J. Dairy Sci. 106:4257–4265
https://doi.org/10.3168/jds.2022-22362

Prevalence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in young calves on Dutch dairy farms

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ABSTRACT

In young calves on dairy farms the animal prevalence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* (ESBL/AmpC-EC) is significantly higher compared with the animal prevalence in young stock and dairy cows. Hitherto it was unknown at what age antimicrobial resistant bacteria appear for the first time in the gut of calves on dairy farms, and how long these infections persist. The aim of this study was to examine the prevalence of ESBL/AmpC-EC, the number of excreted ESBL/AmpC-EC (in cfu/g of feces), as well as the ESBL/AmpC genotypes in young dairy calves (0–21 d of age) and the variation of these parameters between calves of different ages. Next to this, the course of shedding ESBL/AmpC-EC during the first year in dairy calves was studied. In a cross-sectional study, fecal samples from 748 calves, from 0 to 88 d of age, on 188 Dutch dairy farms were collected. The prevalence of calves testing positive for ESBL/AmpC-EC in a phenotypic assay was determined for different age categories (per 2 d of age). Positive samples were subjected to a semiquantitative test to determine the numbers of ESBL/AmpC-EC per gram of feces and for a selection of ESBL/AmpC-EC isolates the ESBL/AmpC genotype was determined. Ten of the 188 farms were selected for a longitudinal study based on the presence of at least 1 female calf with ESBL/Amp-EC in the cross-sectional study. These farms were additionally visited 3 times with a 4-mo interval. All calves that were sampled in the cross-sectional study were, if still present, resampled during the follow-up visits. Results show that from the day of birth ESBL/AmpC-EC can be present in the gut of calves. The phenotypic prevalence of ESBL/AmpC-EC was 33.3% in 0- to 21-d-old calves and 28.4% in 22- to 88-d-old calves. The prevalence of ESBL/AmpC-EC positive calves varied per age category among calves up to 21 d of age: significant increases and decreases at an early age were shown. Results of the longitudinal study show that after 4, 8, and 12 mo the prevalence of ESBL/AmpC-EC positive calves dropped to 3.8% (2/53), 5.8% (3/52), and 2.0% (1/49), respectively. This indicates that early gut colonization in young calves with ESBL/AmpC-EC is transient and does not lead to long-term shedding of these bacteria.

Key words: calves, antimicrobial resistance, ESBL

INTRODUCTION

The emergence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* (**ESBL/AmpC-EC**) in the last decades has been of great concern for both human and animal health organizations (WHO, 2011). These bacteria pose the ability to break down β-lactam antibiotics, resulting in resistance to third- and fourth-generation extended-spectrum cephalosporins and penicillins, which are classified by the World Health Organization as the highest priority critically important antimicrobials for human medicine. In fact, for serious *Salmonella* and *E. coli* infections these antimicrobials are one of few available treatment options in humans (WHO, 2018). The ESBL/AmpC-EC are present in humans, the environment, and (food-producing) animals, including cattle (Korzeniewska et al., 2013; Wu et al., 2013; EFSA, 2014; Laube et al., 2014; von Salviati et al., 2015; Gonggrijp et al., 2016; Hordijk et al., 2019).

In 2013, the animal prevalence of ESBL/AmpC-EC among dairy cattle, with a minimum shedding level of 10^3 cfu/g of feces, in different age categories in dairy herds was established and showed a significant higher

Received June 9, 2022.
Accepted December 9, 2022.
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animal prevalence in calves (19.3%) compared with young stock (0.9%) and dairy cows (0.8%) (Heuvelink et al., 2019). Moreover, the level of shedding ESBL/AmpC-EC in feces (cfu/g) was also higher in calves compared with older cattle. Earlier studies described the prevalence and dynamics of ESBL/AmpC-EC in dairy and veal calves. Results of these studies show that the prevalence of calves shedding ESBL/AmpC-EC is high during the first weeks of their lives and decreases with increasing age. The colonization of the gut of young calves with antimicrobial resistant *E. coli* appears to be transient (Hoyle et al., 2004; Hordijk et al., 2013a; Bastard et al., 2021; Homeier-Bachmann et al., 2022). The high prevalence of calves shedding ESBL/AmpC-EC indicates that calves could play an important role in the dynamics of ESBL/AmpC-EC on dairy farms. However, only limited information is available about the colonization of these antibiotic resistant bacteria in the gut of young calves at a day-to-day level during the first weeks of their lives, raising the questions about the age at which colonization occurs and how long this colonization persists in the gut.

The aim of this study was to examine the prevalence of ESBL/AmpC-EC, the number of excreted ESBL/AmpC-EC (in cfu/g of feces), as well as the ESBL/AmpC genotypes in young dairy calves (0–21 d of age) and the variation of these parameters between calves of different ages. Next to this, the course of shedding ESBL/AmpC-EC during the first year in dairy calves was studied.

MATERIALS AND METHODS

From October until December 2013, a cross-sectional study was performed that was followed by a longitudinal study on a subset of the herds in the cross-sectional study. The cross-sectional study was part of a larger study that was described earlier by Heuvelink et al. (2019). Calves sampled in this study were housed on Dutch nonorganic dairy farms. During sampling the calves were restrained as little as possible by the farmer to limit the amount of possible discomfort.

Cross-Sectional Study

In the cross-sectional study, 1,000 randomly selected nonorganic dairy farmers were asked by letter to submit fecal samples from all calves in the age category 0 to 21 d present in their herd. Of these, 188 farmers submitted 748 fecal samples of calves, who were 0 to 88 d old. Samples were collected through rectal palpation by the private veterinary practitioner. The samples were transported to the laboratory at refrigerator temperature (2–8°C) where bacteriological examination started within 24 h after collection of the samples.

Longitudinal Study

Ten herds were randomly selected from 33 herds with an ESBL/AmpC-EC positive fecal sample from at least 1 female calf and with at least 5 female calves being sampled in the cross-sectional study, and included in the longitudinal study. These 10 herds were visited by the private veterinary practitioner every 4 mo for a period of 1 yr, leading to 3 samplings in addition to the first sampling. Calves present on these 10 herds were sampled during the cross-sectional study (first sampling) and resampled 4 (second sampling), 8 (third sampling), and 12 mo later (fourth sampling) and are referred to as resampled calves. When the number of resampled calves dropped below 5 at the second or third sampling of the longitudinal study, due to early removal of calves from the farm, new calves between 0 and 21 d of age were sampled. These calves are referred to as additional calves, and they were also sampled at the subsequent samplings.

Laboratory Diagnostics

In the cross-sectional as well as the longitudinal study, all fecal samples were individually examined. On all of these samples a phenotypic assay was performed, followed by a semiquantitative assay on samples testing positive in the phenotypic assay and with sufficient material left. After the semiquantitative assay, a random selection of the ESBL/AmpC-EC positive samples was generated by statistical software (STATA 15.0; StataCorp, 2017) and subsequently screened for ESBL/pAmpC genotypes (Figure 1). The phenotypic assay, semiquantitative assay, and genotypic assay are described in brief below and in detail by Heuvelink et al. (2019).

Phenotypic Assay: Isolation of ESBL/AmpC-EC. Fecal samples were streaked either directly or after selective enrichment in Luria-Bertani broth (Becton Dickinson) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich), onto MacConkey agar (Oxoid Ltd.) supplemented with 1 mg/L cefotaxime. After overnight aerobic incubation at 37°C, presumptive *E. coli* colonies were selected for confirmation by using the MALDI-Biotyper (Bruker Daltonics GmbH). Combination disc diffusion tests using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson) were used to examine the confirmed *E. coli* isolates for ESBL or AmpC production, according to Clinical and Labo-

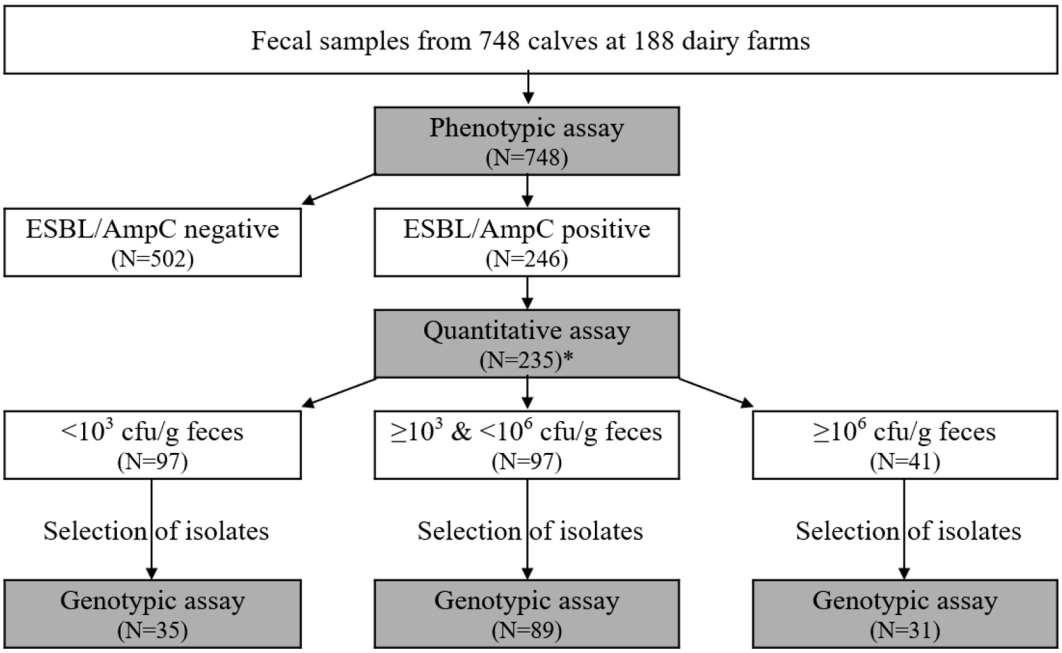

Figure 1. Overview of the laboratory assays applied on the fecal samples. *Eleven samples were not tested in the quantitative assay due to an insufficient amount of feces. ESBL/AmpC-EC = extended-spectrum and AmpC β-lactamase-producing *Escherichia coli*.

ratory Standards Institute guidelines (CLSI, 2011). To detect AmpC phenotypes, a cefoxitin disc (30 µg, Becton Dickinson) was included in the test. *Escherichia coli* isolates from the longitudinal study were not examined for differentiation between ESBL and AmpC production using the disc diffusion test, due to limited resources.

Quantitative Assay: Quantification of ESBL/AmpC-EC. All ESBL/AmpC-EC positive samples were subjected to a (semi)quantitative determination of ESBL/AmpC-EC and total *E. coli*, using the track-dilution technique (Jett et al., 1997). The number of ESBL/AmpC-EC and total *E. coli* were calculated based on the highest 10-fold dilution with typical *E. coli* colonies. To distinguish between calves shedding ESBL/AmpC-EC due to amplification of these bacteria during passage through the gut, calves with ESBL/AmpC-EC colonization in the gut and calves shedding extremely high numbers of ESBL/AmpC-EC due to colonization in the gut, the samples were categorized as containing low ($<10^3$ cfu/g feces), moderate (10^3 to 10^5 cfu/g feces), and high ($\geq 10^6$ cfu/g feces) numbers of ESBL/AmpC-EC and *E. coli* isolates (Chase-Topping et al., 2008).

Genotypic Assay: Identification of ESBL/AmpC Genes. Per ESBL/AmpC-pos calf, 1 to 3 isolates were screened for the presence of different β-lactamase genes and plasmid-mediated AmpC genes (pAmpC) by using the *E. coli* Genotyping Combined microarray (Alere Technologies GmbH). Further identification of the ESBL/AmpC gene types was performed by PCR amplification followed by sequencing using different primers, as described previously (Gonggrijp et al., 2016).

Statistical Analysis

All statistical analyses were carried out in STATA 15.0 (StataCorp, 2017). *P*-values ≤ 0.05 were considered significant and $0.05 < P\text{-values} \leq 0.1$ were considered tendencies.

Cross-Sectional Study. The data of the qualitative and quantitative assays of the cross-sectional study were combined in one data set. Birthdates of calves were added to the data set to classify the calves in the following age categories: 0 to 4 d, 5 to 9 d, 10 to 14 d, 15 to 20 d, and >20 d. Descriptive statistics were used to determine the prevalence of ESBL/AmpC-EC positive calves, ESBL-EC positive calves, and AmpC-EC positive calves, in total and per age category. The variable containing the number of excreted ESBL/AmpC-EC (in cfu/g of feces) was transformed to 3 binary variables containing the prevalence of calves shedding low, moderate, or high levels of ESBL/AmpC-EC (as described above). These prevalences and the identification of ESBL/pAmpC genes were examined per age category of calves using descriptive statistics.

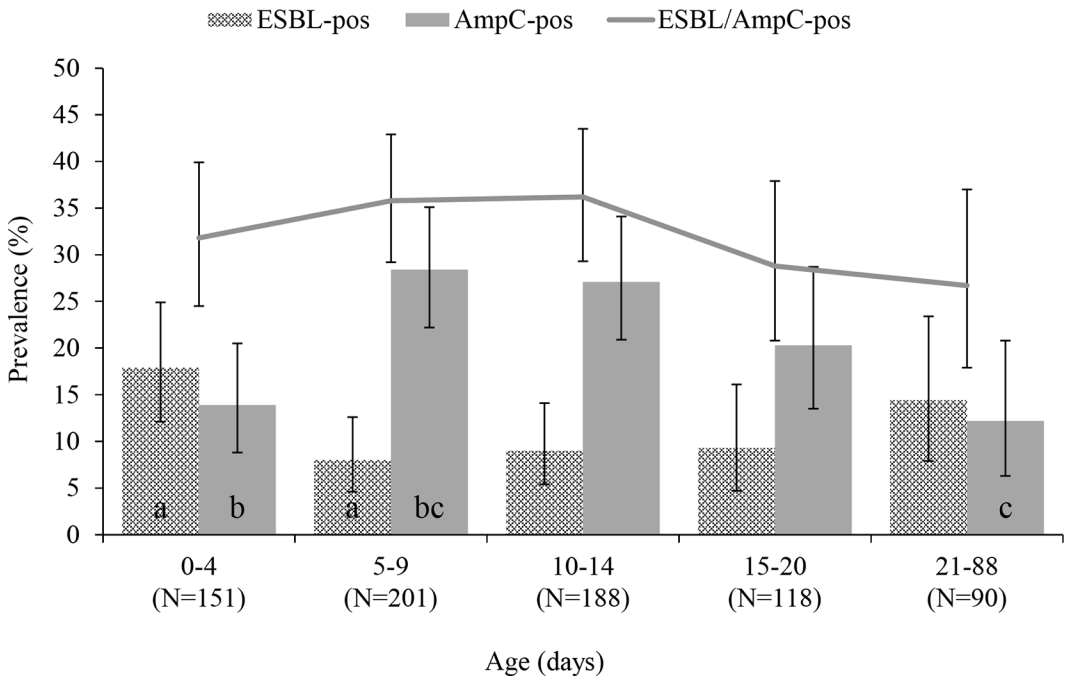

Figure 2. Prevalences (and corresponding 95% CI) of calves testing positive for extended-spectrum or AmpC β-lactamase-producing *Escherichia coli* (or both) in the phenotypic assay (ESBL-pos, AmpC-pos, and ESBL/AmpC-pos, respectively). (a) Significant differences in the prevalence of ESBL-pos calves ($P < 0.02$). (b, c) Significant differences in the prevalence of AmpC-pos calves ($P < 0.01$ and $P < 0.02$, respectively).

To determine if the prevalence of ESBL/AmpC-EC positive calves differed between age categories, multilevel mixed-effects logistic regression analyses were conducted (Stata command: melogit). Data were analyzed on animal level taking herd of origin into account as a random effect. In this way, the pairwise comparisons between age categories were corrected for possible correlations in the prevalence of ESBL/AmpC-EC positive calves between calves originating from the same herd. The same models were used to determine if the prevalence of ESBL-EC positive calves, AmpC-EC positive calves, and levels of shedding ESBL/AmpC-EC differed between age categories. Differences between age categories in the carriage of ESBL and AmpC genes in *E. coli* isolates from calves were studied using proportion tests (Stata command: prtesti). All of the results of pairwise comparisons between age categories were corrected for the use of multiple comparisons according to Bonferroni's method [Stata command: pwcompare, mcompare(bonferroni)].

Longitudinal Study. To describe the results of the phenotypic and genotypic assays of the samples of calves from the longitudinal study, only descriptive statistics were used. The results of all included calves were analyzed and at each moment in time (age <22 d, at 4, 8, or 12 mo of age) it was determined whether a calf shed ESBL/AmpC-EC and, if so, which genes were identified. These results could give an indication of the duration of shedding ESBL/AmpC-EC with a certain genotype and the possible selection of ESBL/AmpC-EC with another genotype in time. Due to the limited number of calves that were included in the longitudinal study, no regression analyses were conducted on these data.

RESULTS

Cross-Sectional Study

Prevalence and Age Distribution of ESBL/AmpC-EC Positive Calves. A total of 748 calves originating from 188 farms were sampled: median 3 individual calves per herd (mean: 4, minimum: 1, maximum: 15 calves). The median age of all calves was 10 d (mean: 11, minimum: 0, maximum: 88 d). Seven calves were sampled on their day of birth and 651 calves were between 1 and 20 d of age. Per age category a median of 72 calves were sampled (mean: 66, minimum: 39, maximum: 85 calves). The remaining 90 calves were between 21 and 88 d of age (Figure 2). This group of older calves, which was sampled despite the protocol, were included in the study but not further divided by age.

The prevalences of calves testing positive for ESBL-EC (**ESBL-pos**) and calves testing positive for AmpC-

Table 1. Results of the multivariable multilevel logistic regression model of the association between calves testing positive for extended-spectrum β-lactamase-producing *Escherichia coli* in the phenotypic assay (ESBL-pos) and age of the calves[1]

	ESBL-pos		
Parameter	OR	95% CI	*P*-value
Age category (d)			
5–9 vs. 0–4 (referent)	0.15	0.03–0.80	0.014
10–14 vs. 0–4 (referent)	0.30	0.07–1.35	0.244
15–20 vs. 0–4 (referent)	0.38	0.07–2.12	1.000
21–88 vs. 0–4 (referent)	0.67	0.11–4.19	1.000
10–14 vs. 5–9 (referent)	1.95	0.39–9.65	1.000
15–20 vs. 5–9 (referent)	2.50	0.37–16.85	1.000
21–88 vs. 5–9 (referent)	4.41	0.56–34.84	0.441
15–20 vs. 10–14 (referent)	1.29	0.21–7.83	1.000
21–88 vs. 10–14 (referent)	2.26	0.33–15.57	1.000
21–88 vs. 15–20 (referent)	1.76	0.22–14.09	1.000
Cluster herd	21.12	8.15–54.71	<0.001

[1]Odds ratios (OR), 95% CI, and the *P*-values of the comparisons between age categories are corrected according to Bonferroni's method.

Table 2. Results of the multivariable multilevel logistic regression models of the association between calves testing positive for AmpC-producing *Escherichia coli* in the phenotypic assay (AmpC-pos) and age of the calves[1]

	AmpC-pos		
Parameter	OR	95% CI	*P*-value
Age category (d)			
5–9 vs. 0–4 (referent)	4.80	1.35–17.06	0.005
10–14 vs. 0–4 (referent)	2.57	0.73–9.05	0.355
15–20 vs. 0–4 (referent)	2.11	0.48–9.28	1.000
21–88 vs. 0–4 (referent)	0.63	0.10–3.94	1.000
10–14 vs. 5–9 (referent)	0.54	0.19–1.54	0.975
15–20 vs. 5–9 (referent)	0.44	0.12–1.58	0.719
21–88 vs. 5–9 (referent)	0.13	0.02–0.76	0.011
15–20 vs. 10–14 (referent)	0.82	0.22–3.09	1.000
21–88 vs. 10–14 (referent)	0.25	0.04–1.40	0.237
21–88 vs. 15–20 (referent)	0.30	0.05–1.97	0.727
Cluster herd	10.85	5.72–20.58	<0.001

[1]Odds ratios (OR), 95% CI, and the *P*-values of the comparisons between age categories are corrected according to Bonferroni's method.

EC (**AmpC-pos**) in the phenotypic assay were established next to a combination of these 2 prevalences: the prevalence of calves testing positive for ESBL- or AmpC-EC (or both) in the phenotypic assay (**ESBL/AmpC-pos**). This last prevalence (ESBL/AmpC-pos) was 32.9% (95% CI: 29.5–36.4%) among all sampled calves (Heuvelink et al., 2019). The prevalence of ESBL/AmpC-pos calves varied per age category [i.e., between 26.7% (d 21–88) and 36.2% (d 10–14); Figure 2]. One ESBL/AmpC-pos calf already tested positive for AmpC-EC in the phenotypic assay (AmpC-pos) on the day of birth (14.3%, 95% CI: 0.4–57.9%) and 3 out of 26 calves (11.5%, 95% CI: 2.4–30.2%) tested positive in the phenotypic assay for ESBL/AmpC-EC 1 d after birth: 2 for ESBL-EC (ESBL-pos) and 1 for AmpC-EC. Among the thirty-nine 2-d-old calves, on the final day of colostrum feeding, 11 calves (28.2%, 95% CI: 15.0–44.9%) tested positive in the phenotypic assay for ESBL/AmpC-EC (6 for ESBL-EC and 5 for AmpC-EC). The results of the multilevel mixed models showed that no significant differences were found in the prevalence of ESBL/AmpC-pos calves between calves of different age categories. On the other hand, herd of origin did have a significant (random) effect: the prevalence of ESBL/AmpC-pos calves was significantly more correlated among calves originating from the same herd compared with calves originating from different herds.

Among calves 0 to 4 d of age, the prevalence of ESBL-pos and the prevalence of AmpC-pos calves were comparable (17.9% and 13.9%, respectively). However, after d 4 of age these prevalences divided and showed different patterns among the different age categories (Figure 2). The prevalence of ESBL-pos calves dropped after d 4 of age, resulting in significantly lower odds for excreting ESBL-EC among calves 5 to 9 d of age ($P < 0.02$) compared with calves 0 to 4 d of age (Table 1). After this drop, the prevalence of ESBL-pos calves slowly increased again among calves of older age and was not significantly different anymore compared with the prevalence of ESBL-pos calves among the youngest calves (Figure 2).

In contrast to the prevalence of ESBL-pos calves, the prevalence of AmpC-pos calves did not decrease after 4 d of age but instead it increased, resulting in significantly higher odds for excreting AmpC-EC among calves 5 to 9 d of age ($P < 0.01$) compared with calves younger than 5 d of age (Table 2). After this peak the prevalence of AmpC-pos calves decreased again, resulting in significantly lower odds for excreting AmpC-EC among calves older than 20 d compared with calves 5 to 9 d of age ($P < 0.02$). However, the prevalence of AmpC-pos calves among calves older than 20 d was not significantly different from the same prevalence among the youngest calves (0–4 d of age) and also comparable to the prevalence of ESBL-pos calves among calves older than 20 d (Figure 2).

Semiquantitative Measurement of Excretion of E. coli and ESBL/AmpC-EC. The total number of *E. coli* (regardless of the ESBL/AmpC status) in the fecal samples varied little among the ESBL/AmpC-pos calves and did not vary with age: 85.1% (95% CI: 79.9–89.4%) of the calves excreted a high level of *E. coli* ($\geq 10^6$ cfu/g), 11.9% (95% CI: 8.1–16.8%) a moderate level (10^3 to 10^5 cfu/g), and 3.0% (95% CI: 1.2–6.0%) a low level of *E. coli* ($<10^3$ cfu/g). In contrast, the level of shedding of ESBL/AmpC-EC showed more variation among the ESBL/AmpC-pos calves: 17.4% (95% CI:

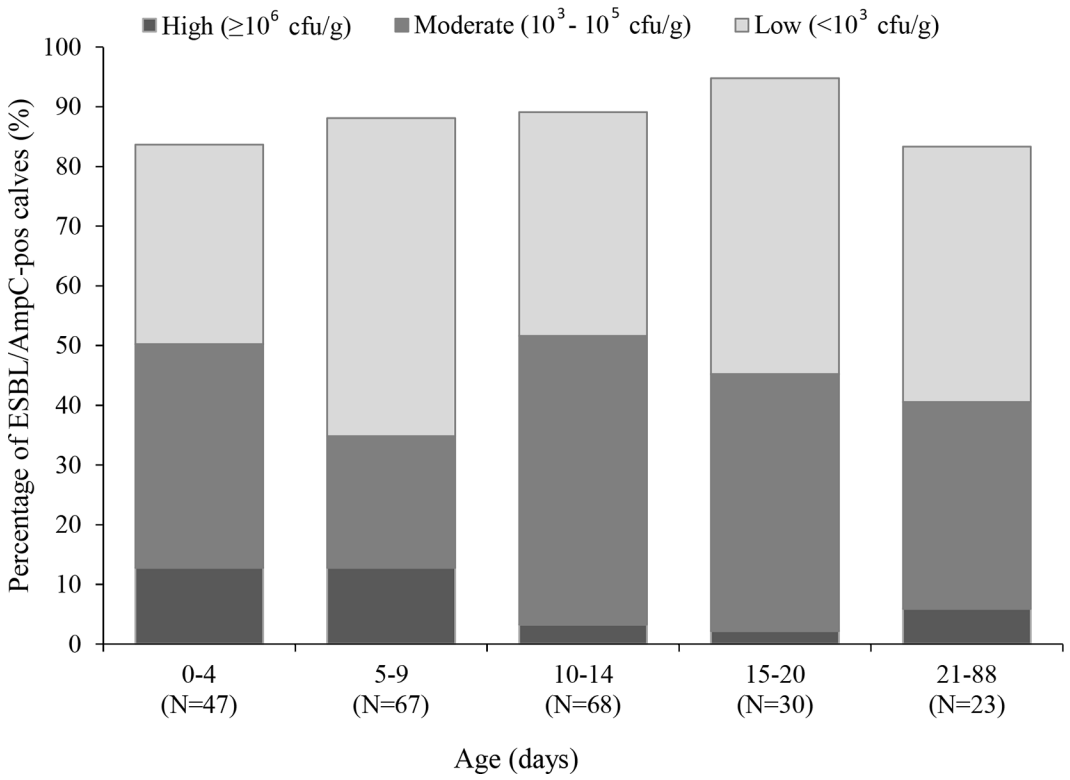

Figure 3. Percentage of calves testing positive for extended-spectrum and AmpC β-lactamase (ESBL/AmpC)-producing *Escherichia coli* in the phenotypic assay (ESBL/AmpC-pos) shedding low (<10^3 cfu/g of feces), moderate (10^3 to 10^5 cfu/g of feces), or high (≥10^6 cfu/g of feces) levels of ESBL/AmpC-producing *Escherichia coli*. Percentages are calculated using multilevel models, taking herd of origin into account as a random effect.

12.8–22.9%) had a high, 41.3% (95% CI: 34.9–47.9%) a moderate, and 41.3% (95% CI: 34.9–47.9%) a low level of excretion (Heuvelink et al., 2019). The moderate excretion levels of ESBL/AmpC-EC also varied with age (Figure 3): 10- to 14-d-old calves showed a tendency toward higher odds for shedding moderate levels of ESBL/AmpC-EC (10^3 to 10^5 cfu/g) compared with 5- to 9-d-old calves ($P < 0.06$; Table 3). There was, however, no significant variation in the excretion of low and high levels of ESBL/AmpC-EC (<10^3 cfu/g) between the different age categories.

Table 3. Results of the multivariable multilevel logistic regression models of the association between calves testing positive for extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* (ESBL/AmpC-EC) in the phenotypic assay, shedding moderate (10^3 to 10^5 cfu/g of feces) levels of ESBL/AmpC-EC, and age of the calves[1]

	Shedding moderate levels of ESBL/AmpC-EC		
Parameter	OR	95% CI	*P*-value
Age category (d)			
5–9 vs. 0–4 (referent)	0.47	0.12–1.80	1.000
10–14 vs. 0–4 (referent)	1.57	0.43–5.75	1.000
15–20 vs. 0–4 (referent)	1.26	0.26–6.07	1.000
21–88 vs. 0–4 (referent)	0.89	0.16–4.98	1.000
10–14 vs. 5–9 (referent)	3.31	0.99–11.05	0.054
15–20 vs. 5–9 (referent)	2.66	0.60–11.90	0.661
21–88 vs. 5–9 (referent)	1.87	0.33–10.54	1.000
15–20 vs. 10–14 (referent)	0.81	0.18–3.524	1.000
21–88 vs. 10–14 (referent)	0.57	0.11–2.96	1.000
21–88 vs. 15–20 (referent)	0.70	0.10–4.74	1.000
Cluster herd	4.71	1.46–15.28	<0.001

[1]Odds ratios (OR), 95% CI, and the *P*-values of the comparisons between age categories are corrected according to Bonferroni's method.

Since the total number of excreted *E. coli* showed only little variation between calves and did not vary with age, the results of the analyses of the ratio between the total number of excreted *E. coli* and the excretion of ESBL/AmpC-EC did not differ from the above described results and are therefore not shown.

Genotypic Assay. Per age category (0–4, 5–9, 10–14, 15–20, and 21–88 d of age) isolates from 18 to 43 calves (median 36 calves) were screened for the presence of β-lactamase and pAmpC genes. When comparing isolates from the different age categories, results show that isolates originating from calves 0 to 4 d of age more often carried ESBL/pAmpC genes compared with isolates from calves 5 to 9 d of age ($P < 0.01$) and isolates from calves 10 to 14 d of age ($P < 0.08$; Table 4). Furthermore, 25.0% (95% CI: 12.1–42.2%) of

Table 4. Extended-spectrum β-lactamase and plasmid-mediated AmpC genes identified in phenotypically confirmed extended-spectrum/AmpC β-lactamase-producing *Escherichia coli* isolates from 155 individual calves on 63 dairy farms, aged 0–4, 5–9, 10–14, 15–20, and 21–88 d

			Age category (d)				
ESBL/pAmpC	Gene group	Gene type	0–4 (n = 36)	5–9 (n = 40)	10–14 (n = 43)	15–20 (n = 18)	21–88 (n = 18)
ESBL	$bla_{\text{CTX-M-1}}$	$bla_{\text{CTX-M-1}}$	9	4	6	0	4
		$bla_{\text{CTX-M-15}}$	2	0	0	1	1
		$bla_{\text{CTX-M-22}}$	2	1	0	0	1
		$bla_{\text{CTX-M-32}}$	1	0	2	0	2
		$bla_{\text{CTX-M-55}}$	1	1	0	1	0
	$bla_{\text{CTX-M-2}}$	$bla_{\text{CTX-M-2}}$	3	0	1	3	1
	$bla_{\text{CTX-M-9}}$	$bla_{\text{CTX-M-14}}$	2	0	0	1	0
		$bla_{\text{CTX-M-65}}$	1	0	0	0	0
	bla_{TEM}	$bla_{\text{TEM-052c}}$	0	1	0	0	0
pAmpC	bla_{CMY}	$bla_{\text{CMY-2}}$	0	0	2	1	1
No. of animals with ESBL/pAmpC genes			21	7	11	7	10

the isolates originating from calves 0 to 4 d of age carried ESBL genes from the gene family $bla_{\text{CTX-M-1}}$. This was significantly higher ($P < 0.05$) compared with the percentages of isolates carrying ESBL genes from the gene family $bla_{\text{CTX-M-1}}$ originating from calves 15 to 21 d of age (0.0%, 95% CI: 0.0–15.4%).

Longitudinal Study

In total, 73 calves of 10 farms were selected from the cross-sectional study (sampling 1) to be sampled again during the longitudinal study, the resampled calves. The number of resampled calves per farm varied between 5 and 11 in the cross-sectional sampling. Not all 73 calves were sampled 4 times because at 1 sampling moment 4 calves were on pasture, and 24 calves left the farm early to go to veal calf operations or calf rearing facilities. At 4 farms 1 calf and on 1 farm 2 calves were added (additional calves) at sampling 2 (after 4 mo). Of these 6 additional calves, 4 were sampled 3 times (at an age of <22 d, 4 mo, and 8 mo) and 2 only 2 times (at an age of <22 d and 8 mo) because they were on pasture on 1 sampling moment and therefore could not be sampled. This resulted in 79 calves that were sampled at an age of <22 d, 53 calves at an age of 4 mo, 52 calves at an age of 8 mo, and 49 calves that were sampled at an age of 12 mo (Table 5).

At an age of <22 d, 46 of the 79 calves (58.2%) tested positive for ESBL- or AmpC-EC (or both) in the phenotypic assay. After 4, 8, and 12 mo the prevalence of ESBL/AmpC-pos calves dropped to 3.8% (2/53), 5.8% (3/52), and 2.0% (1/49), respectively. Of the 46 ESBL/AmpC-pos calves of <22 d old only 5 tested positive a second time at older ages: 1 at an age of 4 mo, 3 at an age of 8 mo, and 1 after 12 mo (Table 6). The ESBL or pAmpC genotypes (or both) detected in *E. coli* isolates from samples of 1 calf were the same at an age of <22 d and 8 mo ($bla_{\text{CTX-M-2}}$). In isolates of 2 other calves that tested positive twice, different ESBL/pAmpC genes were detected when the calves grew older. From the remaining 2 calves, not enough material was left for the identification of ESBL/pAmpC genes.

DISCUSSION

The aim of this study was to examine the prevalence of ESBL/AmpC-EC, the number of excreted ESBL/AmpC-EC (in cfu/g of feces), as well as the ESBL/

Table 5. Seventy-nine calves on 10 dairy farms included in the longitudinal study, testing positive (Pos) or negative (Neg) for extended-spectrum and AmpC β-lactamase (ESBL/AmpC)-producing *Escherichia coli* in the phenotypic assay at an age of <22 d, 4 mo, 8 mo, and 12 mo (NTS = not sampled)

	Age of sampled calves			
Item	<22 d	4 mo	8 mo	12 mo
No. of calves				
1	Pos	Pos	Neg	Neg
3	Pos	Neg	Pos	Neg
1	Pos	Neg	Neg	Pos
23	Pos	Neg	Neg	Neg
3	Pos	Neg	Neg	NTS
2	Pos	Neg	NTS	Neg
1	Pos	Neg	NTS	NTS
1	Pos	NTS	Neg	Neg
11	Pos	NTS	NTS	NTS
1	Neg	Pos	Neg	Neg
16	Neg	Neg	Neg	Neg
1	Neg	Neg	Neg	NTS
1	Neg	Neg	NTS	Neg
2	Neg	NTS	Neg	NTS
12	Neg	NTS	NTS	NTS
No. of calves sampled				
79	79	53	52	49

AmpC genotypes in young dairy calves (0–21 d of age) and the variation of these parameters between calves of different ages. The phenotypic prevalence of ESBL/AmpC-EC was 33.7% in calves of 0 to 20 d and 26.7% in calves of 21 to 88 d of age. In calves younger than 21 d the phenotypic prevalence of ESBL/AmpC-EC varied per age category between 26.7% (d 21–88) and 36.2% (d 10–14). One calf already tested positive for ESBL/AmpC-EC in the phenotypic assay on the first day of life, which raises the question whether the gut was colonized with resistant bacteria before or directly after birth or whether this result was due to contamination of the fecal sample. The prevalence of ESBL-pos and AmpC-pos calves increased rapidly in the first 4 d of age, followed by a significant drop of the prevalence of ESBL-pos calves among calves 5 to 9 d old. On the contrary, the prevalence of AmpC-pos calves among these calves significantly increased, followed by a decrease in prevalence of AmpC-pos calves among older calves. Apparently, the gut of calves in the first days of life is highly susceptible to colonization with these resistant bacteria, which the calves either pick up from their environment or receive from contaminated colostrum. Selection of resistant bacteria in the gut due to antimicrobial treatment of the calves can also result in colonization of the gut with ESBL/AmpC-EC (Ewers et al., 2012). Selection in the gut due to the presence of residues of antimicrobials in colostrum and (waste) milk is, in this study, not likely because in the Netherlands farmers are not allowed to provide waste milk (milk from cows treated with antimicrobials that cannot be used for human consumption) and the dry-cow therapy used in the Netherlands mainly consists of penicillins, which do not select for ESBL/AmpC-EC. Furthermore, in several studies (Schmid et al., 2013; Hordijk et al., 2019) no significant association was found between the prevalence of ESBL/AmpC-EC in calves and feeding colostrum from cows treated with dry-cow antimicrobials. Unfortunately, information about antimicrobial treatments, the feeding of calves, or dry-cow treatment of the dams was not available in our study to evaluate these risk factors.

The above-mentioned variation in prevalence of ESBL/AmpC-pos calves per age category also gives an indication of an instable microbiota of the gut of young calves, where *E. coli* isolates, carrying ESBL or pAmpC genes (or both) are only to a certain extent able to colonize the gut for a longer period of time. Earlier studies already demonstrated the highly variable fecal microbiota of calves in the first days of their lives (Alipour et al., 2018; Klein-Jöbstl et al., 2019). However, other studies also showed the transient character of the shedding of ESBL/AmpC-EC, not only in calves but also in older cattle (Hordijk et al., 2013a, 2019). The variability of the microbiota and the transient character of ESBL/AmpC-EC probably both led to our finding that a specific ESBL genotype ($bla_{\text{CTX-M-1}}$) was found to be significantly more frequent in calves 0 to 4 d of age than in calves 5 d of age or older. In calves 5 d of age or older, ESBL/AmpC-EC isolates relatively more often carried other genotypes. In total, the most common ESBL genotypes in calves in this study were $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$. This result is in accordance with an earlier study in dairy herds (Gonggrijp et al., 2016) and partly corresponding with results from other studies in dairy herds (Hordijk et al., 2019) and veal herds (Hordijk et al., 2013b,c; Ceccarelli et al., 2019) in which *E. coli* isolates most frequently carried $bla_{\text{CTX-M-1}}$ followed by $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$.

The longitudinal study revealed a low prevalence of ESBL/AmpC-pos calves at an age of 4, 8, and 12 mo in calves ESBL/AmpC-pos in the first 21 d of their lives. The same results were found in another longitudinal study in 3 veal calf herds (Hordijk et al., 2013a) in which the ESBL/AmpC-EC prevalence in veal calves also declined from 18 to 26% at the moment the calves arrived at the veal farm to 0% in 2 farms and to 1.4% in 1 farm, 10 wk after arrival. The results of both studies indicate that in the end, the carriage of ESBL or pAmpC genes (or both) does not have a competitive advantage for *E. coli* isolates and the gut of calves is overgrown by nonresistant *E. coli* isolates.

This study shows that dairy calves at a young age can carry ESBL/AmpC-EC, but the colonization in their guts is temporary. More research is needed to find the source of the ESBL/AmpC-EC that colonize the gut.

CONCLUSIONS

It has been demonstrated that calves can be colonized by ESLB/AmpC-EC in the first days of their lives. In addition, the prevalence of ESBL- and AmpC-pos calves significantly varies per age category. The levels of shedding ESBL/AmpC-EC and the ESBL/pAmpC genotypes also vary per age category. These results confirm the variability of the microbiota of young calves and the transient colonization of the gut with ESBL/AmpC-EC, which prevents long-term shedding of these isolates.

ACKNOWLEDGMENTS

This study was financed by the Dutch Commodity Board for Dairy (PZ; Zoetermeer, the Netherlands). The authors thank all farmers for their participation and commitment to this study. The authors have not stated any conflicts of interest.

REFERENCES

Alipour, M. J., J. Jalanka, T. Pessa-Morikawa, T. Kokkonen, R. Satokari, U. Hynönen, A. Iivanainen, and M. Niku. 2018. The composition of the perinatal intestinal microbiota in cattle. Sci. Rep. 8:10437. https://doi.org/10.1038/s41598-018-28733-y.

Bastard, J., M. Haenni, E. Gay, P. Glaser, J. Y. Madec, L. Temime, and L. Opatowski. 2021. Drivers of ESBL-producing *Escherichia coli* dynamics in calf fattening farms: A modelling study. One Health 12:100238. https://doi.org/10.1016/j.onehlt.2021.100238.

Ceccarelli, D., A. Kant, A. van Essen-Zandbergen, C. Dierikx, J. Hordijk, B. Wit, D. J. Mevius, and K. T. Veldman. 2019. Diversity of plasmids and genes encoding resistance to extended spectrum cephalosporins in commensal *Escherichia coli* from Dutch livestock in 2007–2017. Front. Microbiol. 10:76. https://doi.org/10.3389/fmicb.2019.00076.

Chase-Topping, M., D. Gally, C. Low, L. Matthews, and M. Woolhouse. 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. Nat. Rev. Microbiol. 6:904–912. https://doi.org/10.1038/nrmicro2029.

EFSA (European Food Safety Authority and European Centre for Disease Prevention and Control). 2014. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. EFSA J. 12:3590.

Ewers, C., A. Bethe, T. Semmler, S. Guenther, and L. H. Wieler. 2012. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: A global perspective. Clin. Microbiol. Infect. 18:646–655. https://doi.org/10.1111/j.1469-0691.2012.03850.x.

Gonggrijp, M. A., I. M. G. A. Santman-Berends, A. Heuvelink, G. Buter, G. van Schaik, J. Hage, and T. J. G. M. Lam. 2016. Prevalence and risk factors for extended-spectrum β-lactamase- and AmpC-producing *Escherichia coli* in dairy farms. J. Dairy Sci. 99:9001–9013. https://doi.org/10.3168/jds.2016-11134.

Heuvelink, A. E., M. A. Gonggrijp, G. Buter, C. C. ter Bogt-Kappert, G. van Schaik, A. Velthuis, and T. J. G. M. Lam. 2019. Prevalence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in Dutch dairy herds. Vet. Microbiol. 232:58–64. https://doi.org/10.1016/j.vetmic.2019.04.005.

Homeier-Bachmann, T., J. F. Kleist, A. K. Schütz, and L. Bachmann. 2022. Distribution of ESBL/AmpC-*Escherichia coli* on a dairy farm. Antibiotics (Basel) 11:940. https://doi.org/10.3390/antibiotics11070940.

Hordijk, J., E. A. J. Fischer, T. van Werven, S. Sietsma, L. van Gompel, A. J. Timmerman, M. P. Spaninks, D. J. J. Heederik, M. Nielen, J. A. Wagenaar, and J. A. Stegeman. 2019. Dynamics of fecal shedding of ESBL- or AmpC-producing *Escherichia coli* on dairy farms. J. Antimicrob. Chemother. 74:1531–1538. https://doi.org/10.1093/jac/dkz035.

Hordijk, J., D. J. Mevius, A. Kant, M. E. Bos, H. Graveland, A. B. Bosman, C. M. Hartskeerl, D. J. Heederik, and J. A. Wagenaar. 2013a. Within-farm dynamics of ESBL/AmpC-producing *Escherichia coli* in veal calves: A longitudinal approach. J. Antimicrob. Chemother. 68:2468–2476. https://doi.org/10.1093/jac/dkt219.

Hordijk, J., J. A. Wagenaar, A. Kant, A. van Essen-Zandbergen, C. Dierikx, K. Veldman, B. Wit, and D. J. Mevius. 2013b. Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC-producing *Escherichia coli* isolated from veal calves at slaughter. PLoS One 8:e65681. https://doi.org/10.1371/journal.pone.0065681.

Hordijk, J., J. A. Wagenaar, A. Van De Giessen, C. Dierikx, A. Van Essen-Zandbergen, K. Veldman, A. Kant, and D. J. Mevius. 2013c. Increasing prevalence and diversity of ESBL/AmpC-type beta-lactamase genes in *Escherichia coli* isolated from veal calves from 1997 to 2010. J. Antimicrob. Chemother. 68:1970–1973. https://doi.org/10.1093/jac/dkt132.

Hoyle, D. V., H. I. Knight, D. J. Shaw, K. Hillman, M. C. Pearce, J. C. Low, G. J. Gunn, and M. E. Woolhouse. 2004. Acquisition and epidemiology of antibiotic-resistant *Escherichia coli* in a cohort of newborn calves. J. Antimicrob. Chemother. 53:867–871. https://doi.org/10.1093/jac/dkh177.

Jett, B. D., K. L. Hatter, M. M. Huycke, and M. Gilmore. 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques 23:648–650. https://doi.org/10.2144/97234bm22.

Klein-Jöbstl, D., N. M. Quijada, M. Dzieciol, B. Feldbacher, M. Wagner, M. Drillich, S. Schmitz-Esser, and E. Mann. 2019. Microbiota of newborn calves and their mothers reveals possible transfer routes for newborn calves' gastrointestinal microbiota. PLoS One 14:e0220554. https://doi.org/10.1371/journal.pone.0220554.

Korzeniewska, E., A. Korzeniewska, and M. Harnisz. 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. Ecotoxicol. Environ. Saf. 91:96–102. https://doi.org/10.1016/j.ecoenv.2013.01.014.

Lahey database. Accessed Mar. 2012. http://www.lahey.org/studies.

Laube, H., A. Friese, C. von Salviati, B. Guerra, and U. Rösler. 2014. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. Vet. Microbiol. 172:519–527. https://doi.org/10.1016/j.vetmic.2014.06.008.

Mulvey, M. R., E. Bryce, D. A. Boyd, M. Ofner-Agostini, A. M. Land, A. E. Simor, and S. Paton. 2005. Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. Antimicrob. Agents Chemother. 49:358–365. https://doi.org/10.1128/AAC.49.1.358-365.2005.

Schmid, A., S. Hörmansdorfer, U. Messelhäusser, A. Käsbohrer, C. Sauter-Louis, and R. Mansfeld. 2013. Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* on Bavarian dairy and beef cattle farms. Appl. Environ. Microbiol. 79:3027–3032. https://doi.org/10.1128/AEM.00204-13.

StataCorp. 2017. Stata Statistical Software: Release 15. StataCorp LP.

von Salviati, C., H. Laube, B. Guerra, U. Roesler, and A. Friese. 2015. Emission of ESBL/AmpC-producing *Escherichia coli* from pig fattening farms to surrounding areas. Vet. Microbiol. 175:77–84. https://doi.org/10.1016/j.vetmic.2014.10.010.

WHO. 2011. Tackling antibiotic resistance from a food safety perspective in Europe. Accessed Dec. 23, 2021. https://apps.who.int/iris/bitstream/handle/10665/326398/9789289014212-eng.pdf.

WHO. 2019. Critically important antimicrobials for human medicine. 6th revision. World Health Organization.

Wu, G., M. J. Day, M. T. Mafura, J. Nunez-Garcia, J. J. Fenner, M. Sharma, A. van Essen-Zandbergen, I. Rodríguez, C. Dierikx, K. Kadlec, A.-K. Schink, M. Chattaway, J. Wain, R. Helmuth, B. Guerra, S. Schwarz, J. Threlfall, M. J. Woodward, N. Woodford, N. Coldham, and D. Mevius. 2013. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. PLoS One 8:e75392. https://doi.org/10.1371/journal.pone.0075392.

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