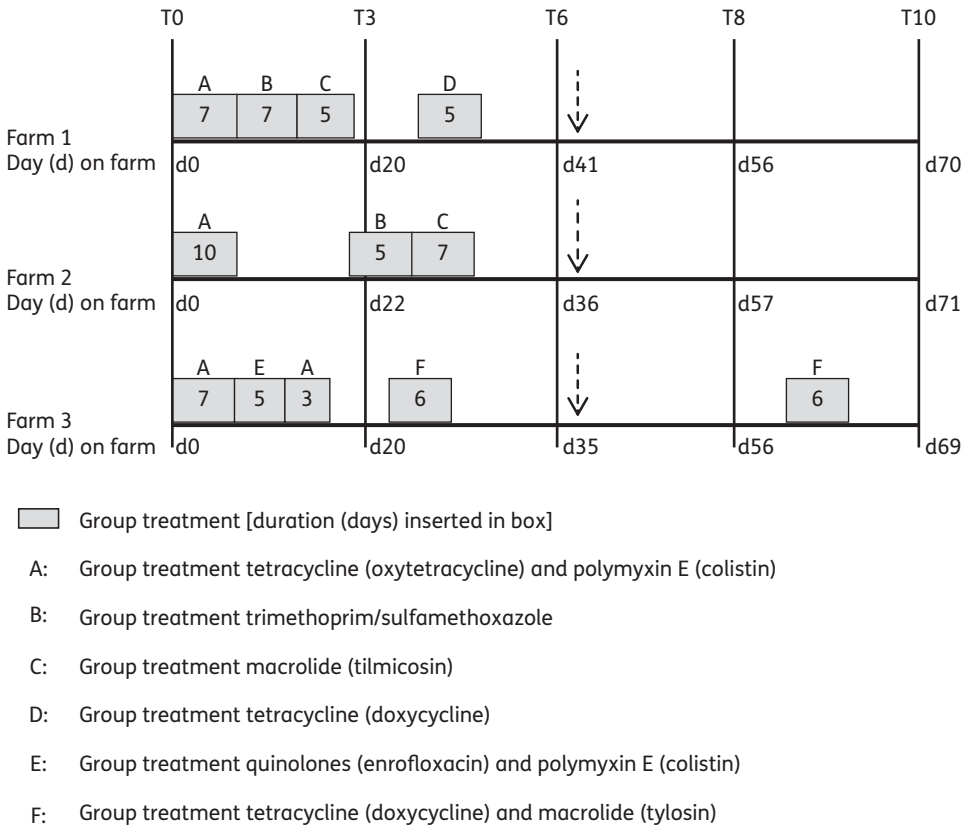

Figure 1. Sampling moments and antimicrobial treatments per farm. Broken arrows represent calves released from individual pens (1 day after T6). This figure was modified, with permission, from Graveland *et al.*[52] Group treatment [duration (days) inserted in box]: A, tetracycline (oxytetracycline) and polymyxin E (colistin); B, trimethoprim/sulfamethoxazole; C, macrolide (tilmicosin); D, tetracycline (doxycycline); E, quinolones (enrofloxacin) and polymyxin E (colistin); F, tetracycline (doxycycline) and macrolide (tylosin).

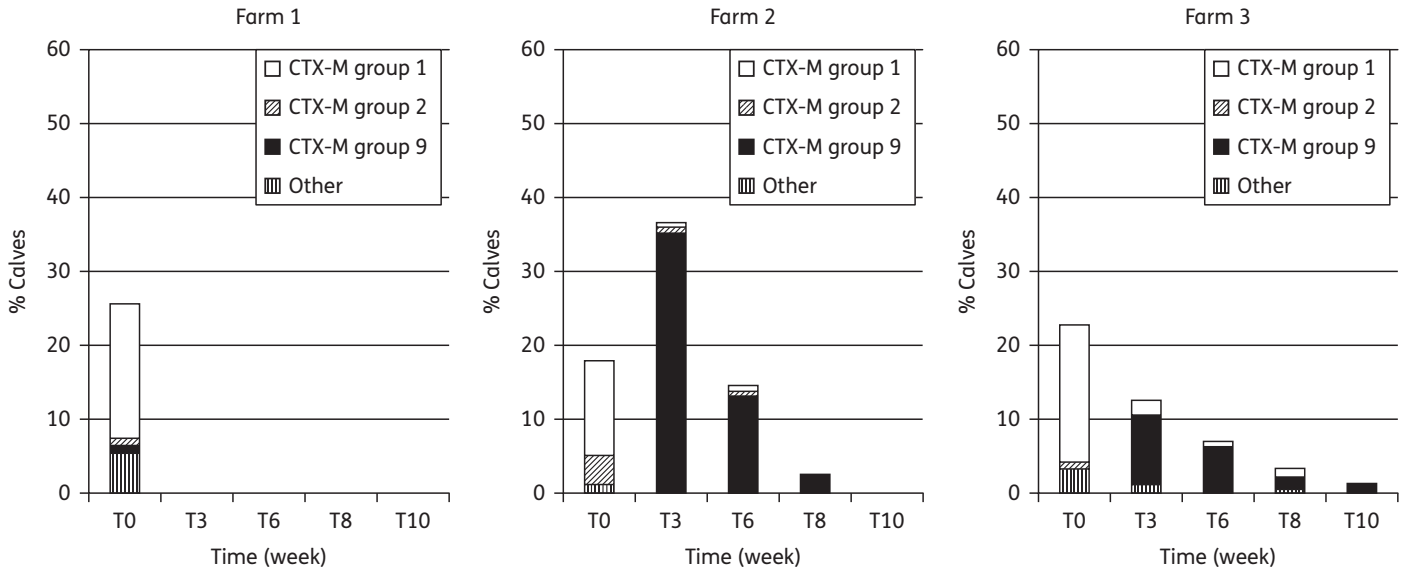

Figure 2. Prevalence and resistance genes of ESBL/AmpC-producing *E. coli*. The number of calves sampled on each farm was as follows: farm 1, $n=109$; farm 2, $n=150$; and farm 3, $n=140$.

Table 1. Molecular characterization of ESBL/AmpC-producing *E. coli* on farm 1

Week	Gene	*n*[a]	Plasmid	*E. coli* MLST		Non-wild-type susceptibility[b]
				ST	CC	
T0	$bla_{CTX-M-1}$	1	Frep	new[c]	—	**AMP-CTX-CAZ**-CHL-SMX-TET-**GEN**-KAN
		1	I1	88	23	**AMP-CTX-CAZ**-CIP-NAL-TMP-SMX-TET-KAN
		1	N	744	—	**AMP-CTX-CAZ**-CIP-NAL-TMP-SMX-TET-KAN-STR
		1	N	973	—	**AMP-CTX-CAZ**-SMX-TET-GEN-KAN-STR
	$bla_{CTX-M-2/97}$[d]	1	I1	new[c]	—	**AMP-CTX-CAZ**-TMP-SMX
	$bla_{CTX-M-14}$	1	I1	952	—	**AMP-CTX-CAZ**-CIP-NAL-**CHL-FFN-TMP-SMX-TET**-STR
	$bla_{CTX-M-15}$	1	FIA	648	—	**AMP-CTX-CAZ**-CIP-NAL-**TMP-SMX-TET-GEN-KAN**-STR
		2	Frep; FIA	361	—	**AMP-CTX-CAZ**-CIP-NAL-**CHL**-TMP-SMX-**TET-GEN-KAN**-STR
	bla_{CMY-2}	1	I1	1480	—	**AMP-CTX-CAZ**-NAL-FFN-TMP-SMX-TET-KAN-STR
	ampC-4[d]	1	—	973	—	AMP-CTX-CHL-FFN-TMP-SMX-TET-KAN-STR
	ampC-18[d]	1	—	58	155	AMP-CTX-CIP-NAL-CHL-FFN-TMP-SMX-TET-STR
	ampC-3[d]	1	—	58	155	AMP-CTX-CAZ

CC, clonal complex; FFN, florfenicol; SMX, sulfamethoxazole.
[a]Number of isolates from the selection for molecular analysis.
[b]Non-wild-type susceptibility was detected for all antibiotics listed. Antibiotics in bold indicate those to which the corresponding transformants showed non-wild-type susceptibility. EUCAST epidemiological cut-off values were used.
[c]This ST was not listed on http://mlst.ucc.ie/.
[d]Chromosomal *ampC* type, as previously described.[28] This isolate also harboured bla_{TEM-1}.

$bla_{CTX-M-14}$-carrying IncF plasmid, showed only non-wild-type susceptibility to β-lactam antibiotics, indicating that no other resistance genes were located on this plasmid. On farm 3, $bla_{CTX-M-14}$ was associated with two different *E. coli* STs. The first was identical to the one found on farm 2, a combination of $bla_{CTX-M-14}$ on an F2:A-:B- plasmid in an *E. coli* ST57, with non-wild-type susceptibility to the same antibiotics (Table 3). The second was $bla_{CTX-M-14}$ on an IncK plasmid in an *E. coli* ST10, also showing non-wild-type susceptibility to the same antibiotics as shown for the ST57 isolates (Table 3). Also, the transformants only harbouring $bla_{CTX-M-14}$ on IncK plasmids showed only non-wild-type susceptibility to β-lactam antibiotics.

Finally, a low prevalence of $bla_{CTX-M-1}$ was detected on farm 2 and farm 3 at T3 and T6 (Tables 2 and 3). On farm 3, a low prevalence of $bla_{CTX-M-15}$ was detected at T6 and T8, and of $bla_{TEM-52c}$ at T3 and T8 (Table 3). Some of these low-prevalence genes at farm 3 were clonally related and showed a similar combination of ESBL gene with plasmid and *E. coli* ST. These clonal variants were not detected at T0.

Discussion

This study showed that all three farms had a comparable prevalence of calves positive upon arrival (T0) for ESBL/AmpC-producing *E. coli* of ∼20% (Figure 2). In general, Dutch veal calves are collected from many different dairy farms. Approximately 50% are derived from domestic dairy farms and 50% from other European countries. These animals are brought together in collection and distribution centres and are subsequently transported to fattening farms, which were investigated in this study. This structure of the veal-calf industry might explain the diversity in resistance genes in association with different plasmids and *E. coli* found at T0 on all farms. After T0, all farms showed a different pattern of prevalence. Farm 1 became negative, while farm 2 and farm 3 differed in prevalence at T3. Despite the different patterns, clonally related ESBL/AmpC-carrying *E. coli* STs were observed on two of the three farms at T3 and later. Until T6, all animals were housed individually, indicating a minimal movement of animals within the stable. Moreover, no clustering of positive animals was observed within the stables. These clonal variants were not observed at T0, which suggests a source on the farm. These clonal variants may have been introduced in the past, because the stables were cleaned between production rounds but not thoroughly disinfected. Simultaneous introduction of these clones in the veal-calf farms through the young calves is less likely, since all animals originated from different Dutch dairy farms.

The data provided in this study showed that $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ genes were most abundant at the beginning of the production cycle. Previous data showed that these genes were also most prevalent at the end of the production cycle, at slaughter.[36] The data obtained at slaughter were obtained in a cross-sectional study among 100 herds. We therefore have no information on when the animals were colonized with ESBL/AmpC-producing *E. coli*, or the persistence of ESBL/AmpC carriage in the animals included in that study. The present study included only three farms, which limits the possibility of extrapolating these data to the entire sector. Further study is therefore required to assess the persistence of ESBL/AmpC-producing *E. coli* in veal calves, and to assess whether recolonization occurs within a production cycle or subsequent production cycles.

On farm 2 and farm 3, at T3 and subsequent sampling moments, the majority of ESBL/AmpC-producing *E. coli* harboured $bla_{CTX-M-14}$ (Tables 2 and 3). In these isolates, the combination of the $bla_{CTX-M-14}$ gene, located on an IncF F2:A-:B- plasmid, in *E. coli* ST57, and a similar pattern of non-wild-type susceptibility for the whole panel of antimicrobials tested, clearly indicates a clonal

Table 2. Molecular characterization of ESBL/AmpC-producing *E. coli* on farm 2

Week	Gene	*n*[a]	Plasmid	*E. coli* MLST		Non-wild-type susceptibility[b]
				ST	CC	
T0	$bla_{CTX\text{-}M\text{-}1}$	1	B/O	117	—	**AMP-CTX-CAZ**-TMP-SMX-TET-GEN-KAN-STR
		1	B/O	88	23	**AMP-CTX-CAZ**-TMP-TET-KAN-CST
		1	I1	58	155	**AMP-CTX-CAZ**-TMP-**SMX**
		1	B/O; P	10	10	**AMP-CTX-CAZ-TET**
	$bla^{c}_{CTX\text{-}M\text{-}2/97}$	1	Frep	362	—	**AMP-CTX-CAZ**-CHL-TMP-SMX-TET-KAN-STR
		1	Frep	69	69	**AMP-CTX-CAZ**-CIP-NAL-CHL-TMP-SMX-TET-GEN-KAN-STR
		1	HI2	34	10	**AMP-CTX-CAZ**-CHL-**TMP-SMX-TET**-STR
	$bla_{CTX\text{-}M\text{-}15}$	1	I1	58	155	**AMP-CTX-CAZ**-TMP-SMX-TET-KAN-STR
		1	Frep; FIA	410	23	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-**TET-KAN**
		1	Frep; FIA	410	23	**AMP-CTX-CAZ**-CIP-NAL-**TET-KAN**
	$bla_{CTX\text{-}M\text{-}32}$	1	NT[d]	224	—	**AMP-CTX-CAZ**-CIP-NAL-**CHL-TMP-SMX-TET-KAN**-STR
	$bla_{CTX\text{-}M\text{-}32}$	1	B/O; P	10	10	**AMP-CTX-CAZ-TET**
	ampC-3[e]	1	—	448	448	AMP-CTX-CAZ-CIP-NAL-SMX-TET-STR
T3	$bla_{CTX\text{-}M\text{-}1}$	1	B/O	648	—	**AMP-CTX-CAZ**-TMP-SMX-TET-GEN-KAN-STR-CST
		1	Frep	648	—	**AMP-CTX-CAZ**-TMP-SMX-TET-GEN-KAN-STR-CST
	$bla_{CTX\text{-}M\text{-}14}$	9	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-GEN-KAN-STR-CST
T6	$bla_{CTX\text{-}M\text{-}1}$	1	B/O	162	469	**AMP-CTX-CAZ**-CIP-NAL-CHL-TMP-SMX-TET-KAN-STR
	$bla_{CTX\text{-}M\text{-}14}$	7	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-GEN-KAN-STR-CST
T8	$bla_{CTX\text{-}M\text{-}14}$	4	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-GEN-KAN-STR-CST

CC, clonal complex; FFN, florfenicol; SMX, sulfamethoxazole.
[a]Number of isolates from the selection for molecular analysis.
[b]Non-wild-type susceptibility was detected for all antibiotics listed. Antibiotics in bold indicate those to which the corresponding transformants showed non-wild-type susceptibility. EUCAST epidemiological cut-off values were used.
[c]Based on the primers used in this study, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}97}$ cannot be distinguished.
[d]This plasmid was not typeable by PBRT.
[e]Chromosomal *ampC* type, as previously described.[28] This isolate also harboured $bla_{TEM\text{-}1}$.

spread on these veal-calf production farms (Tables 2 and 3). The combination of $bla_{CTX\text{-}M\text{-}14}$ on this specific IncF plasmid has been reported before, in China and Korea, in *E. coli* of animal origin (including cattle) as well as in human faecal samples, human urinary tract infections and human blood culture samples.[37–39] In the USA, $bla_{CTX\text{-}M\text{-}14}$ has also been associated with IncF plasmids isolated from dairy cattle, showing a clonal spread within herds, based on PFGE analysis.[40] The $bla_{CTX\text{-}M\text{-}14}$-harbouring variant on farm 3, located on an IncK plasmid in *E. coli* ST10 (Table 3), also showed non-wild-type susceptibility to the whole panel of antimicrobials tested, similar to the IncF variant. In the UK, $bla_{CTX\text{-}M\text{-}14}$ genes were also found on IncK plasmids in a longitudinal field study on a dairy farm and in isolates obtained from turkeys and humans.[16,41] Furthermore, in *E. coli* isolates of human origin in Spain, the spread of $bla_{CTX\text{-}M\text{-}14}$ was mainly driven by IncK plasmids.[42,43] Interestingly, in Spain, $bla_{CTX\text{-}M\text{-}14}$ was not only associated with IncK plasmids but also with *E. coli* ST10,[43,44] as we found in our study. Apparently, the combination of $bla_{CTX\text{-}M\text{-}14}$ on IncK plasmids is widespread in Europe among both humans and animals, and was found in various *E. coli* STs, of which ST10 was reported frequently. The uropathogenic *E. coli* ST131, reported worldwide and commonly associated with $bla_{CTX\text{-}M\text{-}15}$ but also $bla_{CTX\text{-}M\text{-}14}$, was not found in our study.

All isolates selected for molecular characterization were also screened for susceptibility to eight classes of antimicrobials. At T0, 60% or less of the transformants harbouring an ESBL-carrying plasmid also showed non-wild-type susceptibility for antimicrobials other than β-lactams. This indicates that these resistance determinants were likely to be located on the same plasmid.

At T0, the isolates carrying $bla_{CTX\text{-}M\text{-}14}$ were not detected on farms 2 and 3, indicating that either they were present below the detection level or were present in the stable and colonized the calves after the sampling moment at T0. A possible explanation for the sudden shift in the most prevalent ESBL genes found between T0 and T3, and later at farm 2 and farm 3, might be the group treatments with oxytetracycline and colistin upon arrival at the farm (Figure 1), since both clonal variants had reduced susceptibility to both antimicrobials. However, since this is an observational study, this cannot be confirmed. In addition, this effect was not observed at farm 1, where the animals received the same treatment upon arrival. At T3, the calves were still housed in individual

Table 3. Molecular characterization of ESBL/AmpC-producing *E. coli* on farm 3

Week	Gene	*n*[a]	Plasmid	*E. coli* MLST		Non-wild-type susceptibility[b]
				ST	CC	
T0	$bla_{CTX\text{-}M\text{-}1}$	1	B/O	117	—	**AMP-CTX-CAZ**-CIP-NAL-SMX-TET-KAN-STR
		1	NT[c]	224	—	**AMP-CTX-CAZ**-CIP-NAL-**CHL-TMP-SMX-TET**-STR
		1	N	617	10	**AMP-CTX-CAZ**-CIP-NAL-SMX-KAN-STR
		1	B/O	117	—	**AMP-CTX-CAZ**-CHL-FFNTMP-SMX-TET-KAN-STR
		1	N	88	23	**AMP-CTX-CAZ**-SMX-TET-STR
		1	Frep	448	448	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-**TMP**-SMX-TET-GEN-KAN-STR
	$bla_{CTX\text{-}M\text{-}2/97}$[d]	1	N	1291	—	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-GEN-KAN-STR
	$bla_{CTX\text{-}M\text{-}15}$	1	I1	88	23	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-CST-STR
		1	Frep	410	23	**AMP-CTX-CAZ**-CIP-NAL-**TMP-SMX-TET-KAN**
T3	$bla_{CTX\text{-}M\text{-}1}$	1	N	354	354	**AMP-CTX-CAZ**-CIP-NAL-TMP-SMX-TET-CST-STR
		1	Frep	117	—	**AMP-CTX-CAZ**-CIP-NAL-CHL-TMP-SMX-TET-CST-GEN-KAN-STR
	$bla_{CTX\text{-}M\text{-}14}$	3	K	10	10	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
		2	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
	$bla_{TEM\text{-}52c}$	2	I1	1011	—	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
T6	$bla_{CTX\text{-}M\text{-}1}$	1	N	354	354	**AMP-CTX-CAZ**-CIP-NAL-TMP-SMX-TET-CST-STR
	$bla_{CTX\text{-}M\text{-}14}$	1	K	10	10	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
		3	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
	$bla_{CTX\text{-}M\text{-}15}$	1	I1	59	59	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-STR
T8	$bla_{CTX\text{-}M\text{-}14}$	2	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
	$bla_{CTX\text{-}M\text{-}15}$	1	I1	59	59	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-STR
	$bla_{TEM\text{-}52c}$	1	I1	1011	—	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR
T10	$bla_{CTX\text{-}M\text{-}14}$	2	F2:A-:B-	57	350	**AMP-CTX-CAZ**-CIP-NAL-CHL-FFN-TMP-SMX-TET-CST-GEN-KAN-STR

CC, clonal complex; FFN, florfenicol; SMX, sulfamethoxazole.
[a]Number of isolates from the selection for molecular analysis.
[b]Non-wild-type susceptibility was detected for all antibiotics listed. Antibiotics in bold indicate those to which the corresponding transformants showed non-wild-type susceptibility. EUCAST epidemiological cut-off values were used.
[c]This plasmid was not typeable with PBRT.
[d]Based on the primers used in this study, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}97}$ cannot be distinguished.

boxes and the isolates carrying $bla_{CTX\text{-}M\text{-}14}$ were distributed all over the stable. The prevalence decreased on both farms after T3, when more group treatments were administered using antibiotics to which these $bla_{CTX\text{-}M\text{-}14}$-carrying isolates possessed non-wild-type susceptibility (Figure 1 and Tables 2 and 3). If the group treatments with oxytetracycline and/or colistin were the driving force behind this increase of isolates carrying $bla_{CTX\text{-}M\text{-}14}$ at T3, a second increase was to be expected after the subsequent treatments.

Several studies at dairy farms have also shown there is an inverse relationship between the prevalence of antibiotic-resistant bacteria in the gut flora and animal age in young calves, with or without the selection pressure of antimicrobial treatment.[20,22,45] All studies suggest that a 'higher fitness' of the resistant strains caused this effect. Another factor that may contribute to selection pressure is waste milk that may contain antimicrobial residues being fed to calves. Waste milk is unfit for human consumption, and comprises colostrum, milk from cows with mastitis or milk from cows treated with antimicrobial or non-antimicrobial medicines that may leave a residue in the milk.[46] A survey in England and Wales on antimicrobial usage and waste-milk feeding practices on dairy farms showed that on 83% of the included farms, waste milk was fed to calves.[46] Cefquinome and ceftiofur were among the most commonly used antimicrobials on the farms included in the survey. A recent study in England and Wales confirmed the relationship between the usage of waste milk with cefquinome and the occurrence of CTX-M-producing *E. coli* in dairy calves.[47]

Further study is needed to fully understand what causes this increase and the subsequent decrease in the prevalence of these multiresistant strains shown in the present study. Furthermore, it is noteworthy that from the three farms that were studied in this paper, two (unrelated) farms were positive for *E. coli* with a non-wild-type susceptibility to colistin. Reports on colistin resistance in *E. coli* are scarce.[48–51] Studies that performed additional susceptibility testing showed that the vast majority of the colistin-resistant isolates were multiresistant,[49,51] as was shown in this study.

In summary, this study showed that the within-farm prevalence of calves positive for *E. coli* producing different ESBL/AmpC genes may vary greatly over a relatively short time span, and under antimicrobial pressure even decrease to undetectable levels. The changes in prevalence during the first 6 weeks occurred while the

animals were housed in individual pens, limiting contact between animals, indicating other driving forces may influence the spread and (re)colonization of ESBL/AmpC-carrying *E. coli*. The fact that clonally related multiresistant $bla_{CTX\text{-}M\text{-}14}$-producing *E. coli* variants were found on two unrelated farms suggests circulation in the veal-calf production system, possibly introduced to the farms in the past. These isolates were similar to those previously described in both animals and humans.

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Transparency declarations

None to declare.

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