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Prevalence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in Dutch dairy herds

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ABSTRACT

This study was conducted to assess: (1) a change in between-herd prevalence of extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* (ESBL/AmpC-EC) between 2011 and 2013, the period during which the antimicrobial policy in animal husbandry in the Netherlands changed significantly, and (2) the prevalence of ESBL/AmpC-EC in individual calves, young stock, and dairy cows in the Netherlands.

In 196 randomly selected conventional dairy herds, faecal samples were collected from calves (maximum n = 15), and randomly selected young stock (n = 5) and dairy cows (n = 15). Additionally, fresh faecal samples were collected from five different places on the floors where the dairy cows were housed. Samples were screened for *E. coli* with non-wild type susceptibility for cefotaxime and isolates were phenotypically confirmed as ESBL/AmpC-producing by disc diffusion, using cefotaxime and ceftazidime with and without clavulanic acid, and cefoxitin. Samples containing ESBL/AmpC-EC were examined semi-quantitatively.

In 59.6% of the dairy herds one or more samples tested positive for ESBL/AmpC-EC. The between-herd prevalence based on floor samples in 2013 (18.0%) was significantly lower than the prevalence in 2011 based on comparable samples (32.7%). The individual animal prevalence of ESBL/AmpC-EC, with a minimum shedding level of 10^3 cfu/g of faeces, was 19.3% in calves, 0.9% in young stock, and 0.8% in dairy cows.

Although ESBL/AmpC-EC was found in the majority of dairy herds, the herd prevalence declined significantly between 2011 and 2013. Calves were found to have both, a much higher individual animal prevalence and a higher level of shedding than young stock and cows.

1. Introduction

Since the late 1990s, extended-spectrum β-lactamase (ESBL)-producing and AmpC β-lactamase-producing *Enterobacteriaceae*, in particular *Escherichia coli*, have emerged rapidly (Pitout and Laupland, 2008). ESBLs and AmpCs are two distinct types of enzymes, both conferring resistance to bacteria to a variety of ß-lactam antibiotics, including penicillins, 2nd-, 3rd-generation cephalosporins and monobactams (Jacoby and Munoz-Price, 2005). The resistance mechanism is enzymatic hydrolysis of the ß-lactam ring of these antibiotics, resulting in inactive antimicrobial compounds (Bush and Jacoby, 2010). ESBL/AmpC-producing bacteria are frequently co- or multiresistant, exhibiting resistance to other antimicrobial classes such as fluoroquinolones, aminoglycosides, and trimethoprim-sulphamethoxazole (Jacoby and Munoz-Price, 2005; Pitout, 2010). The high mortality of humans infected with ESBL/AmpC-producing bacteria have made them a big threat to human health worldwide (Pitout, 2010; Rottier et al., 2012).

Initially, ESBL/AmpC-producing bacteria were only found in humans, but in recent years numerous reports of ESBL/AmpC-positive isolates were published, including reports on ESBL-producing *Enterobacteriaceae* (mainly *E. coli* and *Salmonella*) in food-producing animals and food (Carattoli, 2008; EFSA, 2011; Ewers et al., 2012). The use of antimicrobials in animals, and more specifically the use of 3rd- and 4th-generation cephalosporins has been reported to be correlated to the emergence of ESBL/AmpC-producing bacteria in animal populations (Tragesser et al., 2006; Snow et al., 2012; Liebana et al., 2013).

In the Netherlands, at the end of 2008 a covenant was signed by the main stakeholders in livestock farming to improve the antimicrobial resistance in animals. A comprehensive strategy was developed and carried out to reduce antimicrobial usage in livestock and to stimulate prudent use of antimicrobials. This resulted in a significant decrease in

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the use of antimicrobials in cattle and other livestock species in the Netherlands, with the use of 3rd- and 4th-generation cephalosporins having decreased towards negligible levels (Lam et al., 2017; SDa, 2018). The question remains whether the decreased use of antimicrobials in dairy cattle led to a reduction of the prevalence of ESBL/AmpC-producing *E. coli* (ESBL/AmpC-EC).

During a cross-sectional study conducted in 2011, the between-herd prevalence of ESBL-/AmpC-EC in dairy herds in the Netherlands was estimated at 41.0% (Gonggrijp et al., 2016). In that study, no association was found between the total antimicrobial use and the ESBL/AmpC herd status. The use of 3rd- and 4th-generation cephalosporins was, however, associated with an increased odds of having a positive ESBL/AmpC herd status.

In a study conducted at the end of 2013, two years after our previous study (Gonggrijp et al., 2016), the between-herd prevalence of ESBL/AmpC-EC was again assessed using the same methods as in 2011, with the aim to compare the two prevalence rates, set against the restrictive use of antimicrobials, and specifically of highest priority critically important antimicrobials such as 3rd- and 4th-generation cephalosporins introduced between 2011 and 2013. A second goal of this study was to estimate the individual animal prevalence of ESBL/AmpC-EC in calves, young stock, and dairy cows, as well as the extent of shedding by individual animals. ESBL/AmpC gene types identified were compared among age groups and with those found previously in *E. coli* isolates from dairy cattle, other food-producing animals and humans.

2. Materials and methods

2.1. Selection of herds and collection of faecal samples

Between October and December 2013, faecal samples were collected at 196 randomly selected conventional (non-organic) dairy farms in the Netherlands. At each farm, individual faecal samples were collected from all calves present, preferably younger than 22 days, with a maximum of 15 calves per herd, from five randomly selected young stock (aged 1–2 years) and from fifteen randomly selected dairy cows (aged ≥2 years). The samples were taken through rectal palpation by the private veterinarian. In addition to these rectal samples from individual animals, freshly voided faecal samples were collected from five different places on the floors of the barn where the dairy cows were housed. The samples were transported to the laboratory under chilled conditions and bacteriological examination started within 24 h after collection.

2.2. Isolation of ESBL/AmpC-EC

Calf faecal samples were examined individually. Following mixing of the faeces using a sterile cotton swab, the same swab was streaked onto MacConkey agar No. 3 (Oxoid Ltd., Basingstoke, UK) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (MacC + cef) and then, the swab was transferred into 10 mL Luria-Bertani broth (Becton Dickinson, Franklin Lakes, New Jersey, USA) supplemented with 1 mg/L cefotaxime (LBB + cef). Both inoculated plates and broths were incubated aerobically at 37 °C. After overnight incubation, morphologically presumptive *E. coli* colonies on the MacC + cef plates were confirmed as such by using the MALDI-Biotyper (Bruker Daltonics GmbH, Bremen, Germany). For samples that did not yield *E. coli* colonies on the MacC + cef plates, the overnight cultures in LBB + cef were streaked onto new MacC + cef plates. Following overnight incubation at 37 °C, typical *E. coli* colonies were confirmed as described above. Confirmed *E. coli* isolates were examined for ESBL or AmpC production by the combination disc diffusion test using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson) according to CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI, 2011). Additionally, a cefoxitin disc (30 µg, Becton Dickinson) was included in the test to detect AmpC phenotypes. Isolates (intermediate-) resistant to cefoxitin according to CLSI criteria (zone diameter ≤17 mm) were classified as AmpC-producers. Phenotypically positive ESBL/AmpC-EC isolates were stored at −80 °C in Microbank vials (Pro-lab Diagnostics, Austin, Texas, USA), one to three per sample.

Faecal samples from young stock, adult cattle, and the floors were examined as pools of five per type of sample, resulting in one pooled sample of young stock faeces, three pooled samples of cow faeces, and one pooled sample of faeces from the floor. To this end, the five swabs used for mixing of the faeces, were streaked onto a single MacC + cef plate (five parallel streaks onto one-third of the plate) and then the same five swabs were transferred into a single tube with 25 mL LBB + cef (five swabs in one tube). The samples were further spread across the MacC + cef plate by phase streaking using a sterile loop. The inoculated plates and broths were processed according to the same procedure as described above for the individually examined samples of calf faeces.

After mixing with an equal volume of 20%-glycerol peptone, all faecal samples collected from calves, young stock, and cows were stored individually at −20 °C pending semi-quantitative examination of ESBL/AmpC-EC.

2.3. Quantification of ESBL/AmpC-EC

Calf samples that yielded ESBL/AmpC-EC by direct selective plating or plating following selective enrichment were subjected to a semi-quantitative determination of ESBL/AmpC-EC. Also all individual samples from young stock and adult cattle that were part of positive pools were examined semi-quantitatively.

The extent of shedding of ESBL/AmpC-EC was estimated by applying the so-called track-dilution technique (Jett et al., 1997), using square petri plates (100 x 100 mm) with tryptone bile X-glucuronide (TBX) agar (Oxoid) supplemented with 1 mg/L cefotaxime (TBX + cef). In brief, the faecal suspensions in 20%-glycerol peptone stored at −20 °C were thawed, diluted 1:5 in 0.1% peptone salt solution (the 10^{-1} suspension), and subsequently serially diluted tenfold up to 10^{-5}. Then, by using a multichannel pipette, 10 µl of each dilution was deposited onto the TBX + cef agar surface along one side of the square plate that was tipped onto its side (at a 45° angle). By tipping the plate the spots were allowed to migrate in parallel tracks across the agar surface. The dilutions were additionally spotted onto the agar surface of a square plate filled with unsupplemented TBX agar to estimate the total number of *E. coli* present in the samples, in order to determine the fraction of ESBL/AmpC-EC of the total *E. coli* count. Both the TBX + cef and unsupplemented TBX plates were incubated aerobically, overnight at 37 °C.

The ESBL/AmpC-EC count and total *E. coli* count were calculated based on the highest dilution with typical *E. coli* colonies on the TBX + cef agar and the unsupplemented TBX agar, respectively. Based on the number of ESBL/AmpC-EC per g of faeces, the samples were categorized as containing low (< 10^3 cfu/g), moderate (10^3–10^5 cfu per g), and high (≥10^6 cfu/g) numbers of ESBL/AmpC-EC. The fraction of ESBL/AmpC-EC of the total *E. coli* count was calculated by dividing the ESBL/AmpC-EC count by the total *E. coli* count. Presumptive *E. coli* colonies from the TBX + cef agar plate were identified by using the MALDI-Biotyper, starting the selection of colonies from the highest dilution, and confirmed isolates were stored at −80 °C in Microbank vials (one to three per sample).

2.4. Identification of ESBL/AmpC genes

Identification of ESBL/AmpC genes was performed by micro-array, PCR and sequence analysis. DNA isolation was performed with the DNeasy Blood & Tissue kit (Qiagen Benelux B.V., Venlo, Nederland). Stored isolates were cultured onto MacC + cef and following overnight incubation at 37 °C, three to four colonies were picked from the plate

and suspended in 180 µL ATL buffer of the kit. Then, the procedure was continued according to the protocol for "tissue samples" described in the manual of the manufacturer, starting from step 2. Finally, the DNA was eluted in 200 µL elution buffer.

By using the *E. coli* Genotyping Combined micro-array (Alere Technologies GmbH, Jena, Germany) and following the instructions of the manufacturer, samples were screened for the presence of different ESBL and plasmid-mediated AmpC (pAmpC) gene families.

Further identification of β-lactamase gene types for samples that responded positively in the array for the bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{SHV}, $bla_{OXA\text{-}1}$ and bla_{CMY} gene groups was performed by PCR amplification and sequencing, as described previously (Gonggrijp et al., 2016). DNA purification and sequencing was performed by Macrogen Europe, Amsterdam, the Netherlands. Consensus sequences were obtained with DNA Baser version 3.5.4. (Heracle BioSoft SRL, Pitesti, Romania). For each β-lactamase family alignments were made in MEGA (Molecular Evolutionary Genetics Analysis) version 6 (Center for Evolutionary Medicine and Informatics, Tempe, USA) (Tamura et al., 2011). Identification of β-lactamase gene types was performed using BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and by comparing sequences to references in Genbank and the Lahey database (Lahey, 2015).

Initially, *E. coli* isolates with AmpC phenotypes that could not be attributed to the presence of a pAmpC gene of the bla_{CMY} gene group were tested for mutations in the AmpC promoter/attenuator region as described by Mulvey et al. (2005). Sequences were compared to references in Genbank and the Lahey database (Lahey, 2015).

2.5. Statistical analysis

All statistical analyses were carried out in STATA 13.0 (StataCorp, 2014).

Based on results of the qualitative examination of individual calf samples and of pooled samples from young stock, dairy cows and floors, herds were categorized as phenotypically positive (isolation of ESBL/AmpC-EC from at least one of these samples) or unsuspected (none of the samples yielding ESBL/AmpC-EC) for ESBL/AmpC-EC. In this part of the analysis, only herds with complete sample sets were included.

The between-herd prevalence of the current study was compared with the between-herd prevalence determined in 2011 by Gonggrijp et al. (2016) based on the same type of samples, using a two-sample test of proportions (prtesti). In the 2011-study, manure samples were examined, that had been taken from the manure scraper (n = 26 herds) or, if not present, from a pool sample made by thoroughly mixing samples collected from five different places on the floor (n = 55 herds). For a fair comparison of prevalence rates, the percentage of phenotypically positive floors determined in the current study was compared with the percentage of phenotypically positive pooled floor samples in the 2011-study.

The individual calf prevalence was calculated based on the qualitative isolation method. The individual animal prevalence in young stock and dairy cows was calculated using the semi-quantitative examination instead of the qualitative assay. The latter was performed on pooled samples only, due to limited resources. Hence, the estimated animal prevalence of ESBL/AmpC-EC in young stock and dairy cows only includes animals with at least 10^3 cfu/g faeces. Samples with less ESBL/AmpC-EC than the detection limit of the semi-quantitative method (i.e. 10^3 cfu/g) were considered negative but may contain up to 999 cfu/g (since samples tested semi-quantitatively were part of pools positive by the qualitative assay). To be able to compare the individual animal prevalence for the three age groups, the individual calf prevalence was also calculated by considering animals that shed less than 10^3 cfu/g of ESBL/AmpC-EC as unsuspected.

3. Results

The total collection of faecal samples comprised individual faecal samples from 748 calves, 965 young stock, and 2920 dairy cows, as well as 194 pooled floor samples, originating from a total of 196 herds.

3.1. Prevalence and quantification of ESBL/AmpC-EC at herd level

For 13 herds, the set of sample types collected was not complete. In three of these herds, no calves were present at the moment of sampling. In five herds, animals were sampled as being calves but either their age was not recorded or all animals sampled were older than one year. In three herds, no young stock (aged 1–2 years) was sampled. And of two herds, floor samples were lacking. Of 109 (59.6%, 95% confidence interval (CI): 52.5–66.7%) of the remaining 183 herds, one or more samples tested phenotypically positive for ESBL/AmpC-EC. Isolates of 50 (27.3%, 95% CI: 20.9–33.8%) of the 183 herds were all phenotypically identified as AmpC-producers, of 29 herds (15.8%, 95% CI: 10.6–21.1%) as ESBL-producers, and in 30 herds (16.4%, 95% CI: 11.0–21.8%) both ESBL and AmpC were detected. Of 106 (57.9%, 95% CI: 50.8–65.1%) of the 183 herds, one or more of the samples from calves, young stock or cows tested positive for ESBL/AmpC-EC. The between-herd prevalence of the various combinations of phenotypically positive and unsuspected samples of these three groups of animals is summarized in Table 1. Based on results of individual calves, pools of young stock, and pools of cows, 48.6%, 15.3%, and 23.0% of herds tested positive, respectively.

Of 34 (18.6%, 95% CI: 12.9–24.2%) of the 183 herds, the pool of five samples from the floors was found phenotypically positive for ESBL/AmpC-EC; floor samples collected from the remaining 149 herds tested negative and were therefore considered unsuspected of ESBL/AmpC-EC. Of 50.3% (95% CI: 42.3–58.4%) of these 149 herds, one or more animal groups tested phenotypically positive. More specifically, of 12.8% (95% CI: 7.4–18.1%) of the 149 herds with negative floor samples, ESBL/AmpC-EC were present in dairy cows (Table 2).

Based on floor samples solely and including herds with incomplete sets of individual animal samples (n = 194 herds), the herd prevalence in the current study was assessed at 18.0% (95% BI: 12.6–23.5%). This prevalence is statistically significantly ($p < 0.03$) lower than the prevalence of 32.7% (95% CI: 20.3–45.1%) based on floor samples determined in 2011 (Gonggrijp et al., 2016).

Samples from phenotypically positive calves and individual samples from young stock and adult cattle that were part of ESBL/AmpC-EC-positive pool samples were subjected to a semi-quantitative determination of ESBL/AmpC-EC. Of 45.0% (95% CI: 34.1–55.9%) of herds, all samples tested semi-quantitatively, contained low numbers of ESBL/AmpC-EC only. For 27.5% (95% CI: 17.7–37.3%) of herds, both samples with low and samples with moderate numbers of ESBL/AmpC-EC were

Table 1
Frequency of various outcomes of bacteriological culture of faecal samples collected from calves, young stock, and dairy cows for ESBL/AmpC-EC, at herd level (n = 183 herds).

No. of herds (%)	Calves	Young Stock	Cows
77 (42.1%)	Unsuspected	Unsuspected	Unsuspected
49 (26.8%)	Positive	Unsuspected	Unsuspected
17 (9.3%)	Positive	Unsuspected	Positive
12 (6.6%)	Unsuspected	Unsuspected	Positive
12 (6.6%)	Positive	Positive	Positive
11 (6.0%)	Positive	Positive	Unsuspected
4 (2.2%)	Unsuspected	Positive	Unsuspected
1 (0.5%)	Unsuspected	Positive	Positive
183 (100.0%)	89 (48.6%)	28 (15.3%)	42 (23.0%)

Positive: isolation of phenotypically confirmed ESBL/AmpC-EC from at least one of these samples; Unsuspected: none of the samples yielded ESBL/AmpC-EC.

Table 2
Number of herds (%) with faecal samples collected from calves, young stock, and dairy cows phenotypically positive for ESBL/AmpC-EC, split up for herds with samples collected from floors tested positive and negative for ESBL/AmpC-EC.

	No. of herds (%) with positive samples from		
	Calves	Young stock	Cows
Herds with positive floor samples (n = 34)	25 (73.5%)	11 (32.4%)	23 (67.6%)
Herds with negative floor samples (n = 149)	64 (43.0%)	17 (11.4%)	19 (12.8%)

encountered. For 17.5% (95% CI: 9.2–25.8%) of herds, samples with low, samples with moderate, as well as samples with high numbers were found. Of the remaining 10.0% (95% CI: 3.4–16.6%) of herds, samples were categorized as either containing all moderate, all high, or both moderate and high numbers of ESBL/AmpC-EC.

3.2. Prevalence and quantity of ESBL/AmpC-EC in calves

Of 748 calves sampled, 681 were aged younger than 22 days and 67 were, despite the protocol, between 22 and 88 days of age. Because the prevalence of ESBL/AmpC-EC in calves younger than 22 days and in calves between 22 and 88 days did not differ (results below), all 748 calves were included in the study.

Per herd, median three calves (mean: 4, min: 1, max: 15) were included. The median age of the calves was 10 days (mean: 11, min: 0, max: 88). Of the calves, 246 (32.9%; 95% CI: 29.5–36.3%) tested phenotypically positive for ESBL/AmpC-EC. Isolates from 162 (65.9%, 95% CI: 59.9–71.8%) calves were phenotypically identified as AmpC-producers. Isolates from 82 (33.3%, 95% CI: 27.4–39.2%) calves were phenotypically identified as ESBL-producers. And isolates from two (0.8%, 95% CI: 0.09–2.9%) calves were phenotypically identified as both ESBL- and AmpC-producing. Positive calves originated from 90 farms. The prevalence of ESBL/AmpC-EC among the 681 calves aged younger than 22 days was 33.3% (95% CI: 29.8–36.9%) and among the 67 calves aged between 22 and 88 days 28.4% (95% CI: 17.6–39.2%).

Of the 246 positive calf samples, 235 were subjected to the semi-quantitative examination of ESBL/AmpC-EC. Of the remaining 11 positive samples insufficient material was left for this test. Results of the semi-quantitative examination are summarized in Table 3. To calculate the prevalence of calves shedding at least 10^3 cfu/g of ESBL/AmpC-EC in their faeces, it was taken into account that only 235 of 246 positive calves were tested semi-quantitatively. Therefore, the number of calves with $\geq 10^3$ cfu/g of ESBL/AmpC-EC (n = 138) was multiplied by 246 divided by 235, to give the estimated total number of calves in the study shedding $\geq 10^3$ cfu/g of ESBL/AmpC-EC (n = 144). This results in an estimated prevalence of calves shedding at least 10^3 cfu/g of ESBL/AmpC-EC of 19.3% (95% CI: 16.4–22.1%) (Table 3).

For samples containing moderate numbers of ESBL/AmpC-EC, the fraction of ESBL/AmpC-EC relative to the total *E. coli* count had a median of 0.6% (mean: 5%, min: 0.01%, max: 100%). For samples with the highest numbers of ESBL/AmpC-EC this fraction could not be calculated, because samples were not diluted far enough to allow estimating *E. coli* counts.

3.3. Prevalence and quantity of ESBL/AmpC-EC in young stock

A total of 965 young stock were sampled in 193 dairy herds (five animals per herd). In three herds no young stock was sampled because the day these herds were visited, these animals were in a meadow not close to the farm. Of 29 (15.0%, 95% CI: 10.0–20.1%) of the 193 herds the pool of five samples was phenotypically positive for ESBL/AmpC-EC. Isolates from 18 (62.1%, 95% CI: 44.4–79.7%) of the 29 pools were phenotypically identified as ESBL-producers and from 11 (37.9%, 95% CI: 20.3–55.6%) pools as AmpC-producers.

All 145 individual samples of positive pools were tested to determine numbers of ESBL/AmpC-EC using the semi-quantitative method. Results are summarized in Table 3. The prevalence of ESBL/AmpC-EC in young stock defined as the percentage of animals shedding at least 10^3 cfu/g of ESBL/AmpC-EC in their faeces was 0.9% (95% CI: 0.3–1.5%) (Table 3).

For the nine samples with moderate numbers of ESBL/AmpC-EC, the fraction of ESBL/AmpC-EC relative to the total *E. coli* count had a median of 0.1% (mean: 1%, min: 0.01%, max: 10%).

3.4. Prevalence and quantity of ESBL/AmpC-EC in dairy cows

A total of 2920 dairy cows were sampled; in 194 herds 15 cows and in two herds five cows. Of 45 (23.0%, 95% CI: 17.1–28.8%) of the 196 herds one or more pools of five samples tested phenotypically positive for ESBL/AmpC-EC, 79 pools in total. Of 22 of the 45 herds one pool tested positive for ESBL/AmpC-EC, of 12 herds two pools and of 11 herds three pools. Isolates from 41 (51.9%, 95% CI: 40.9–62.9%) of the 79 positive pools were phenotypically identified as ESBL-producers, from 37 (46.8%, 95% CI: 35.8–57.8%) pools as AmpC-producers, and from one (1.3%, 95% CI: 0.03–6.9%) pool as both ESBL- and AmpC-producing.

Of the 395 individual samples of dairy cows being part of a positive pool, 373 were tested individually for the presence of ESBL/AmpC-EC using the semi-quantitative method described. Of the other 22 samples insufficient material was left. Results are summarized in Table 3. The prevalence of ESBL/AmpC-EC in dairy cows defined as the percentage of animals shedding at least 10^3 cfu/g of ESBL/AmpC-EC in their faeces was 0.8% (95% CI: 0.5–1.2%) (Table 3).

Table 3
Results of semi-quantitative examination of individual samples from phenotypically ESBL/AmpC-EC-positive calves and of individual samples from young stock and cows that were part of ESBL/AmpC-EC-positive pool samples, and the estimated prevalence of ESBL/AmpC-EC ($\geq 10^3$ cfu/g faeces) in individual calves, young stock, and dairy cows.

	Semi-quantitative examination				Estimated animal prevalence (%) (95% CI)[a]
	No. of individual animals tested	No. of animals (%) with low, moderate or high ESBL/AmpC-EC counts			
		Low ($< 10^3$ cfu/g)	Moderate (10^3-10^5 cfu/g)	High ($\geq 10^6$ cfu/g)	
Calves	235	97 (41.3%)	97 (41.3%)	41 (17.4%)	19.3[b] (16.4–22.1%)
Young stock	145	136 (93.8%)	9 (6.2%)		0.9 (0.3–1.5%)
Cows	373	350 (93.8%)	23 (6.2%)		0.8 (0.5–1.2%)

[a] Percentage of individual animals shedding $\geq 10^3$ cfu ESBL/AmpC-EC per g faeces of the total number of calves (n = 748), young stock (n = 965), and cows (n = 2920) included in the study, calculated based on results of the semi-quantitative examinations.

[b] Taking into account that only 235 of 246 positive calves were tested semi-quantitatively, the estimated total number of calves in the study shedding $\geq 10^3$ cfu/g of ESBL/AmpC-EC is 144 ((97 + 41)*246/235).

For the 23 samples containing moderate numbers, the fraction of ESBL/AmpC-EC relative to the total *E. coli* count had a median of 0.1% (mean: 1%, min: 0.01%, max: 10%).

3.5. Identification of ESBL/AmpC genes

From 35 calves with low and 120 calves with moderate or high numbers of phenotypically confirmed ESBL/AmpC-EC in their faeces, isolates were screened for the presence of ESBL/AmpC genes. The median and mean number of isolates per calf was three. These 155 calves were randomly selected from the positive calves. *E. coli* isolates from 93 of the 155 calves were phenotypically identified as AmpC-producing, from 61 calves as ESBL-producing, and isolates from one calf as both ESBL- and AmpC-producing. In *E. coli* isolates from four of the 94 calves with faecal *E. coli* with an AmpC phenotype (including the calf shedding isolates with both an ESBL- and AmpC phenotype), the plasmid-encoded bla_{CMY-2} gene was detected. In isolates from 52 of the 62 calves with faecal *E. coli* with an ESBL phenotype (including the calf shedding isolates with both an ESBL- and AmpC phenotype), ESBL genes belonging to the β-lactamase gene families bla_{CTX-M} or bla_{TEM} were detected. ESBL/AmpC genes detected in multiple isolates from the same calf were all found to be identical. In total, isolates from 56 (36.1%, 95% BI: 28.6–43.7%) of the 155 calves carried ESBL genes belonging to the gene families bla_{CTX-M} or bla_{TEM} or the pAmpC gene bla_{CMY-2}. The frequency of identification of the different genes is shown in Table 4.

Among the ESBL/AmpC-EC isolates from individual young stock (n = 8) and dairy cows (n = 23) screened for ESBL/AmpC genes, no isolates were identified as both ESBL- and AmpC-producing. In isolates from 10 of the 12 individual animals (six young stock and six cows) testing positive for *E. coli* isolates with an ESBL phenotype, ESBL genes were detected (Table 4). Isolates from one of the 19 animals (two young stock and 17 cows) with isolates with an AmpC phenotype tested positive for the pAmpC gene bla_{CMY-2}. Like observed for calves, ESBL/AmpC genes detected in multiple isolates (one to three) from the same young stock and dairy cow were found to be identical.

Many of the phenotypically ESBL/AmpC-EC were positive for small-spectrum β-lactamases (bla_{TEM-1a}, bla_{TEM-1b}, $bla_{TEM-1b/TEM-79}$, $bla_{TEM-52c}$ and/or bla_{OXA-1}) (results not shown). None of the individual animal isolates was positive for the bla_{SHV} gene group. For some of the isolates with an AmpC phenotype with negative results in the micro-array, mutations in the AmpC promoter/attenuator region were detected (results not shown). Chromosomally encoded AmpC genes found in these isolates belonged to types 3, 5, 11, 14, and 18. No further tests have been performed to clarify the genetic basis of the ESBL/AmpC phenotype of isolates with negative screening results.

4. Discussion

This study was primarily conducted to assess the prevalence of ESBL/AmpC-EC, two years after a study conducted in 2011 (Gonggrijp et al., 2016), to determine whether the restrictive use of 3rd- and 4th-generation cephalosporins in the Netherlands coincided with a decrease of the between-herd prevalence of ESBL/AmpC-EC.

The between-herd prevalence determined in the present study, based on the same type of faecal samples as in the 2011-study, significantly decreased from 32.7%–18.0%. Although in our current study, the floor samples were pooled in the laboratory, while in the 2011-study, the pooling was performed at the farm, we believe that it is fair to conclude that the between-herd prevalence of ESBL/AmpC-EC within the two-year period studied declined. The reduced use of antimicrobials in dairy farming, specifically of 3rd- and 4th-generation cephalosporins (Lam et al., 2017), likely played a key role in this, given the earlier described relation between the use of these antimicrobials and the prevalence of ESBL/AmpC-EC (Tragesser et al., 2006; Snow et al., 2012; Gonggrijp et al., 2016).

The second objective was to assess the prevalence at the level of individual calves, young stock, and dairy cows. The animal prevalence of ESBL/AmpC-EC, defined as the percentage of animals shedding at least 10^3 cfu/g of ESBL/AmpC-EC in their faeces, was much higher in young calves than in other age groups. In addition to a higher prevalence among calves, also the numbers of ESBL/AmpC-EC shed by calves were much higher than those shed by young stock and cows. While none of the young stock and cow samples examined individually by the semi-quantitative method contained numbers equal to or higher than 10^6 cfu/g, 17.4% of positive calf samples contained high numbers of ESBL/AmpC-EC ($\geq 10^6$ cfu/g), i.e. 5.7% of the total number of calves included in the study. These results are supported by several studies reporting an inverse relationship between the prevalence of antimicrobial drug-resistant bacteria, such as ESBL/AmpC-EC, and animal age (Howe and Linton, 1976; Khachatryan et al., 2004; Liebana et al., 2006; Horton et al., 2016). The high prevalence in younger animals seems not necessarily to be related to recent use of antimicrobials. Several studies suggest that antimicrobial resistant *E. coli* have a higher fitness in the calf enteric environment compared to susceptible *E. coli* (Berge et al., 2005; Khachatryan et al., 2006; Edrington et al., 2012). Khachatryan et al. (2006) hypothesized that the greater fitness advantage of antimicrobial resistant *E. coli* in calves is the result of a linkage between resistance genes and genes conferring selective advantage in neonatal intestines. The age-associated decline in prevalence of antimicrobial resistant *E. coli* is suggested to be the result of losing their competitive advantage during maturation of the digestive system, in part as the result of being resistant; bacterial flora diversifies and increases in numbers resulting in a slow removal of antimicrobial

Table 4
ESBL/pAmpC genes identified in phenotypically confirmed ESBL/AmpC-EC isolates from individual calves (n = 155), young stock (n = 8) and dairy cows (n = 23). Results are presented at animal level, because ESBL/AmpC genes detected in multiple (one to three) isolates from the same animal were found to be identical.

			Calf Phenotype			Young stock Phenotype		Cow Phenotype	
ESBL/pAmpC	Gene group	Gene type	ESBL (n = 61)	AmpC (n = 93)	ESBL&AmpC (n = 1)	ESBL (n = 6)	AmpC (n = 2)	ESBL (n = 6)	AmpC (n = 17)
ESBL	$bla_{CTX-M-1}$	$bla_{CTX-M-1}$	22		1	5		3	
		$bla_{CTX-M-15}$	4						
		$bla_{CTX-M-22}$	4					1	
		$bla_{CTX-M-32}$	5						
		$bla_{CTX-M-55}$	3						
	$bla_{CTX-M-2}$	$bla_{CTX-M-2}$	8						
	$bla_{CTX-M-9}$	$bla_{CTX-M-14}$	3					1	
		$bla_{CTX-M-65}$	1						
	bla_{TEM}	$bla_{TEM-052c}$	1						
pAmpC	bla_{CMY}	bla_{CMY-2}		4					1
No. of animals with ESBL/pAmpC genes			51	4	1	5	0	5	1

resistant *E. coli* from the intestinal tract (Edrington et al., 2012).

We found that a between-herd prevalence which is solely based on faeces collected from floors of barns where dairy cows are housed, is an underestimation of the herd level prevalence of ESBL/AmpC-EC. Of around 60% of the herds, at least one of the samples directly collected from animals tested positive, whereas only 18.6% of the herds floor samples were found positive. Studies performed in other European countries report herd prevalence rates ranging from around 30 to almost 90% (Snow et al., 2012; Friese et al., 2013; Schmid et al., 2013; Hille et al., 2017). If in our study herd prevalence would have been based on testing pools of cow faeces, it would have been estimated at 23.0%. When a minimum number of shedding of 10^3 cfu of ESBL/AmpC-EC per gram of faeces of individual dairy cows had been used as a cut-off, the cow prevalence would be as low as 0.8%. This underlines the importance of choosing appropriate sample types and bacteriological methods when screening dairy herds for ESBL/AmpC-EC. When choosing sampling methods, the goal of a study, such as comparison with earlier or parallel studies, has to be taken into account. Our findings show that prevalence rates reported in literature not only depend on farm management practices and policy on antimicrobial use, but also on laboratory method and sample types.

Although in some herds no ESBL/AmpC-EC were isolated from any of the individual cow samples collected, these bacteria were isolated from the floors. This may be due to the number of dairy cows sampled, or to positive floor samples originating from faecal spread from positive young stock or calves, for example transmitted by boots. Additionally, it cannot be excluded that the bacteria have been introduced from the environment. These findings also show that classifying a herd or a group of animals, and probably even an individual animal, as negative for ESBL/AmpC-EC, likely is incorrect. We therefore prefer the term unsuspected of ESBL/AmpC-EC.

The ESBL/AmpC gene types encountered among isolates from calves, young stock, and dairy cows have been found previously in isolates from humans, companion animals, and livestock (Ewers et al., 2012; Baede et al., 2015; Ibrahim et al., 2016; Michael et al., 2017). The most common ESBL genes in animal isolates are $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-14}}$, followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ (EFSA, 2011). Among the genes encoding AmpC-type β-lactamases, $bla_{\text{CMY-2}}$ is the most common (EFSA, 2011). All ESBL gene types identified were also detected in the 2011-study (Gonggrijp et al., 2016), with the exception of $bla_{\text{CTX-M-22}}$ and $bla_{\text{CTX-M-65}}$. Also in the 2011-study, the most frequently identified ESBL gene was $bla_{\text{CTX-M-1}}$. The ESBL type $bla_{\text{CTX-M-15}}$ that is predominantly found in human isolates was detected in isolates from four calves (of one herd).

Negative results for ESBL genes for phenotypically confirmed ESBL-EC can be explained by the presence of ESBL genes that were not screened for, or the contribution of other, hitherto unknown mechanisms. Negative results for pAmpC genes for phenotypically confirmed AmpC-EC most likely can be explained by carriage of a chromosomal AmpC gene with expression-enhancing mutations in the promoter region (Tracz et al., 2007). Since plasmid-mediated diffusion of β-lactamases contributes to the rapid dissemination of these enzymes among bacteria, we focused on the detection of pAmpC genes.

Future studies should be aimed at determining sources and dynamics of ESBL/AmpC-EC transmission in dairy herds, including research on the age at which calves start excreting ESBL/AmpC-EC, stop excreting or start excreting lower levels. To determine the potential clonality of ESBL/AmpC-EC from the same herds, both isolates and the plasmids they carry need to be characterized in that type of studies.

5. Conclusion

Between 2011 and 2013, the period during which the use of 3rd- and 4th-generation cephalosporins was minimized, the between-herd prevalence of ESBL/AmpC-EC in Dutch dairy herds declined significantly. Calves were found to have both, a much higher individual animal prevalence and a higher level of shedding than young stock and cows. The most sensitive approach to find ESBL/AmpC-EC in Dutch dairy herds is through collecting samples from individual young calves.

Conflict of interest

None.

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