

## Increasing prevalence and diversity of ESBL/AmpC-type $\beta$ -lactamase genes in *Escherichia coli* isolated from veal calves from 1997 to 2010

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**Objectives:** Several studies on faecal carriage of extended-spectrum  $\beta$ -lactamase (ESBL)/AmpC-producing *Escherichia coli* have been performed in cattle, but little is known about faecal carriage in veal calves. This study describes the prevalence and molecular characteristics of ESBL/AmpC genes in *E. coli* isolated from faecal samples of veal calves from 1997 to 2010.

**Methods:** Pooled faecal samples were inoculated using selective enrichment broth and subsequently selective MacConkey agar. All isolates with reduced susceptibility to cefotaxime were screened by PCR and sequencing analysis for the presence of ESBL/AmpC genes.

**Results:** The prevalence of *E. coli* with reduced susceptibility to cefotaxime showed a discontinuous increasing trend, ranging from 4% in 1998 and 1999 to 39% in 2010. Promoter mutations of the chromosomal *ampC* gene were present in all years. In 2000, ESBL genes *bla*<sub>CTX-M-1</sub>, *bla*<sub>TEM-52</sub> and *bla*<sub>TEM-20</sub> were first observed. Before 2005 the majority of *E. coli* with reduced susceptibility to cefotaxime harboured *ampC* promoter mutations. From 2005 onwards the majority harboured *bla*<sub>CTX-M</sub> genes, of which *bla*<sub>CTX-M-1</sub> was the most abundant, followed by *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub>. The diversity of *bla*<sub>CTX-M</sub> genes gradually increased from one variant in 2000 to six variants in 2010. The prevalence of *bla*<sub>TEM-52</sub> was relatively low, but it was detected from 2000 onwards. *bla*<sub>CMY</sub> and *bla*<sub>SHV</sub> were found sporadically.

**Conclusions:** The prevalence and molecular diversity of genes encoding cefotaxime resistance in *E. coli* isolated from veal calves over a 14 year period showed an increasing trend. From 2005 onwards, *bla*<sub>CTX-M</sub> genes were most abundant, especially *bla*<sub>CTX-M-1</sub>.

**Keywords:** antimicrobial resistance, cattle, faecal carriage, retrospective

### Introduction

Extended-spectrum cephalosporins (ESCs) are used in both human and veterinary medicine. Resistance to ESCs may lead to therapy failure and is therefore of great concern.<sup>1</sup> Resistance to ESCs in Enterobacteriaceae is mainly caused by production of extended-spectrum  $\beta$ -lactamases (ESBLs) or AmpC  $\beta$ -lactamases.<sup>2</sup>

Faecal carriage of ESBL/AmpC-producing bacteria in cattle has been reported previously.<sup>3–6</sup> However, cattle are kept for different purposes in livestock production. Dairy farms are predominantly closed production systems, while at veal calf farms virtually all animals originate from different farms. Also, the housing facilities, farm management and the lifespan of the animals differ greatly. Furthermore, dairy cattle are generally less exposed to antimicrobials.<sup>7</sup> These differences may lead to

differences in prevalence and epidemiology of ESBL/AmpC-producing bacteria. In particular, data on faecal carriage of ESBL/AmpC-producing bacteria in veal calves are limited. The aim of this study was to retrospectively determine the emergence, trends and molecular characteristics of cefotaxime-resistant *Escherichia coli* in faecal samples obtained from veal calves in the Netherlands from 1997 to 2010.

### Materials and methods

#### Sampling design and method

Faecal samples were collected at farms by the Netherlands Food and Consumer Product Safety Authority (NVWA) from 1997 to 2010 to monitor zoonotic foodborne pathogens in food-producing animals.

Farms were randomly selected by the Foundation for Quality Guarantee of the Veal Sector (SKV), not taking animal age into account. On each farm one herd (a group of animals of similar age and maintained in the same open space) was sampled. Each faecal sample consisted of a fresh pooled sample of at least 100 g, picked from the floor equally distributed over the animal house. Samples collected from 1997 to 2005 were stored at the National Institute for Public Health and Environment (RIVM) in a 1:1 (w/v) suspension of faeces in tryptic soy broth with 30% glycerol at  $-80^{\circ}\text{C}$ . Samples collected from 2006 to 2010 were stored at the Central Veterinary Institute (CVI) in a 10% (w/v) suspension of faeces in buffered peptone water with 30% glycerol at  $-20^{\circ}\text{C}$ .

### *Escherichia coli* isolates

Each faecal sample was inoculated in 1 mL of Luria–Bertani broth (Becton Dickinson) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany) (LB+) using a sterile cotton swab and incubated overnight at  $37^{\circ}\text{C}$ . From these overnight cultures MacConkey agar plates (product number 212123, Becton Dickinson) supplemented with 1 mg/L cefotaxime (MC+) were inoculated and incubated overnight at  $37^{\circ}\text{C}$ . From each MC+ plate showing growth, one typical pink colony was selected for further analysis, and confirmed as *E. coli* by a check for tryptophan hydrolysis and subsequently by analysis on a MALDI Biotyper (Bruker Daltonics).

### ESBL/AmpC gene identification

All isolates were screened by PCR and sequence analysis for the presence of *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CMY</sub> and mutations in the promoter region of the chromosomally encoded *ampC* gene using primers and conditions as described previously.<sup>8</sup> The isolates were also screened for *bla*<sub>OXA</sub> group 1, 2 and 10.<sup>9</sup> All positive PCR products were sequenced using a Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems, USA) with additional primers for *bla*<sub>TEM</sub> (TEM-Fseq, 5'-GCCAACTACTTCTGCAACG) and *bla*<sub>CMY</sub> (CMY-F-838, 5'-TGGCGTATTGGCGATATGTA; CMY-R-857, 5'-TACATATCGCCAATACGCCA). All sequences were analysed using Sequencher v4.9 (Gene Codes Corporation, USA) and BioNumerics v6.6 (Applied Maths, Belgium). All mutations in the promoter region of the chromosomally encoded *ampC* gene were identified as described by Mulvey *et al.*<sup>10</sup> All isolates for which no ESBL/AmpC gene could be determined were screened on the Check-MDR CT-101 array platform (Check-Points, The Netherlands) according to the manufacturer's protocol.

### Statistical analysis

Prevalence data were analysed by a linear regression model with intercept and slope using the open source R statistics software.

### Results

The prevalence of cefotaxime-resistant *E. coli* in both datasets (1997–2005 and 2006–10) showed a discontinuous increasing trend, ranging from 4% in 1998 and 1999 to 39% in 2010 (Table 1). A relatively high prevalence of  $\geq 24\%$  was observed in 2002, 2004, 2005, 2009 and 2010. In contrast, a relatively low prevalence of  $\leq 10\%$  was observed in 1997–99, in 2003 and in 2008. The first sample set (1997–2005) showed an increase of 3.6% per year ( $P=0.004$ ). The second set (2006–10) showed an increasing trend of 6% per year ( $P=0.09$ ).

The diversity of ESBL/AmpC genes isolated from veal calves from 1997 to 2010 gradually increased (Table 1). Both the number of gene families and the diversity within gene families increased. In all years from 1997 to 2010, promoter mutations

of the chromosomal *ampC* gene were found. The most commonly found *ampC* promoter mutation was *ampC* type 3 (Table 1).

The vast majority of ESBL genes found in veal calves from 2000 onwards belonged to the *bla*<sub>CTX-M</sub> gene family (Table 1). In 2010, 65% of all isolates with non-wild-type cefotaxime susceptibility carried genes of the *bla*<sub>CTX-M</sub> gene family, of which *bla*<sub>CTX-M-1</sub> was most abundant (39% of all non-wild-type isolates). Other resistance genes belonging to the gene families of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CMY</sub> were found with a relatively low prevalence (Table 1).

In three isolates no ESBL/AmpC resistance gene could be determined. In these isolates, only the narrow-spectrum  $\beta$ -lactamase *bla*<sub>OXA-1</sub> was identified. Additional testing using the Check-MDR CT101 array also gave a negative result. These isolates were therefore designated 'unknown' (Table 1).

### Discussion

This study showed an increase in the prevalence and molecular diversity of genes encoding cefotaxime resistance in *E. coli* from veal calves over a 14 year period. Since the two sets of faecal samples were stored under different conditions, we cannot fully exclude a possible bias between the two sets of samples.

To our knowledge, similar studies on faecal carriage covering a large number of years have not been performed in other countries. In contrast to other studies, we used an enrichment supplemented with cefotaxime to increase the sensitivity of the isolation method. Using different sampling methods and different populations (e.g. dairy cows, veal calves or a mixed population) makes comparing prevalence data rather difficult. The best comparable study was performed in Switzerland, where 63 calves (maximum of 2 per farm) were included in the study and sampled at slaughter in 2009, 2010 and 2011. In that study 25% of the faecal samples were positive for *bla*<sub>CTX-M</sub>-producing *E. coli*, using EE broth enrichment and subsequently Brilliance ESBL agar.<sup>11</sup> This is similar to the prevalence we found in 2009. In contrast, the prevalence in other cattle (young cows, fattening bulls and bullocks) in this Swiss study was 1.6%, confirming the possible differences in prevalence between different kinds of cattle. Furthermore, in the UK in 2007–08, 7% of farms (mixed cattle population) that submitted faecal samples for diagnostic purposes were positive for *bla*<sub>CTX-M</sub>-producing *E. coli*.<sup>6</sup> These samples were cultured on selective plates that did not include a selective enrichment broth. In Poland no ESBL/AmpC-producing *E. coli* were found in 2009 (no enrichment; agar supplemented with 2 mg/L cefotaxime; cattle not further specified).<sup>12</sup> In a study performed in France in 2009, a prevalence of 5% was shown in a mixed population of healthy adults and diseased calves (ESBL screening agar supplemented with 4 mg/L cefotaxime or 4 mg/L ceftazidime).<sup>13</sup>

Mutations in the promoter region of the chromosomal *ampC* were found in all examined years (Table 1). All *ampC* variants except that from 2009 harboured mutations at one or more positions that have been previously described to cause a 'strong' promoter ( $-42$ ,  $-32$ ,  $-18$ ,  $-11$ ,  $-1$ ,  $+17$  to  $+37$  or  $+58$ ). Mutations at positions  $-42$  and  $-32$  are especially thought to have a large effect on promoter strength.<sup>14</sup> Our data show that, of all mutations in the chromosomal *ampC*, *ampC* type 3

**Table 1.** ESBL/AmpC genes detected in *E. coli* isolated from faecal samples from veal calves

	Year													
	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Number of faecal samples screened	49	49	49	47	51	50	23	50	50	152	170	160	180	182
Number (%) with non-wild-type cefotaxime susceptibility	3 (6)	2 (4)	2 (4)	6 (13)	7 (14)	13 (26)	2 (9)	14 (28)	18 (36)	16 (11)	33 (19)	16 (10)	44 (24)	71 (39)
ESBL/AmpC genes, number (%) <sup>a</sup>														
chromosomal <i>ampC</i> <sup>b</sup>														
<i>ampC</i> type 3	1 (33)	2 (100)	1 (50)	2 (33)	7 (100)	7 (54)	2 (100)	9 (64)	5 (28)	5 (31)	10 (30)	2 (13)	11 (25)	20 (28)
<i>ampC</i> type 34	1 (33)													
<i>ampC</i> type ND <sup>d</sup>	1 (33)		1 (50)	1 (17)		1 (8)			1 (6)				1 (2)	1 (1)
pAmpC														
<i>bla</i> <sub>CMY-2</sub>									1 (6)		2 (6)		1 (2)	2 (3)
ESBLs														
<i>bla</i> <sub>CTX-M-1</sub>				1 (17)		2 (15)		1 (7)	3 (17)	4 (25)	8 (24)	5 (31)	12 (27)	28 (39)
<i>bla</i> <sub>CTX-M-2/97</sub> <sup>e</sup>						1 (8)		1 (7)	2 (11)	1 (6)	4 (12)	1 (6)	3 (7)	3 (4)
<i>bla</i> <sub>CTX-M-14</sub>											1 (3)	1 (6)	6 (14)	7 (10)
<i>bla</i> <sub>CTX-M-15</sub>								1 (7)	2 (11)	2 (13)	3 (9)	3 (19)	4 (9)	3 (4)
<i>bla</i> <sub>CTX-M-32</sub>											1 (3)		2 (5)	2 (3)
<i>bla</i> <sub>CTX-M-79</sub>														2 (3)
<i>bla</i> <sub>TEM-52</sub>				1 (17)		2 (15)		1 (7)	2 (11)	3 (19)	2 (6)	3 (19)	2 (5)	
<i>bla</i> <sub>TEM-20</sub>				1 (17)										
<i>bla</i> <sub>SHV-12</sub>													2 (5)	2 (3)
combinations														
<i>bla</i> <sub>CTX-M-15</sub> + <i>bla</i> <sub>SHV-12</sub>														1 (1)
<i>bla</i> <sub>CTX-M-1</sub> + <i>ampC</i> type 3									1 (6)					
<i>bla</i> <sub>CTX-M-1</sub> + <i>ampC</i> type ND <sup>d</sup>									1 (6)					
<i>bla</i> <sub>TEM-52</sub> + <i>ampC</i> type 3											1 (3)			
<i>bla</i> <sub>TEM-52</sub> + <i>ampC</i> type ND <sup>d</sup>											1 (3)			
unknown <sup>c</sup>								1 (7)		1 (6)		1 (6)		

<sup>a</sup>Number and percentage of isolates with non-wild-type cefotaxime susceptibility harbouring this resistance gene.

<sup>b</sup>*ampC* types are designated as described by Mulvey et al.<sup>10</sup>

<sup>c</sup>No ESBL/AmpC gene was found; only the narrow-spectrum  $\beta$ -lactamase gene *bla*<sub>OXA-1</sub> was identified.

<sup>d</sup>ND, not defined, as described by Mulvey et al.<sup>10</sup> All ND variants, except from 2007 and 2009, harboured a mutation at position -42 or -32. The following mutations were observed in the ND variants from 2007 and 2009: 2007, -18 G→A, -11 C→T, -1 C→T and +58 C→T; and 2009, +57 C→G.

<sup>e</sup>Based on the primers used in this study, no distinction could be made between these two variants.

was most abundant. This was similar to a Canadian study in which samples of human clinical origin were analysed.<sup>10</sup>

This study also showed an increasing diversity in plasmid-mediated ESBL/AmpC gene families, as well as an increasing number of gene variants within a gene family (Table 1). The first ESBL genes in this study were found in 2000. These were *bla*<sub>CTX-M-1</sub>, *bla*<sub>TEM-52</sub> and *bla*<sub>TEM-20</sub>. To our knowledge, in other countries *bla*<sub>CTX-M-1</sub> was not found in cattle until 2003.<sup>15,16</sup> Furthermore, *bla*<sub>TEM-52</sub> was not reported in cattle until 2004, isolated from Danish cattle meat products imported from Germany, and *bla*<sub>TEM-20</sub> was reported in cattle meat from Tunisia in 2007.<sup>17,18</sup> From 2007 to 2010 the vast majority of ESBLs in our study were *bla*<sub>CTX-M-1</sub>, followed by either *bla*<sub>CTX-M-15</sub> in 2008 or *bla*<sub>CTX-M-14</sub> in 2009 and 2010. The high prevalence of these three *bla*<sub>CTX-M</sub> gene variants is in agreement with what is found in cattle in the rest of Europe, as has been summarized by Ewers *et al.*<sup>19</sup> In contrast to the low prevalence of TEM ESBLs, TEM  $\beta$ -lactamases, especially *bla*<sub>TEM-1</sub>, were highly dispersed (not shown).

In summary, in the years 1997–2010 both the prevalence and the diversity of different ESBL/AmpC-producing *E. coli* in veal calves increased. Further research is required to assess transmission between animals in relation to the health risks for the animals themselves, and to assess the relative importance of the food transmission route towards humans.

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## Transparency declarations

None to declare.

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