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Detection and Characterization of Verocytotoxin-Producing *Escherichia coli* by Automated 5′ Nuclease PCR Assay

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In recent years increased attention has been focused on infections caused by isolates of verocytotoxin-producing *Escherichia* coli (VTEC) serotypes other than O157. These non-O157 VTEC isolates are commonly present in food and food production animals. Easy detection, isolation, and characterization of non-O157 VTEC isolates are essential for improving our knowledge of these organisms. In the present study, we detected VTEC isolates in bovine fecal samples by a duplex 5′ nuclease PCR assay (real-time PCR) that targets *vtx1* and *vtx2*. VTEC isolates were obtained by colony replication by use of hydrophobic-grid membrane filters and DNA probe hybridization. Furthermore, we have developed 5′ nuclease PCR assays for the detection of virulence factors typically present in VTEC isolates, including subtypes of three genes of the locus of enterocyte effacement (LEE) pathogenicity island. The 22 assays included assays for the detection of verocytotoxin genes (*vtx1*, *vtx2*), pO157-associated genes (*ehxA*, *katP*, *espP*, and *etpD*), a recently identified adhesin (*saa*), intimin (*eae*, all variants), seven subtypes of *eae*, four subtypes of *tir*, and three subtypes of *espD*. A number of reference strains (VTEC and enteropathogenic *E. coli* strains) and VTEC strains isolated from calves were tested to validate the PCR assays. The expected virulence profiles were detected for all reference strains. In addition, new information on the subtypes of LEE genes was obtained. For reference strains as well as bovine isolates, a consistent relationship between subtypes of the LEE genes was found, so that a total of seven different combinations of these were recognized (corresponding to the seven subtypes of *eae*). Isolates with 15 different serogroup-virulence profiles were isolated from 16 calves. Among these, 53% harbored LEE and 73% harbored factors carried by the large virulence plasmid. One LEE-negative isolate had the gene for the adhesin Saa. The most common virulence profile among the bovine isolates was *vtx1*, *eae*-ζ, *tir*-α, *ehxA*, and *espP*. This panel of assays offers an easy method for the extensive characterization of VTEC isolates.

A large number of different serotypes of verocytotoxin (VT)-producing *Escherichia coli* (VTEC)—also known as Shiga toxin-producing *E. coli*—have been identified as causative agents of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (http://www.sciencenet.com.au/vtectable.htm). The most well known VTEC serotype is *E. coli* O157:H7, which has been implicated in many large outbreaks of HC and HUS. However, VTEC strains of other serotypes have increasingly been implicated in sporadic cases and outbreaks of serious illness, e.g., serotypes O26, O111, O103, and O145 (51). VTEC strains known to be pathogenic for humans as well as VTEC strains with an unknown potential to cause HC and HUS are commonly isolated from healthy cattle. Among the most important sources of human infection are direct contact with cattle and other ruminants and contaminated bathing water, beef products, unpasteurized milk, vegetables, fruits, and drinking water (48).

The focus on *E. coli* O157 instead of other VTEC serotypes has been further enhanced by the ease of isolation of *E. coli* O157 due to fast and sensitive methods based on immunomagnetic capture and selective or indicative media. In contrast, the isolation of other VTEC serotypes from human patients and potential reservoirs and sources has been hampered by the lack of such methods to isolate all relevant VTEC strains. PCR detection of VT genes in fecal samples and foods has, however, been widely used (12, 34). However, if a bacterial isolate is wanted, e.g., for the important further analysis of its virulence profile, a subsequent step of isolation is needed. VTEC isolates are usually considerably outnumbered by other *E. coli* strains, especially in fecal samples from healthy carriers of VTEC, e.g., cattle. As no selective or indicative medium exists for strains of all VTEC serotypes, most approaches for the isolation of such strains are laborious, e.g., PCR screening of a large number of colonies, and have a low rate of success. However, colony replication by use of hydrophobic-grid membrane filters (HGMFs) and DNA probe hybridization has been used with success for the isolation of VTEC strains from animal fecal samples (9, 47).

The frequent presence of VTEC in cattle and other ruminants calls for further analysis of the pathogenic potential for humans of VTEC strains isolated from nonhuman sources. An increasing number of putative virulence genes of VTEC have been characterized. At present, the most relevant virulence factors are considered to be the VTs and variants of these, the pathogenicity island locus of enterocyte effacement (LEE), and factors situated on the large virulence plasmid of many VTEC strains. Production of VT is the single most important factor, which is considered essential for the development of HC and HUS (30). The two main toxins are VT1 and VT2; however, a number of VT2 variants exist, and these possess various biological effects (30). The LEE region of *E. coli* O157 strain EDL933 has 54 genes (38) and codes for proteins for intimate adhesion to the intestinal epithelium and the generation of the

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characteristic attaching-and-effacing lesions. LEE encodes a type III secretion system, the outer surface protein intimin (coded by *eae*), the translocated receptor Tir, and several secreted effector proteins (Esp) (11, 29, 38)

Most of the frequently isolated VTEC serotypes causing HC and HUS possess LEE, or at least *eae*, as tests for other genes in the region are usually not conducted. However, important exceptions exist; e.g., it was found that strains of VTEC O91 and O113 responsible for an HUS outbreak lacked the LEE region (36). Recent findings have shown that these LEE-negative strains have another adhesin, designated Saa, which is probably encoded on the large virulence plasmid (35). The large virulence plasmid of two *E. coli* O157 strains has been fully sequenced (7, 28); and a number of putative virulence factors have been identified on this plasmid, e.g., enterohemolysin (*ehxA*), katalase-peroxidase (*katP*) (5), a type II secretion system (*etp*) (42), and a serine protease (*espP*) (6).

VTEC strains isolated from cattle, food, and other animal sources have various virulence profiles; and to assess the potential virulence of VTEC isolates from these sources, it is important to be able to examine them for the presence of virulence genes. Furthermore, large biologically important variations have been identified in several of these virulence factors, especially VT2 and the LEE region. For example, differences in lengths, insertion sites, and gene sequences have been found for the LEE region (11, 37, 38, 53); and specific variants of intimin have been found to be related to VTEC strains pathogenic for humans, whereas other intimin variants have been found to be related to human or animal enteropathogenic *E. coli* (EPEC) strains (1, 33). It has recently been shown that the intimin variant influences the site of colonization; e.g., intimin-γ from *E. coli* O157 appears to restrict colonization to Peyer's patches of the human intestine (39).

PCR is widely used for the detection of virulence factors, and PCR techniques are generally known to be sensitive and specific methods. Gel electrophoresis is most widely used for detection of the amplified product. However, this method lacks specificity; and other post-PCR processing steps should be performed to ensure specific detection, e.g., probe hybridization or restriction fragment analysis, which is time-consuming and not conducive to rapid, high-throughput automated schemes. TaqMan 5′ nuclease assays allow automated PCR amplification, detection, and analysis. This approach uses dual labeled fluorogenic hybridization probes incorporated into PCR and exploits the 5′→3′ exonuclease activity of *Taq* DNA polymerase to hydrolyze these probes during the DNA polymerization step (18, 24). The probe is labeled with a reporter dye and a quencher dye, and for the intact probe, the quencher dye suppresses the fluorescent emission of the reporter dye because of the spatial proximity of the probe. If hybridization occurs, the probe is cleaved by the 5′ nuclease activity of the DNA polymerase during extension of the primer (24). This separates the reporter dye from the quencher dye and generates an increase in the fluorescence signal of the reporter dye. Repeated PCR cycles result in exponential amplification of the PCR product and a corresponding increase in fluorescence intensity. The development of reporter signals is monitored throughout the PCR by a fluorometer and eliminates the need for post-PCR sample handling. The 5′ nuclease assays have recently been used for the detection and characterization of pathogens, e.g., for the detection of *Vibrio cholerae* (27), identification of *Salmonella* (19), and detection of virulence factors in porcine *E. coli* strains (14); and recently, assays that detect up to four virulence factors of *E. coli* O157 and other VTEC strains have been reported (40, 43).

TABLE 1. Validation of the 5′ nuclease detection assays by testing positive and negative control strains[a]

Target gene	Positive control strain	Negative control strain(s)
vtx1	EDL933	E2348/69
vtx2	E32511 (*vtx2*, *vtx2c*)	E2348/69, DVI-94/1 (*vtx2e*)
eae	EDL933	B2F1
eae-α	E2348/69	RDEC-1, DVI-828, EDL933, D276/1/1, TB154A, DVI-797
eae-β	RDEC-1	E2348/69, DVI-828, EDL933, D276/1/1, TB154A, DVI-797
eae-δ	DVI-828	E2348/69, RDEC-1, EDL933, D276/1/1, TB154A, DVI-797
eae-γ	EDL933	E2348/69, RDEC-1, DVI-828, D276/1/1, TB154A, DVI-797
eae-θ	D276/1/1	E2348/69, RDEC-1, DVI-828, EDL933, TB154A, DVI-797
eae-ε	TB154A	E2348/69, RDEC-1, DVI-828, EDL933, D276/1/1, DVI-797
eae-ζ	DVI-797	E2348/69, RDEC-1, DVI-828, EDL933, D276/1/1, TB154A
tir-α, *espD*-α	E2348/69	RDEC-1, EDL933, D276/1/1
tir-β, *espD*-β	RDEC-1	E2348/69, EDL933, D276/1/1
tir-γ, *espD*-γ	EDL933	E2348/69, RDEC-1, D276/1/1
tir-θ	D276/1/1	E2348/69, RDEC-1, EDL933
ehxA	EDL933	E2348/69
katP	EDL933	E2348/69
espP	EDL933	E2348/69
etpD	EDL933	E2348/69
saa	B2F1	EDL933

[a] The serotypes of the strains are listed in Table 2 or Table 4.

The objective of the present study was to develop a reliable method for the detection and isolation of all VTEC strains from animal feces and to develop an easy system for determination of the most important virulence factors in VTEC isolates. The panel of virulence factors in the detection system includes two *vtx* variants; *eae*, *tir*, and *espD* and variants of these (LEE region genes); *saa*; and four genes on plasmid pO157 (*ehxA*, *katP*, *etD*, and *espP*); this plasmid is present in many VTEC serotypes other than O157.

MATERIALS AND METHODS

Bacterial strains. *E. coli* reference strains were used as positive and negative controls for the panel of primers and probes used for the detection of virulence factors (Table 1). In addition, other well-characterized *E. coli* O157 strains were included (Table 2). Strains 493/89, TB154A, RDEC-1, and 90-1787 were kindly provided by Tom Whittam, The National Food Safety and Toxicology Center, Michigan State University. The other strains were from our in-house collection.

Preparation of DNA from pure cultures. Bacterial strains were grown (18 to 24 h at 37°C) on blood agar plates, and one loopful (approximately 10 μl) of bacterial culture was suspended in 200 μl of sterile distilled water and lysed at 100°C for 10 min.

Probe and primer design. Primer and probe sets were designed for detection of the following VTEC- and EPEC-related virulence factors: genes for the two main VT subtypes (*vtx1* and *vtx2*); four plasmid-borne genes (*ehlyA*, *katP*, *espP*, and *etpD*); three genes of the LEE pathogenicity island (*eae*, *tir*, and *espD*), including variants of these; and the gene encoding the STEC autoagglutinating adhesin, Saa (*saa*). Primer Express Software (version 2.0; Applied Biosystems, Foster City, Calif.), together with the corresponding guidelines (User's Manuel; Applied Biosystems), was used to design the primers and probes for the TaqMan PCR. BLAST N database searches were done, and the primer and probe sequences were designed to be specific for a region that had no homology with

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TABLE 2. Results of characterization of reference strains and other well-described strains

Serotype[a]	Origin	Strain	Toxins		LEE-related genes				Plasmid-borne genes				
			vtx1	*vtx2*	*eae*	*eae* subtype	*tir* subtype	*espD* subtype	*ehxA*	*katP*	*espP*	*etpD*	*saa*
O127:H6	Human	E2348/69	−	−	+	α	α	α	−	−	−	−	−
X03:NM	Cow	90-1787	−	−	+	α	α	α	−	+	+	+	−
O26:H11	Human	H19	+	−	+	β	β	β	+	+	+	−	−
O15:NM	Rabbit	RDEC-1	−	−	+	β	β	β	−	−	−	−	−
O49:Hnd	Calf	DVI-828	−	−	+	δ	α	α	+	−	+	−	−
O4:NM		B1096/1/1	−	+	+	ε	β	β	+	+	+	−	−
O8:H19		B2619/21	−	+	+	ε	β	β	−	−	−	+	−
O45:H2	Human	A2619-C2	+	−	+	ε	β	β	+	−	−	+	−
O103:H6	Human	TB154A	+	−	+	ε	β	β	+	−	−	+	−
O157:H7	Human	EDL933	+	+	+	γ	γ	γ	+	+	+	+	−
O157:H−	Human	E32511	−	+	+	γ	γ	γ	+	+	+	+	−
O157:H7	Human	CCUG29188	−	−	+	γ	γ	γ	+	+	+	+	−
O157:H−	Human	493/89 (SF)	−	+	+	γ	γ	γ	+	−	−	+	−
O157:H7	Cattle	DVI-104	+	+	+	γ	γ	γ	+	+	+	+	−
O157:H7	Cattle	DVI-106	−	−	+	γ	γ	γ	+	+	+	+	−
O111:Hnd		D276/1/1	+	−	+	θ	θ	−	+	+	+	−	−
O111:H−	Human	C1177-01	+	+	+	θ	θ	−	+	+	+	−	−
O91:H21	Human	B2F1	−	+	−	−	−	−	+	−	+	−	+
O113:Hnd	Calf	DVI-450	−	+	−	−	−	−	+	−	+	−	+
O139:Hnd	Pig	DVI94/1	−	−[b]	−	−	−	−	−	−	−	−	−

[a] NM, nonmotile; nd, not determined.
[b] The strain is positive for *vtx2e*.

other known regions of interest in the database and that covered all relevant strains in the database. The probes and primers, listed in Table 3, were synthesized by DNA Technology (Århus, Denmark). Specifically, the *vtx2*-specific assay included primers and probes specific for all *vtx2* variants except *vt2e*, which is related to edema disease in pigs and which is rarely related to disease in humans.

TaqMan PCR assays. The 5′ nuclease PCR assays were carried out in 20-μl volumes containing 2 μl of template lysate, 600 nM each primer, 200 nM each probe, and the TaqMan Universal Master Mix (Applied Biosystems). The Master Mix contained AmpErase uracil-*N*-glycosylase (UNG), deoxynucleoside triphosphates with dUTPs, 6-carboxy-*S*-rhodamine as an internal passive fluorogenic reference, and optimized buffer components. Multiplex as well as single-reaction assays were performed with these concentrations. To optimize the work routine and minimize waste of the Master Mix, batches of mix in 70 to 80 tubes with one or two factors (for nonmultiplex or multiplex PCR) were prepared and stored at −20°C. A few minutes before use the required numbers of tubes were taken from the freezer and template was added.

Thermal cycling consisted of initial steps at 50°C for 2 min, which is required for optimal AmpErase UNG enzyme activity, and 95°C for 10 min, to activate the AmpliTaq Gold DNA polymerase and to deactivate the AmpErase UNG enzyme. This was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. An ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used for amplification and fluorescence measurement.

Post-PCR analysis. The fluorescent intensity of each dye was measured with the ABI Prism 7700 Sequence Detection System at every temperature step and cycle during the reaction. Data acquisition and analysis were handled by Sequence Detector software (version 1.7; Applied Biosystems). Briefly, a normalized reporter value (R_n) was calculated by dividing the reporter dye intensity by the passive reference dye intensity; and the change in R_n (ΔR_n), an indication of the magnitude of the signal generated by the PCR, was determined. The cycle threshold (C_t) value is the first cycle at which a statistically significant increase in ΔR_n is detected and is based on an arbitrary threshold of the average standard deviation of ΔR_n during the early cycles. The C_t value is inversely proportional to the amount of target DNA. Generally, samples with ΔR_n values exceeding the threshold and a clear indication of probe cleavage (judged by the multicomponent analysis) were considered positive. However, when VT-specific assays were run with enriched fecal samples, a C_t value less than 30 was used as a selection criterion, as initial trials showed that VTEC strains could not be isolated from samples with higher C_t values.

Cattle fecal samples. Two dairy cattle farms were visited once during June 2001, and 20 calves ages 2 to 6 months were sampled from each farm. These farms had participated in a longitudinal VTEC O157 study during the preceding year and had been positive for VTEC O157 in the summer and autumn of 2000 but were negative in January 2001.

Detection of VT genes and isolation of VTEC from fecal samples. Screening for *vtx*-positive fecal samples was performed by enrichment followed by TaqMan PCR for *vtx1* and *vtx2* (multiplex PCR). Ten grams of feces was suspended in 90 ml of buffered peptone water, and the suspension was incubated overnight at 37°C. A 0.2-ml aliquot of enrichment culture supernatant was added to 0.8 ml of tryptic soy broth, and the mixture was centrifuged at 12,000 × *g* for 3 min. The pellet was resuspended in 0.2 ml of sterile distilled water and boiled for 10 min to lyse the cells. The suspension was centrifuged at 1,700 × *g* for 5 min, and the supernatant was used as the template. The TaqMan PCR assay was performed as described above by using probes and primers for the detection of both *vtx1* and *vtx2* in each reaction. The enrichment cultures showing a positive reaction in the PCR assay for *vtx1* and/or *vtx2* were used for isolation of VTEC by the HGMF replica method, as described by Cobbold and Desmarchelier (9). Briefly, 1 ml of a 10^{-5} dilution of the enrichment culture was mixed with 8 ml of saline, and the mixture was filtered through HGMFs with 1,600 separate cells (ISO-GRID membranes; pore size, 0.45 μm; Acumedia Manufacturers Inc., Baltimore, Md.) by using a spread filter (Filtaflex Ltd., Almonte, Ontario, Canada). The filters were placed on modified hemorrhagic colitis (mHC) agar (44) and incubated overnight at 37°C. One replica of the colonies on the HGMF was placed onto another HGMF with a replicator (Filtaflex Ltd). The replicate was used for hybridization after overnight incubation on mHC agar (Fig. 1). Colony hybridization was performed with the digoxigenin (DIG) wash and block buffer kit (Roche Diagnostics, Mannheim, Germany), according to the recommendations of the manufacturer. DIG-labeled DNA probes were made by use of the PCR DIG labeling kit (Roche Diagnostics) with primers MK1 and MK2 (22). The *vtx* genes of two *E. coli* O157 strains, EDL933 (*vtx1 vtx2*) and E32511 (*vtx2*), served as templates for the production of probes, and these were pooled and used for the detection of both *vtx1* and *vtx2*.

Serotyping and VT subtyping of *E. coli* isolates. The O serogroups of the VTEC isolates were determined with antisera against *E. coli* antigens O1 to O175 as described previously (13). The variants of the *vtx* genes were determined by restriction fragment length polymorphism analysis of the *vtx* genes as described previously (31). This method is based on PCR detection of all *vtx* genes (25) followed by digestion with *Hinc*III (3) and distinguishes between *vtx1*, *vtx2*, *vtx2c* (including *vtx2d1* and *vtx*-OX3/b-031), *vtx2d2*, *vtx2e*, etc.

RESULTS

Detection of virulence factors by automated 5′ nuclease assay (TaqMan). Primer and probe combinations were designed for detection of a panel of virulence factors and variants of these relevant for VTEC and EPEC isolates: the two *vtx* genes,

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TABLE 3. Probes and primers used for automated 5′ nuclease PCR assays

Target gene	Primer or probe name	Forward primer, reverse primer, and probe sequence (5′→3′)	Melting temp (°C)	Location within sequence	Size of amplicon (bp)	Reporter dye[a]	EMBL accession no. or serotype strain
vtx1	vt1-F	GGA TAA TTT GTT TGC AGT TGA TGT C	58	359–383	107	FAM	Z36899
	vt1-R	CAA ATC CTG TCA CAT ATA AAT TAT TTC GT	58	465–437			O48:H21
	vt1-P	CCG TAG ATT ATT AAA CCG CCC TTC CTC TGG A	70	425–395			94C
vtx2[b]	vt2-F	GGG CAG TTA TTT TGC TGT GGA	59	779–799	131	TET	X65949
	vt2-R	GAA AGT ATT TGT TGC CGT ATT AAC GA	59	909–882			OX3:H21
	vt2-P	ATG TCT ATCA GGC GCG TTT TGA CCA TCT T	69	814–842			
eae	eae-F2	CAT TGA TCA GGA TTT TTC TGG TGA TA	59	899–924	102	FAM	Z11541
	eae-R	CTC ATG CGG AAA TAG CCG TTA	59	1000–979			O157:H7
	eae-P	AT AGT CTC GCC AGT ATT CGC CAC CAA TAC C	69	966–936			EDL933
eae-α	eae-alfa-F	GAT ACG AAT GGC TAT GCC AAA G	58	2459–2482	60	TET	M58154
	eae-alfa-R	CAT CGC TAA CAC GGG CAC TA	58	2575–2554			O127:H7
	eae-alfa-P	A ACA TCG ACA ACT CCA GGA AAA TCA CTC GT	68	2541–2511			E2348/69
eae-β	eae-beta-F	GGT GAT AAT CAG AGT GCG ACA TAC A	59	3167–3191	93	TET	U60002
	eae-beta-R	GGC ATC AAA ATA CGT AAC TCG AGT AT	58	3259–3234			O15:H−
	eae-beta-P	CCA CAG CAA TTA CAA TAC TAC CCG GTG CA	68	3227–3199			RDEC-1
eae-γ	eae-gamma-F	GAC TGT TAG TGC GAC AGT CAG TGA	58	2267–2291	84	FAM	Z11541
	eae-gamma-R	TTG TTG TCA ATT TTC AGT TCA TCA AA	59	2350–2325			O157:H7
	eae-gamma-P	TGA CCT CAG TCG CTT TAA CCT CAG CC	66	2319–2294			EDL933
eae-δ	eae-delta-F	CAT TAT CCG GTG AAG AAG TGA CTT T	59	98–123	84	FAM	Y13112
	eae-delta-R	CAT AAC CAC TCT GAT CGG TCG TTA	59	181–158			O86:H34
	eae-delta-P	CTT TAG TTT TAT CCA ATG CCC CAA AAT CCG	68	157–128			ICC95
eae-ε	eae-epsilon-F	ATA CCC AAA TTG TGA AAA CGG ATA	58	2528–2551	84	TET	AF116899
	eae-epsilon-R	CAC TAA CAA CAG CAT TAC CTG CAA	58	2611–2588			O103:H2
	eae-epsilon-P	CCA GAT GTC AGT TTT ACC GTA GCC CTA CCA	68	2585–2556			PMK5
eae-ζ	eae-zetha-F	GAT GTC AAA GCA CCT GAA GTT GAA	59	2224–2247	87	TET	AF449417
	eae-zetha-R	CCC TTT GAT TCC AGT TCC TAC AA	58	2310–2288			O111:H9
	eae-zetha-P	TCT TCA CCC CAC TTG CTA TTG ATG ACG G	69	2249–2276			921-B4
eae-θ (*eae*γ2)	eae-theta-F	TGT TAA AGC ACC TGA GGT TAC ATT TT	58	5776–5802	84	FAM	AF025311
	eae-theta-R	TCA CCA GTA ACG TTC TTA CCA AGA A	58	5859–5835			O111:H−
	eae-theta-P	TCA ACC TTG TTG TCA ATT TTC AGT CCA TCA	67	5832–5802			95NR1
ehxA	ehec-F	CGT TAA GGA ACA GGA GGT GTC AGT A	59	41816–41840	142	TET	AF074613
	ehec-R	ATC ATG TTT TCC GCC AAT GAG	59	41957–41937			O157:H7
	ehec-P	TCA TAA GGA ATT CCA CCG GTT CTG AAT TCA	68	41905–41876			EDL933
espD-α	espD-alfa-F	TGG AAA ATT TAA CTC GAC AAA GTG AGT	60	998–1024	83	FAM	Y09228
	espD-alfa-R	GTT TTG CAG CTC GAG AGT AGC TT	58	1080–1058			O127:H7
	espD-alfa-P	CAG CTC TGC CTT CGC ACT CTG ACT T	66	1053–1029			E2348/69
espD-β	espD-beta-F	CTG GCC GAT GTA TTT GTT GAG A	59	32309–32330	94	TET	AF200363
	espD-beta-R	GCA CAG TTG TCC CAA TAG AAC GT	59	32402–32380			O15:H-
	espD-beta-P	CGT AAA TCC ATT TGC CGT TGC CG	68	32371–32349			RDEC-1
espD-γ	espD-gamma-F	GGC GTT ACG AAC GGT ATT CG	59	4196–4215	64	FAM	Y13068
	espD-gamma-R	GGA GTT GCG CAG CCT CAT TA	60	4259–4240			O157:H7
	espD-gamma-P	TCA ACG CAG TTG TCC CGG CA	67	4238–4219			EDL933
tir-α	tir-alfa-F	GGT AGT GGG CAT CCG ATG GT	61	553–572	80	TET	AF013122
	tir-alfa-R	TCT GGG TCT AAT TTG GCC AGT ATT	60	632–609			O127:H7
	tir-alfa-P	CAC TGT CGC ATC AGA TAT CGC GGA A	68	573–597			E2348/69
tir-β	tir-beta-F	GCG CAG GGC ATG CTA TG	58	569–585	98	FAM	AF045568
	tir-beta-R	CTT CGT CTC CTT TGT ATC CTT TGG	59	666–643			O15:H-
	tir-beta-P	CAC GGT TGC TTC AGA TAT CGC CGA	68	588–611			RDEC-1
tir-γ	tir-gamma-F	CTA TGC TGG AAG TGT CAA AGA AAG TC	58	5381–5406	87	TET	AE005595
	tir-gamma-R	ATA CCC CTG GGT CAG AAG ATA CC	59	5467–5445			O157:H7
	tir-gamma-P	AGC CAT CGA GCT ACG TCT GCT CTC C	67	5419–5442			EDL933
tir-θ(γ2)	tir-theta-F	CTA GCG CAG TGG TTA ATC CAT ATG	59	2564–2587	137	FAM	AF025311
	tir-theta-R	ATT CCC TGA GAA ATT TTG AAT GAC G	61	2700–2676			O111:H−
	tir-theta-P	CAC CGG CTC GTC AGG CAG AAG A	68	2615–2636			95NR1

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TABLE 3—*Continued*

Target gene	Primer or probe name	Forward primer, reverse primer, and probe sequence (5′→3′)	Melting temp (°C)	Location within sequence	Size of amplicon (bp)	Reporter dye[a]	EMBL accession no. or serotype strain
katP	katP-F	GAA GTC ATA TAT CGC CGG TTG AA	59	1914–1936	73	FAM	X89017
	katP-R	GTC ATT TCA GGA ACG GTG AGA TC	59	1986–1964			O157:H7
	katP-P	AGC CTC ATT GAT AAA GCC AGT CAG CTG	66	1937–1963			EDL933
etpD	etpD-F	AA CGT GTG GAT AAG GTG GGT AAT C	59	27225–27248	67	FAM	AF074613
	etpD-R	TGT TGC GGA CGC GTA GGT	60	27289–27272			O157:H7
	etpD-P	CGT TGC CAC GGT CCC GCT	66	27253–27270			EDL933
espP	espP-F	GAT TAC AGC ACG CAT TCA TGG TAT	59	14668–14691	73	TET	AF074613
	espP-R	TCC AGG CAT CCT CAG TGA CA	59	14740–14721			O157:H7
	espP-P	TAG CCC GCT TCT GCA CCG G	65	14711–14693			EDL933
saa	Saa-F	TGC CGC TGG TAT AAT TTT TCG	59	2669–2689	85	FAM	AF325220
	Saa-R	AAC GCC TGT TCC ATG TTG TG	58	2753–2734			O113:H21
	Saa-P	ACC AGC GAA AAC ACC GTC GTC AGA CTA	68	2691–2717			98NK2

[a] FAM, 6-carboxyfluorescein; TET, 6-carboxy-tetrachlorofluorescein. The quencher dye is 6-carboxytetramethylrhodomine.
[b] Excluding *vtx2e*.

five plasmid-borne genes, and the three genes of the LEE pathogenicity island, including seven variants of *eae*, four variants of *tir*, and three variants of *espD* (Table 3). These primer and probe combinations were designed to detect all relevant sequences found in GenBank but not irrelevant genes. Primers and probes for variant *vtx2e* (related to edema disease in pigs) were not included in the *vtx2*-specific assay.

General primer and probe sets was designed to detect all known *eae* variants, and in addition, primer and probe sets were designed for the detection of seven *eae* variants. The nomenclature established previously was used (1, 33, 46), so that *eae*-β2 and *eae*-γ2 identified by Oswald et al. (33) are termed *eae*-δ and *eae*-θ, respectively, and variant eae-ζ is based on the *eae* sequences of O84 and O111:H9 (46). Due to the high level of variation, general primers and probes could not be designed for the other selected genes of the LEE region (*tir* and *espD*), but primers and probes for the detection of known variants of these were made (Table 3). The nomenclature for *tir* and *espD* follows the same principle as that for *eae*, as described by China et al. (8), but with the separation of *tir*-γ into two variants: *tir*-γ and *tir*-θ. As *tir* sequences are available from only a limited number of strains, primers and a probe could be designed for the detection of only four variants of *tir*, although it must be expected that there are more variants (the sequences of the *tir* genes from strains with *eae*-δ, *eae*-ε, and *eae*-ζ are not available). All *E. coli tir* sequences in GenBank should be detected by one of the four assays specific for *tir* variants (perfect match of primer and probe sequences). Primers and probes for the detection of three *espD* subtypes (subtypes α, β, and γ) were designed, and these detected all but one sequence in GenBank. Information on the strain with a different *espD* sequence is scarce (diffusely adherent EPEC O8:H−; GenBank accession no. Y17874; no other genes of this strain have been sequenced), and therefore, it is unknown how this variant relates to other LEE variants.

Each of the 22 detection assays was validated by testing positive and negative control strains (Table 1), which were selected on the basis of the presence or absence of the various factors by the use of other methods, as described in the literature. All TaqMan assays were found to give the expected results. Examples of the amplification plots for detection of *saa*, *eae*, and the *eae* subtypes are shown in Fig. 2.

The whole panel of detection assays was tested with a number of well-characterized *E. coli* strains (mainly VTEC and EPEC strains) to further validate the method and to obtain more complete virulence profiles for these strains (Table 2). All sorbitol-nonfermenting O157:H7 and O157:H− strains had the LEE profile *eae*-γ, *tir*-γ, and *espD*-γ and the four plasmid-borne factors (*ehxA*, *katP*, *espP*, and *etpD*). These isolates possessed *vtx1* and/or *vtx2*. Sorbitol-fermenting O157:H7 strain 493/89 was negative for two of the plasmid-borne factors, *katP* and *espP*. Different combinations of virulence factors were found among the strains of the other VTEC and EPEC serotypes. For example, VTEC O26:H11 strain H19 was found to have the β subtype of the LEE genes and *ehxA*, *katP*, and *espP* on the large plasmid. VTEC O91:H21 strain B2F1 was LEE negative and positive for the adhesin Saa. EPEC strain E2348/69 (O127:H6) had the LEE-related genes *eae*-α, *tir*-α, and *espD*-α but was negative for all other factors tested.

It was unknown beforehand whether any of the assays for the *tir* subtypes would react with strains with *eae* subtype δ, ε, or ζ and whether any assays for the *espD* subtypes would be positive for strains with *eae* subtype δ, ε, θ, or ζ. It was found

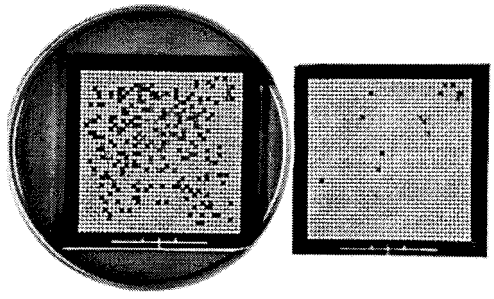

FIG. 1. (Left) mHC agar plate with an HGMF placed on top showing the growth of approximately 230 *E. coli* colonies in the 40-by-40 grid of the HGMF. (Right) Replicate of the filter on the left after hybridization with DIG-labeled *vtx*-specific probes. Fourteen *E. coli* colonies were found to be *vtx* positive in this example.

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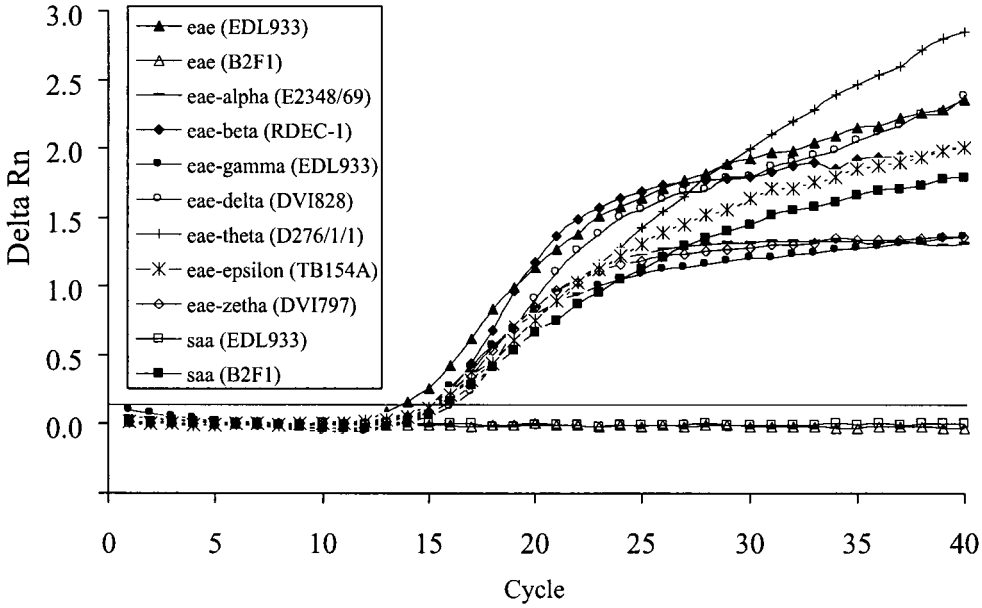

FIG. 2. Examples of amplification plots from real-time PCRs for detection of *saa*, *eae*, and *eae* variants. The amplification plots for detection of *eae* and *saa* for the positive and negative control strains are shown. For the seven *eae* variants, the results for the positive control strains are shown.

that both the control strain positive for *eae*-ε and other reference strains with this *eae* variant were positive by the *tir*-β- and the *espD*-β-specific assays (Table 2). The strain with *eae*-δ was positive by the assays for the α variants of *tir* and *espD*, and the strain with *eae*-ζ was positive by the assay for *tir*-α but negative by the assays for all three *espD* variants. The strains with the θ variants of *eae* and *tir* were negative by the *espD*-specific assays.

Detection and isolation of VTEC from cattle feces by TaqMan PCR and colony hybridization. A method was developed for the detection and isolation of VTEC strains in cattle feces. The method was based on screening of enriched samples by a multiplex TaqMan PCR assay for *vtx1* and *vtx2* (Table 3) and isolation of VTEC from the positive samples by the use of grid membranes with 1,600 separate cells. *vtx*-positive colonies were identified by hybridization with *vtx*-specific probes (Fig. 1). The detection and isolation method was used with 40 fecal samples from 2- to 6-month-old calves from two dairy cattle herds. Screening by the TaqMan PCR showed that 35 (88%) of these samples were positive for *vtx*: 14 samples were positive for *vtx1*, 10 were positive for *vtx2*, and 11 were positive for both *vtx1* and *vtx2*. Sixteen of the positive samples were selected for isolation of VTEC. Samples were selected to represent those with all three combinations of *vtx* subtypes, and in addition, samples with a strong reaction by the TaqMan PCR assay were selected. With the use of one dilution of enrichment culture filtered through an HGMF, *vtx*-positive colonies were found in 15 of the 16 samples after hybridization. In most cases, several colonies were positive on each HGMF. Four to five positive colonies were subcultured and tested by the *vtx1*- and *vtx2*-specific TaqMan assay. However, not all isolates were found to be *vtx* positive after a pure culture was obtained, indicating that a colony growing in one cell of the grid membrane in some cases consisted of several different strains. At least one confirmed VTEC isolate was obtained from 14 of the 15 hybridization-positive samples.

Characterization of VTEC isolates. The VTEC isolates obtained from fecal samples from calves were further characterized by using the panel of 22 factors, O serogrouping, and *vtx* subtyping. When more than one isolate from the same sample had the same O serogroup and the same *vtx* profile, only one of these isolates was included in the further analysis, and thus, a total of 19 isolates were fully characterized. Between one and three different O groups and *vtx* profiles could be characterized from each positive sample. VTEC isolates representing five different O groups and/or virulence profiles were isolated from farm A. Ten different profiles were found on farm B (Table 4).

Nine of the 15 (60%) different VTEC strains had *vtx1* only, 5 (33%) strains had one or more *vtx2* genes, and 1 strain had both *vtx1* and *vtx2c*. Eight of the strains (53%) possessed the LEE pathogenicity island (positive for two or three of the three LEE genes tested for), and four different *eae* subtypes were found. Eleven strains (73%) were likely to harbor the large virulence plasmid (one to three of the four genes tested for). One O113 strain had the *saa* gene (Table 4). The most common virulence profile among the isolates from calves in both herds was *vtx*1, *eae*-ζ, *tir*-α, *ehxA*, and *espP*. This profile was found for serogroup O84 and O98 isolates (Table 4). Similar profiles with variation in the plasmid factors only were found for an O8 isolate (*espP*) and a nontypeable isolate (*ehxA*, *espP*, and *etpD*). Three other *eae* subtypes were identified (*eae*-β, -γ, and -ε).

DISCUSSION

The increasing focus on VTEC infections, especially infections caused by VTEC isolates of serotypes other than O157:H7 and O157:H−, has emphasized the necessity of hav-

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TABLE 4. Results of characterization of strains isolated from calves ages 2 to 6 months

Farm and animal no.	O type	Toxin genes	LEE genes	Plasmid-borne gene(s)
Farm A				
7	O8	*vtx1*	*eae*-ζ, *tir*-α	*espP*
7, 11	O98	*vtx1*	*eae*-ζ, *tir*-α	*ehxA*, *espP*
9	O165	*vtx2*, *vtx2c*	*eae*-ε, *tir*-β, *espD*-β	*ehxA*, *katP*, *espP*
3, 4	O172	*vtx1*	*eae*-γ, *tir*-γ, *espD*-γ	*ehxA*, *espP*
5, 8	NT[a]	*vtx1*, *vtx2c*		*espP*
Farm B				
24	O4/156	*vtx2c*		
40	O8	*vtx1*		
21	O15	*vtx1*		*espP*
24	O21	*vtx1*		
40	O68	*vtx2c*	*eae*-β, *tir*-β, *espD*-β	*ehxA*, *espP*
21, 29	O84	*vtx1*	*eae*-ζ, *tir*-α	*ehxA*, *espP*
23	O98	*vtx1*	*eae*-ζ, *tir*-α	*ehxA*, *espP*
21	O113	*vtx2*		*saa*, *ehxA*, *espP*
30	NT	*vtx1*	*eae*-ζ, *tir*-α	*ehxA*, *espP*, *etpD*
26	NT	*vtx2*, *vtx2nt*[b]		

[a] NT, nontypeable.
[b] The *vtx2* gene of this isolate (*vtx2nt*) gave a pattern that has not previously been defined; when the 900-kb PCR product was digested with *Hin*cII, a ca. 800-bp fragment appeared.

ing reliable methods for the detection and isolation of all VTEC strains pathogenic for humans from patients as well as relevant reservoirs, e.g., foods and animal feces. As VTEC isolates usually constitute a minority of the *E. coli* flora in fecal samples of healthy animals and in food samples, the task is to detect and isolate this minority of isolates, which have no common phenotypic traits that can differentiate them from nonpathogenic *E. coli* strains. Therefore, we have developed a method that can be used to screen for *vtx*-positive samples by real-time PCR. Screening is performed with enrichment cultures and is therefore similar to many other published methods based on PCR. However, the advantage is the use of real-time PCR and detection by fluorescence probes, which produce a final result within 2 h. Furthermore, it is important to obtain a bacterial isolate to be able to further characterize the isolate and thereby assess the virulence potential of the organism. Most *vtx*-positive *E.* coli O157:H7 and O157:H− strains have predictable virulence profiles; however, this is not the case for VTEC strains of other serotypes isolated from animals or food. As knowledge of the virulence profiles and the virulence potentials of non-O157 isolates from nonhuman sources is limited, it is important to further characterize these isolates. Only a few efficient methods exist for the isolation of VTEC strains from among a large indigenous *E. coli* flora. In this case, picking a few random colonies for further characterization is usually not sufficient to obtain a reasonable VTEC isolation rate. We have chosen to use the principle of DNA hybridization on grid filters, which has previously been used for the isolation of VTEC strains from enrichment cultures of fecal samples (9, 47). We found that it was possible to isolate one VTEC colony among approximately 500 other coliform colonies, and this method was sufficient to obtain an isolate from the majority of the PCR-positive samples. The fecal samples used in this study were obtained from calves ages 2 to 6 months. The prevalence of *vtx*-positive animals among the 40 calves from two dairy cattle herds was high (88%). This age group has previously been identified to be the group with the highest prevalence of VTEC O157 excretion in Danish cattle herds (32). Studies from other countries have also found a higher prevalence of VTEC O157 in calves than in cows (17). However, the prevalence of non-O157 VTEC strains in calves was higher than that in cows in some studies (50) but not in others (4). In the Danish study, the prevalence of VTEC O157 strains was 8.6% among the 2- to 6-month-old calves; i.e., the prevalence of all VTEC isolates can be expected to be approximately 10 times higher than the prevalence of VTEC O157 in calves in this age group.

We have developed real-time PCR assays for the detection of a wide range of virulence factors relevant primarily for VTEC isolates, but also to some extent for attaching-and-effacing *E. coli* and EPEC. The panel of assays can be used for the detection of *vtx* genes, genes of the LEE region, and genes of the large virulence plasmid. This panel can easily be extended to include other subtypes of specific genes in, for example, the LEE region or new factors when these are identified or when particular genes need to be detected for specific purposes. Our main interest has been to detect the VTEC virulence factors most important at present, but also to detect variants of some of these factors, as it has been shown that there is a strong correlation between, e.g., the *eae* variant, the serotype, and the presence of other virulence factors (33, 45, 52). Although some of the variants of the LEE genes are found only or primarily in EPEC strains, primers and probes for these variants are included to make the most complete characterization system.

Primer and probe design for TaqMan PCR assays needs to be very specific; i.e., it is necessary to find a region of the target gene of, preferably, less than 200 bp from which the sequences for two primers and one probe can be chosen. The sequences of the primers and probe should perfectly match the relevant sequence of the gene that is sought. This makes it difficult to design general assays for all variants of *vtx*, *tir*, and *espD*. On the other hand, the high degrees of specificity of the primers and probes are an advantage when assays for different subtypes are designed and only minor sequence differences separate the subtypes. Multiplex assays can easily be performed, so that all variants of *vtx1* and *vtx2* can be detected in one multiplex assay; i.e., screening for *vtx*-positive samples can be done by the use of one well per sample. Furthermore, the panel of detection assays can be used in two steps when unknown isolates are characterized. First, the isolates are tested for *vtx1*, *vtx2*, and *eae* (the general assay detects all subtypes), and the five plasmid-borne genes. Detection of these eight factors can be performed in four reaction wells. Then, *eae*-positive isolates are tested for the subtype of *eae* and the presence of variants of the other LEE genes (14 genes are detected in seven wells). In this study, we found that all isolates with a specific *eae* subtype had the same combination of *tir* and *espD* subtypes. If future characterization of a large number of LEE-positive isolates confirms this relationship between *eae* subtype and the subtypes of the other LEE-related genes, it might be sufficient to determine only the *eae* subtype.

For each of the 22 PCR assays, the expected results were obtained for the positive and negative control strains. Furthermore, a panel of 20 well-described strains was characterized by the use of all PCR assays to further validate the assays with a

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wider range of strains. The virulence profiles of these strains or other strains with the same O:H-serotype are partly known from the literature, except that the subtypes of the LEE genes have not always been described previously, and in most cases, only the *eae* subtype has been determined. In this study, information on the subtypes of two other LEE genes is added. All sorbitol-nonfermenting *E. coli* O157:H7 and O157:H− strains were shown to have the same profile, i.e., the γ variant of the LEE genes and the four plasmid-borne factors, which are found in the well-described strain *E. coli* EDL933 (7, 33). Sorbitol-fermenting O157:H− strain 493/89 was negative for two of the plasmid-borne factors, *katP* and *espP*, as has been shown to be characteristic of sorbitol-fermenting VTEC O157 strains (21). Another well-described strain, *E. coli* H19 O26:H11, was also found to have the expected profile, i.e., *vtx1*, three of the plasmid-borne factors (*ehxA*, *katP*, and *espP*; it was negative for *etpD*) (41), and the β variant of *eae*, and *tir*, and *espD*, as found in other strains of serotype O26:H11 (1, 8, 16). As expected from the literature, the VTEC O111:H− strains had three of the plasmid-borne genes (41) and the θ subtype of *eae* and *tir*. The presence of *eae*-θ in O111:H8 and O111:H− strains is in accordance with the findings of Oswald et al. (33), but with the renaming made by Tarr and Whittam (46); i.e., γ_2 is θ. As expected, the human and rabbit EPEC strains had LEE-α and LEE-β, respectively, but were negative for *vtx* and pO157 genes.

It has previously been shown that specific genetic variants of the intimin gene are highly related to evolutionary lineages and, thereby, serotypes (1, 33). This has also been shown, but to a more limited extent, for the whole LEE region (38). We found a good concordance between the LEE subtypes, as all strains possessing *eae*-α, *eae*-β, or *eae*-γ also had the corresponding α, β, or γ subtypes of *tir* and *espD*. Furthermore, *eae*-θ strains were positive for *tir*-θ. Strains with *eae*-θ or *eae*-ζ were negative by the three assays specific for *espD*. However, it is most likely that these strains possess a variant of the *espD* gene that is not covered by our three PCR assays. This is substantiated by the fact that large variations in *espD* exist for the few *espD* genes that have been sequenced from different *E. coli* lineages; e.g., there was only 20% identity between *espD* from EPEC strain E2348/69 and *espD* from VTEC strain EDL933 (38). A heterologous relationship was found for three *eae* subtypes *eae*-δ, -ε, and -ζ. No *tir* or *espD* genes have yet been sequenced from strains with these *eae* subtypes. The sequences of the probes and primers for the subtype-specific PCR assays were in the extracellular C-terminal region of intimin, as large variations between intimin subtypes exist in this region. This is also the Tir-binding region of intimin; however, it has recently been shown that only a few amino acids are likely to be critical for Tir binding, and these residues are conserved among the different intimin types (26). Also, the intimin-binding area of Tir, the central portion of Tir, is conserved (23); and likewise, it has been predicted that only a few conserved residues are critical for binding (26). Our primers and probes for Tir are placed in the variable regions outside the central intimin-binding area. We found that all strains with *eae*-ε were positive for *tir*-β and *espD*-β. Tarr and Whittam (46) found that intimin-ε (represented by VTEC O103:H2 strain PMK5) was closely related to intimin-β when the periplasmic domain and the central domain of the protein sequence were analyzed, but for the extracellular domain, intimin-ε and intimin-β were only distally related. The single *eae*-δ strain in our study was positive for *tir*-α and *espD*-α. According to the phylogenetic trees created for the periplasmic and central domains of intimin subtypes, intimin-δ (exemplified by dog EPEC strain 4221) was closely related to intimin-α. Again, this relationship was not found for the extracellular domains (33). None of the reference strains had *eae*-ζ, but all isolates with this subtype obtained from cattle had *tir*-α and were negative by the three *espD*-specific assays. The former is in accordance with the findings of Tarr and Whittam (46), who found that *tir* from an O111:H9 strain (*eae*-ζ) was most closely related to the *tir* sequences of LEE-α strains. However, the *tir* sequence was not published or submitted to GenBank.

A large diversity of VTEC strains was isolated from calves on two cattle farms. VTEC strains were isolated from 16 calves, and the isolates were characterized by the 22-assay panel. At least 15 distinct strains were present, and it can be expected that even more different VTEC isolates would be obtained if more animals and more colonies were selected from these farms. All O serogroups represented by these isolates have previously been isolated from cattle, and furthermore, most of them have been associated with human disease (www.sciencenet.com.au/vtectableu.htm). Most strains had either the *vtx1* gene (60%) or the *vtx2* gene (33%). The *vtx* distribution in this limited material is similar to the findings of a study with 361 non-O157 isolates from beef carcasses in the United States (2). Strains from human patients with VTEC infections more often possess *vtx2* alone or together with *vtx1*. This is especially the case for VTEC strains associated with HUS (15). More than half of the strains (53%) possessed the LEE pathogenicity island. This is greater than the proportion of non-O157 VTEC strains with the *eae* gene found in cattle in many other studies, e.g., in Scotland, the United States, and Spain (17, 12, and 9%, respectively) (2, 4, 20). However, a much higher prevalence of *eae* (70%) was found among VTEC isolates from Germany and Belgium (49). A high prevalence of *eae* is usually found among isolates from human clinical cases, e.g., 70% among non-O157 isolates from Finland (10). All *eae*-positive strains in this study also harbored the large virulence plasmid (the strains were positive for one to three of the plasmid-borne genes tested for); in addition, a few other strains were also positive for some of the plasmid-borne factors (in total, 73%). An LEE-negative O113 strain had the adhesin Saa. O113 was among the serogroups in which Saa was originally identified (35). The strain isolated from a calf in this study had the same virulence profile as an O113 isolate obtained from another Danish cattle farm 1 year earlier (isolate DVI-450; Table 2). As Saa has just recently been described, the prevalence of *saa* among VTEC strains isolated from cattle is unknown. The most common virulence profile among isolates from both herds was *vtx1*, *eae*-ζ, *tir*-α, *ehxA*, and *espP*. This profile was found for strains of serotypes O84 and O98. Five of the eight LEE-positive strains characterized from these two farms had the *eae*-ζ subtype.

We have shown that the 22 real-time PCR assays described here are useful tools for determination of the virulence profiles of VTEC isolates and, to some degree, EPEC isolates as well. Together with the improved method for the detection and isolation of VTEC isolates from fecal samples, it is possible to

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isolate and characterize VTEC isolates from possible sources of human infections and compare the virulence profiles of those isolates to the virulence profiles of isolates from human infections. This comparison is important for assessing the sources of human VTEC infections. It is well known that ruminants often harbor VTEC isolates, but knowledge of the virulence potentials of these isolates is limited. Some knowledge of the virulence profiles of strains pathogenic for humans already exists; however, the subtypes of LEE genes are generally described only for the most common serotypes, and in most cases only the *eae* subtype has then been determined. In this study, information on the subtypes of two other LEE genes is added. We generally found concordance between the LEE subtypes, as all strains with four of the seven *eae* subtypes had the corresponding subtype of *tir* and *espD*, except that *eae*-θ strains were negative for *esp*D. A heterologous relationship was found for three *eae* subtypes, *eae*-δ, -ε, and -ζ. However, as no *tir* or *espD* genes have yet been sequenced for strains with these *eae* subtypes, the variation outside the target region for our probes and primers is unknown. It is noteworthy that for a specific *eae* subtype, the same subtypes of the two other LEE genes were consistently found in all isolates obtained from multiple sources. In general, the O serogroups and virulence profiles of the majority of the cattle isolates obtained in this study indicate that these are likely pathogenic for humans, as these O groups and virulence profiles are also found among human clinical isolates.

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