Prevalence, molecular typing, and antibiotic sensitivity of enteropathogenic, enterohaemorrhagic, and verotoxigenic *Escherichia coli* isolated from veal calves

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ABSTRACT

Cattle are considered to be an important reservoir of enterohaemorrhagic *Escherichia coli* (EHEC) and verotoxigenic *Escherichia coli* (VTEC) strains that can cause disease in humans, and numerous studies of the prevalence of these strains in cattle (focusing mainly on dairy and beef cattle) have been carried out in different regions of Europe, Asia, and America. To date, only a few studies of veal calves have been published focusing on EHEC strains belonging to the O157 serogroup EHEC, whereas EHEC and VTEC can belong to hundreds of different serotypes (many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113 and O145 serogroups). The aim of this study was to investigate the presence of enteropathogenic *Escherichia coli* (EPEC), EHEC, and VTEC strains in veal calves in Belgium and to characterize the positive isolates (serogroups, virulence-associated factor-encoding genes and antibiotic resistance profiles). The prevalence of EPEC, EHEC, and VTEC strains in faecal samples from veal calves in Belgium was found to be 11.7% (6.5% of the calves were found to be positive for EPEC strains, 2.6% for EHEC, and 3.9% for VTEC strains). No O157:H7 EHEC strain was identified, but three calves were found to carry strains belonging to the O26 and O111 serogroups. The results of antibiotic sensitivity tests showed a high level of resistance (83% of strains were resistant or intermediate resistant to five or more antibiotics of the 13 tested antibiotics), which might be caused by the frequent use of antibiotics in veterinary practice.

SAMENVATTING

Prevalentie, moleculaire typering en antibioticagevoeligheid van enteropathogene, enterohemorrhagische en verotoxigene* Escherichia coli *geïsoleerd uit vleeskalveren

Rundvee wordt gezien als belangrijk reservoir van enterohaemorrhagische Escherichia coli *(EHEC)- en verotoxigene* Escherichia coli *(VTEC)-stammen die mensen ziek kunnen maken. In verschillende regio's in Europa, Azië en Amerika is veelvuldig onderzoek gedaan naar het voorkomen van deze stammen bij rundvee (waarbij de nadruk vooral lag op melk- en vleeskoeien). Tot op heden zijn echter slechts enkele studies verschenen bij vleeskoeien. Hierbij lag de nadruk op de EHEC-stammen uit de O157-serogroep EHEC. Maar zowel EHEC als VTEC komen voor in honderden verschillende serotypes, waarvan er veel net zo gevaarlijk zijn voor mensen als de O157 :H7 EHEC, zoals de serogroepen O26, O91, O103, O111, O113 en O145.*

Dit onderzoek had als doel om de aanwezigheid van enteropathogene Escherichia coli *(EPEC)-, EHEC- en VTEC-stammen in vleeskalveren in België in kaart te brengen en de positieve isolaten te karakteriseren (op serogroep, genen voor virulentiegeassocieerde factoren en antibioticaresistentieprofielen). De prevalentie van EPEC-, EHEC- en VTEC-stammen in ontlastingsmonsters van vleeskalveren in België bedroeg 11,7 procent (6,5 procent van de kalveren bleek positief te zijn voor EPEC-stammen, 2,6 procent was positief voor EHEC-stammen en 3,9 procent was positief voor VTEC-stammen). Er werd geen O157 :H7 EHEC-stam geïdentificeerd, maar bij drie kalveren werden stammen aangetroffen uit de O26- en O111-serogroepen. De resultaten van de antibioticagevoeligheidstesten lieten een hoog resistentieniveau zien (83 procent van de stammen waren resistent of gemiddeld resistent tegen vijf of meer van de dertien onderzochte antibiotica). De hoge resistentie wordt mogelijk veroorzaakt door het veelvuldige gebruik van antibiotica in de diergeneeskundige praktijk.*

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC), enterohaemor-

rhagic *Escherichia coli* (EHEC), and verotoxigenic *Escherichia coli* (VTEC) represent three important classes of enteric pathogens that can cause enteritis and enterotoxaemia in humans and animals. These pathogens are defined on the basis of two main virulence properties (28). The main virulence property of EPEC strains is the production of a specific histological and ultrastructural lesion called an "attaching and effacing lesion" (A/E lesion), characterized by the loss (= effacement) of the microvilli of the enterocytes as a consequence of cytoskeleton rearrangements initiated by type III-secreted (T3S) bacterial effectors, and by the intimate (<10 nm) attachment of the bacteria to host enterocytes, via an interaction between an outer membrane protein named intimin and one of the T3S effectors called Tir (for Translocated Intimin Receptor) (25). The main virulence property of VTEC strains is the production of verotoxins (VTS) (or Shiga-like toxins, STXS or SLTS), which are lethal to eukaryotic cells both in vitro (Vero, HeLa, and/or MDBK cells) and in vivo (endothelial cells), by blocking protein synthesis (19). EHEC strains share the main virulence properties of EPEC and VTEC strains: the production of A/E lesions and VTS (STXS). Today, EHEC strains are considered to have evolved from EPEC strains through the acquisition of bacteriophages carrying stx genes encoding for SLT (30, 37).

In contrast to their limited importance in developed countries, EPEC strains are a major cause of infant diarrhoea in developing countries, often associated with high mortality rates (8). EPEC strains infect mainly infants under 2 years of age. EPEC strains are also associated with diarrhoea in most domestic animal species. In bovines, EPEC strains are associated with diarrhoea in 1- to 8-week-old calves (9, 27). The diarrhoea is a consequence of the production of the A/E lesion and of the ensuing inflammatory response of the host (8).

EHEC strains can cause different intestinal and extra-intestinal syndromes in humans: undifferentiated diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), and thrombotic thrombocytopaenic purpura (TP) (29). In developed countries (USA, Canada, UK, France, Japan, etc) EHEC strains are responsible for individual human cases, and for small-to-large outbreaks (13, 24, 31-33, 35). Human infection can occur via consumption of vegetable and other foodstuffs contaminated by faeces from ruminants (mainly cattle), which may be asymptomatic healthy carriers (6, 15). Nevertheless, some EHEC strains are also responsible for undifferentiated diarrhoea in young calves of up to 3 months of age (23, 27, 34).

VTEC strains cause clinical syndromes mainly in humans and piglets but can also be isolated from a wide range of domestic and wild animals, which are healthy asymptomatic carriers (38). VTEC infections are not common in humans but, when they happen, they are frequently associated with HUS. VTEC strains are also responsible for oedema disease in piglets, which occurs up until 2 weeks after weaning (26). While in most cases the source of human infection is foodstuffs contaminated with ruminant faeces, human and porcine VTEC strains are different and no cross-contamination has been reported.

Cattle thus represent an important reservoir of EHEC and VTEC strains that can cause disease in humans (5, 7, 16, 23). Consequently, numerous studies of their prevalence in cattle have been carried out in different regions of Europe, Asia, and America (3, 14, 18, 20, 21, 36), focusing mainly on dairy and beef cattle. To date, only five studies of veal calves have been published, which focused their search on EHEC strains belonging to the O157 EHEC (4, 11, 12, 14, 17), whereas EHEC and VTEC can belong to hundreds of different serotypes, many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113 and O145 serogroups.

The aim of this study was to investigate the presence of EPEC, EHEC, and VTEC strains in veal calves in Belgium using polymerase chain reaction assays (PCRS) targeting the genes coding for intimin adhesin (eae) and for VTS (vt1, vt2, vt2c). The positive isolates were further characterized by PCR for other virulence-associated factor-encoding genes (EHEC-hlyA, bfp) and for five of the most important somatic serogroups (O26, O103, O111, O145, and O157). They were also tested for their antibiotic resistance profiles against 13 frequently used antibiotics.

MATERIALS AND METHODS

Collection of specimens and isolation of *E. coli* strains

All the samples were collected between January and May 2008 on one commercial farm [?company] producing approximately 120,000 veal calves per year. A first group of samples (G1) consisted of rectal swabs or faecal samples from veal calves aged 1 to 20 weeks. The second group of samples (G2) consisted of jejuno-ileal content from 6-month-old veal calves taken at the time of slaughter.

The samples were inoculated onto Gassner agar plates and incubated for 18 hours at 37 °C. Subsequently, five lactose-positive colonies per calf were picked up and transferred into LB broth with 0.1% tryptophan. Bacteria were grown for 8 hours at 37 °C, and Kovacs reagent was added to detect indole production. Indole-positive isolates were stored at -80 °C in 20% glycerol until further characterization.

Genotypic characterization

DNA extraction was carried out as described previously by China et al. (10). Briefly, a pure bacterial culture was grown for 8 hours at 37 °C in LB broth with slight agitation. Then 300 µl was centrifuged for 1 min at 13,000 rpm and the supernatant was discarded. After the addition of 50 µl of sterile water, the suspension was boiled for 10 min. Afterwards, the suspension was centrifuged for 1 min at 13,000 rpm and the supernatant was stored at -20 °C.

All PCR conditions for the detection of the eae, vt1, vt2, wzxO26, fliCH11, rfbO157, wzxO111, wzxO103, wzxO145, vt2c, bfpA, and EHEC-hlyA genes have been described

Primer name	Sequence (5' to 3')	Target gene	Annealing temp. (°C)	Amplicon size (bp)	Reference
B52	AGGCTTCGTCACAGTTG	eaeA	50	570	(China et al., 1996)
B53	CCATCGTCACCAGAGGA				
B54	AGAGCGATGTTACGGTTTG	slt-I	50	388	(China et al., 1996)
B55	TTGCCCCCAGAGTGGATG				
B56	TGGGTTTTTCTTCGGTATC	slt-II	50	807	(China et al., 1996)
B57	GACATTCTGGTTGACTCTCTT				
wzx-wzyO26-F	AAATTAGAAGCGCGTTCATC	wzxO26	56	596	(Durso et al., 2005)
wzx-wzyO26-R	CCCAGCAAGCCAATTATGACT				
fliC-H11-F	ACTGTTAACGTAGATAGC	fliCH11	56	224	(Durso et al., 2005)
fliC-H11-R	TCAATTTCTGCAGAATATAC				
wzxO157-F	CGGACATCCATGTGATATGG	rfbO157	60	259	(Paton & Paton, 1998)
wzxO157-R	TTGCCTATGTACAGCTAATCC				
wzxO111-F	TAG AGA AAT TAT CAA GTT AGT TCC	wzxO111	62	406	(Paton & Paton, 1998)
wzxO111-R	ATA GTT ATG AAC ATC TTG TTT AGC				
wzxO103-F	TTGGAGCGTTAACTGGACCT	wzxO103	57	321	(Fratamico et al., 2005)
wzxO103-R	GCTCCCGAGCACGTATAAG				
wzxO145-F	CCATCAACAGATTTAGGAGTG	wzxO145	59	609	(Feng et al., 2005)
wzxO145-R	TTTCTACCGCGAATCTATC				
slt-IIc-F	GCGGTTTTATTTGCATTAGT	slt-IIc	52	124	(Osek, 2003)
slt-IIc-R	AGTACTCTTTTCCGGCCACT				
bfpA-F	AATGGTGCTTGCGCTTGCTGC	bfpA	56	326	(Gunzburg et al., 1995)
bfpA-R	GCCGCTTTATCCAACCTGGTA				
EHEC-hlyA-F	ACGATGTGGTTTATTCTGGA	EHEC-hlyA	58	165	(Fagan et al., 1999)
EHEC-hlya-R	CTTCACGTGACCATACATAT				

Table 1: primers used in this study.

previously (Table 1). All PCR products were separated by 1.5% agarose gel electrophoresis. Gels were stained with SYBR Green and were visualized under UV light.

A Fisher's exact test was performed to assess statistical differences ($p<0.01$) between the different groups of animals.

Antibiotic susceptibility tests

Susceptibility tests were carried out on the positive isolates for the eae, vt1, and/or vt2 genes, using the disc diffusion method of Bauer et al. (2) on Mueller-Hinton agar (Oxoid,). Zones of inhibition were measured (in millimetres) after overnight incubation at 37 °C and were interpreted according to the CLSI (Clinical and Laboratory Standards Institute) (CLSI, 2008). Thirteen antibiotics used on the farm were tested: cefuroxime (30 µg), ceftiofur (30 µg), ampicillin (10 µg), neomycin (5 µg), enrofloxacin (5 µg), the combination of trimethoprim-sulfamethoxazole (1.25 µg-23.75 µg), tetracycline (30 µg), (Becton Dickinson), florfenicol (30 µg), flumequin (30 µg) (Oxoid), tylosin (150 µg), and the combination of lincomycin-spectinomycin (15 ²g-200 µg) (Neo-Sensitabs).

RESULTS

Prevalence of EPEC, EHEC and VTEC strains in veal calves (Table 2)

One hundred and ninety-five strains of *E. coli* (g1) isolated from the faeces of 39 diarrhoeic and non-diarrhoeic calves (G1) aged between 1 and 20 weeks of age, and 190 strains (g2) isolated from intestinal content of 38 non-diarrhoeic 6-month-old calves (G2) were examined for virulence factors. According to the PCR results, 11.7% of the calves were carriers of one of the three pathotypes (6.5% of the calves were found positive for EPEC strains, 2.6% for EHEC, and 3.9% for VTEC strains). The percentage of carriers did not differ between G1 (5+/39) and G2 (4+/38), between diarrhoeic (1+/8) and non-diarrhoeic (8+/69) calves, or between Belgian Blue (1+/30) and Black and White calves (8+/47) (Fisher Exact Test, $p<0.01$). Eighteen of the isolates from 9 different calves were positive with the multiplex PCR for eae, vt1, and vt2 genes: 5 isolates were eae+vt1+ (EHEC); 3 isolates were vt2c+ (VTEC); 1 isolate was vt1+ (VTEC); and 9 isolates were eae+ (EPEC). The five EHEC strains were isolated from two calves and the four VTEC strains from 3 calves in the G1 group (Table 2). The nine EPEC strains were isolated from the four calves in the G2 group and from one calf in the G1 group.

Typing of the PCR-positive isolates (Table 2)

Of the most frequent EHEC serogroups (O157, O26, O111, O103, and O145), the O26 and O111 serogroups were identified in 5 isolates (all were eae+ and vt1+) and 1 isolate (vt1+), respectively, but no isolates belonging to the O157, 0145 or 0103 serogroup were detected. All isolates of the O26 serogroup were positive for the fliCH11 gene.

All of the EPEC, EHEC, and VTEC isolates tested negative with the PCR for the bfp gene, and 11 strains (the five O26 EHEC, the O111 VTEC and five EPEC) tested positive with the PCR for the EHEC-hlyA gene, all but one isolated from five calves from the G1 group (Table 2).

Antibiotic susceptibility tests (Table 2)

Of the 18 EPEC, EHEC, and VTEC isolates, none was sensitive or resistant to all of the thirteen antibiotics tested. One isolate showed intermediate resistance to one antibiotic and two isolates showed intermediate resistance to two antibiotics, and these three strains were sensitive to all of the other antibiotics tested. Fifteen strains were resistant to up to three of the thirteen antibiotics tested.

DISCUSSION

Cattle are considered to be a major reservoir of EPEC, EHEC, and VTEC strains (6, 7, 16, 22). Numerous studies of their prevalence in cattle have been carried out in different regions of Europe, Asia, and America (3, 14, 18, 20, 21, 36), focusing mainly on dairy and beef cattle. To date, only five other studies of veal calves have been published and focused on EHEC strains belonging to the O157 EHEC (4, 11, 12, 14, 17), whereas EHEC and VTEC can belong to hundreds of different serotypes, many of which are as dangerous to humans as the O157:H7 EHEC, such as strains of the O26, O91, O103, O111, O113, and O145 serogroups. The aim of this study was to determine the prevalence of EPEC, EHEC, and VTEC strains in veal calves in Belgium. To our knowledge, it is the first study of this type on veal calves in Belgium.

The prevalence of EPEC, EHEC, and VTEC strains in faecal samples from Belgian veal calves was 11.7%: 2.6% of the calves were positive for EHEC strains, 6.5% for EPEC, and 3.9% for VTEC strains. No O157:H7 EHEC strain was identified, but three calves (3.9% of the calves) were found to carry strains belonging to the "gang of five" serogroups (O157, O26, O111, O103, O145), which are frequently associated with human disease: five EHEC isolates belonged to the O26 serogroup and one VTEC isolate belonged to the O111 serogroups. Although infrequently infected, veal calves could be considered as a potential vector of EHEC and VTEC strains that can infect humans. However, these O26 and O111 strains may also be more cattle-specific and cause diarrhoea in veal calves. Indeed, EHEC strains belonging to some serogroups, including O26, O111, and O118, are also responsible for undifferentiated diarrhoea in young calves up to 3 months of age (23).

The other EPEC and VTEC strains identified did not belong to these five serogroups dangerous for humans. There are two possibilities: (1) the other serogroups do not infect humans, or do so only occasionally, and so there is a low potential risk of zoonosis, or (2) the other serogroups represent pathogenic serogroups that can lead to human infections. The 2007 annual report of the European Union on zoonotic agents (1) supports the second hypothesis. In 2007, 29% of reported confirmed VTEC cases in humans concerned untyped or strains of untypeable serogroups that did not belong to the gang of five (O157, O26, O111, O103, and O145). However, comparison of the percentage of VTEC strains found in veal calves (6.5% in our study), the percentage of VTEC found on beef carcasses in Belgium (0.4% of 1611 carcasses in 2007), and the percentage of reported confirmed VTEC cases in humans (0.4 cases per 100,000 habitants in 2007) (Anonymous, 2009) shows that food safety practices are well applied in Belgian slaughterhouses and that the zoonotic risk is limited in Belgium.

The results of the antibiotic sensitivity tests were disappointing in view of the high percentage of resistance, which might be explained by the frequent use of the tested antibiotics in veterinary practice. In total, 83% of the strains were multiresistant (resistant to more than two antibiotics) and, in our case, these multiresistant strains were resistant or showed intermediate resistance to five or more antibiotics of the 13 antibiotics tested. On average, strains were resistant to 4.5 ± 2.2 antibiotics and showed intermediate resistance to 1.4 ± 1.3 antibiotics, with 28% of the strains showing resistance or intermediate resistance to eight or more antibiotics. A challenge would be to reduce the use of antibiotics and to use them only when it is strictly necessary, to avoid the emergence of multiresistant pathogenic strains.

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Population	Calf n°	Strain n°	slt	eae	Serogroup	EHEC-hlyA	bfp	Antibiotic resistance pattern
G1	11	11.2	1	+	O26:H11	+	-	A: R; T: R; L/S: I; TTC: R; T/S: R
	11	11.4	1	+	026:H11	+	-	A: R; T: R; L/S: I; TTC: R; T/S: R
	15	15.3	2c	-	NI	-	-	A: R; N: R; T: R; TTC: R; T/S: R
	15	15.5	2c	-	NI	-	-	N: R; T: R; TTC: R; T/S: R
	18	18.1	-	+	NI	+	-	A: R; CX: I; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.3	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.4	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	18	18.5	-	+	NI	+	-	A: R; T: R; L/S: I; TTC: R; FF: I; T/S: R
	20	20.1	1	+	O26:H11	+	-	T:I
	20	20.2	1	+	O26:H11	+	-	CX: I; T: I
	20	20.3.1	1	+	O26:H11	+	-	CX: I; T: I
	20	20.3.2	2c	-	NI	-	-	N: R; T: R; TTC: R; T/S: R
	25	25.1	1 and 2	-	O111	+	-	N: R; T: R; TTC: R; T/S: R
G2	59	59.4	-	+	NI	-	-	A: R; T: R; TTC: R; T/S: R
	63	63.5	-	+	NI	-	-	A: R; N: R; T: I; TTC: R; T/S: R; F: R
	68	68.3	-	+	NI	-	-	A: R; CX: I; T: R; TTC: R; T/S: R
	68	68.4	-	+	NI	-	-	A: R; T: R; TTC: R; T/S: R; F: R; E: R
	70	70.2	-	+	NI	+	-	A: R; N: R; T: I; L/S: R; TTC: R; T/S: R

Table 2: results obtained for each strain (NI: not identified; A: ampicillin; CX: cefuroxim; E: enrofloxacin; FF: florfenicol; F: flumequin; L/S: lincomycin/spectinomycin; N: neomycin; T: tylosin; T/S: trimethoprim/sulfamethoxasol; TTC: tetracyclin; R: resistant; I: intermediate).

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