Applied and Environmental Microbiology, Oct. 2009, p. 6282–6291 Vol. 75, No. 19
0099-2240/09/$08.00+0 doi:10.1128/AEM.00873-09

Virulence Genes and Molecular Typing of Different Groups of *Escherichia coli* O157 Strains in Cattle[▽]

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Received 17 April 2009/Accepted 20 July 2009

Characterization of an *Escherichia coli* O157 strain collection ($n = 42$) derived from healthy Hungarian cattle revealed the existence of diverse pathotypes. Enteropathogenic *E. coli* (EPEC; *eae* positive) appeared to be the most frequent pathotype ($n = 22$ strains), 11 O157 strains were typical enterohemorrhagic *E. coli* (EHEC; *stx* and *eae* positive), and 9 O157 strains were atypical, with none of the key *stx* and *eae* virulence genes detected. EHEC and EPEC O157 strains all carried *eae*-gamma, *tir*-gamma, *tccP*, and *paa*. Other virulence genes located on the pO157 virulence plasmid and different O islands (O island 43 [OI-43] and OI-122), as well as *espJ* and *espM*, also characterized the EPEC and EHEC O157 strains with similar frequencies. However, none of these virulence genes were detected by PCR in atypical O157 strains. Interestingly, five of nine atypical O157 strains produced cytolethal distending toxin V (CDT-V) and carried genes encoding long polar fimbriae. Macro-restriction fragment enzyme analysis (pulsed-field gel electrophoresis) revealed that these *E. coli* O157 strains belong to four main clusters. Multilocus sequence typing analysis revealed that five housekeeping genes were identical in EHEC and EPEC O157 strains but were different in the atypical O157 strains. These results suggest that the Hungarian bovine *E. coli* O157 strains represent at least two main clones: EHEC/EPEC O157:H7/NM (nonmotile) and atypical CDT-V-producing O157 strains with H antigens different from H7. The CDT-V-producing O157 strains represent a novel genogroup. The pathogenic potential of these strains remains to be elucidated.

Escherichia coli O157:H7 is a food- and waterborne zoonotic pathogen with serious effects on public health. *E. coli* O157:H7 causes diseases in humans ranging from uncomplicated diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (30). Typically, enterohemorrhagic *E. coli* (EHEC) strains express two groups of important virulence factors: one or more Shiga toxins (Stx; also called verotoxins), encoded by lambda-like bacteriophages, and a pathogenicity island called the locus of enterocyte effacement (LEE) encoding all the proteins necessary for attaching and effacing lesions of epithelial cells (41). Comparative genomic studies of *E. coli* O157:H7 strains revealed extensive genomic diversity related to the structures, positions, and genetic contents of bacteriophages and the variability of putative virulence genes encoding non-LEE effector proteins (29, 43).

Ruminants and, in particular, healthy cattle are the major reservoir of *E. coli* O157:H7, although the prevalence of O157:H7 strains in cattle may vary widely, as reviewed by Caprioli et al. (12). *E. coli* O157:H7 has been found to persist and remain infective in the environment for a long time, e.g., for at least 6 months in water trough sediments, which may be an important environmental niche.

In Hungary, infections with *E. coli* O157 and other Shiga toxin-producing *E. coli* (STEC) strains in humans in cases of "enteritidis infectiosa" have been notifiable since 1998 on a case report basis. Up to now, the disease has been sporadic, and fewer than 100 ($n = 83$) cases of STEC infection among 2,700 suspect cases have been reported since 2001. However, until the present study, no systematic, representative survey of possible animal sources had been performed.

In this study, our aim was to investigate healthy cattle in Hungary for the presence of strains of *E. coli* O157 and the genes encoding Shiga toxins (stx_1 and stx_2) and intimin (*eae*) and a wide range of putative virulence genes found in these strains. In addition, the phage type (PT) was determined, and pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to further compare the strains at the molecular level. Shiga toxin and cytolethal distending toxin (CDT) production was also examined, and phage induction experiments were conducted. The high incidence of enteropathogenic *E. coli* (EPEC; *eae*-positive) O157:H7 strains and atypical (*eae*- and *stx*-negative) O157 strains indicates that cattle are a major reservoir of not only EHEC O157 but also EPEC O157 and atypical *E. coli* O157 strains. These atypical, non-sorbitol-fermenting O157 strains frequently produced CDT-V and may represent a novel O157 clade as demonstrated by MLST and PFGE.

MATERIALS AND METHODS

Isolation of *E. coli* O157 strains. Altogether, 756 samples—colon specimens ($n = 428$), feces and rectal scrapings ($n = 214$), and milk samples ($n = 114$)—from 542 healthy cattle were collected. The 114 milk and 174 fecal samples were obtained from a large dairy farm (Enying), and all the other samples were taken

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▽ Published ahead of print on 14 August 2009.

TABLE 1. Primers used for non-LEE effector genes

Primer[a]	Sequence	Position in control strain/gene	Amplicon size (bp)	Control strain	Reference(s)
M-EspJf2	ATGTCAATTATAAAAAACTGCTTATC	2668573–2668598	650	*E. coli* Sakai O157	21, 37
M-EspJr2	TTTTTTGAGAGGATATATGTCAAC	2669223–2669200			
M-EspJf1	ATGCCAATCATAAAGAACTGC	771364–771344	650	*E. coli* E2348/69	21, 37
M-EspJr1	TTTTTTGAGTGGGTGGATAT	770714–770733			
M-EspK_{LIC1}	ATGCTTCCTACATCGCAATTAC	1589509–1589488	1,357	*E. coli* Sakai O157	56
M-EspK_{LIC2}	GAATATTTATATGTGGAACC	1588152–1588171			
M-EspM1-pKK-F	ATGCCAGTAAATGCGACAGG	1811677–1811696	588	*E. coli* Sakai O157	4
M-EspM1-pKK-R	ACCCCTGTATAACACGACTCA	1812265–1812245			
M-EspM2-pKK-F	ATGCCGATGAATACTACAGGTATGT	3477573–3477549	587	*E. coli* Sakai O157	4
M-EspM2-pKK-R	TCCCTGTATAGCACGCATCAA	3476986–3477006			
M-EspT-HA-F	ATGCCGGGAACAATAAGCTCCAG	1–23	548	*Citrobacter rodentium* ICC169[b]	11
M-EspT-HA-R	TAGGTTCTCTGAGCCTCCTGAA	569–548			

[a] M, modified.
[b] In the absence of *C. rodentium* control strains, *espT*-specific PCR amplicons were sequenced.

from five slaughterhouses representing different regions of Hungary in the years 2002 and 2003. Samples were transferred to the laboratory on ice and investigated immediately after arrival or kept at −70°C until processing.

For the isolation of *E. coli* O157 and STEC, the following procedures were applied. First, polymyxin B extraction was used to detect Stx with the Seiken verotoxigenic *E. coli* screening kit. In the case of Shiga toxin detection, the samples were plated onto cefixime-tellurite-sorbitol-MacConkey agar and the presence of *stx* genes was investigated by *stx* universal and stx_1- and stx_2-specific PCR analyses of up to 50 colonies.

In addition to being utilized for the isolation of *E. coli* O157 strains, the samples were processed according to the International Organization for Standardization reference method (ISO 16654) using an O157-specific immunomagnetic separation (IMS) kit (Dynal, Oslo, Norway). For enrichment, novobiocin-containing modified tryptic soy broth was applied, and the suspensions were plated onto cefixime-tellurite-sorbitol-MacConkey agar, CHROMagar, and bromothymol blue agar plates. Five to 10 non-sorbitol-fermenting coliform colonies were agglutinated with O157 latex (Oxoid Ltd.) particles and with O26- and O111-specific immune sera. The agglutinating colonies were subcultured and confirmed biochemically to be *E. coli*. Only one *E. coli* O157 colony per sample was selected and analyzed further. One-third of samples were assayed by using O26 and O111 IMS kits. The isolated colonies were tested with O26 and O111 antibodies.

Phenotypic methods. Serotyping of O (lipopolysaccharide) and H (flagellar) antigens was performed with O-specific and H-specific rabbit antisera prepared at the Federal Institute for Risk Assessment (Berlin, Germany) according to standard methods (44). Antigens O1 to O181 and H1 to H56 were investigated, and nonmotile (NM) *E. coli* O157 strains were evaluated by PCR and restriction fragment length polymorphism analyses of RsaI-digested *fliC* PCR products (16).

Phage typing was performed by the method of Khakria et al. (33) at the National Center for Epidemiology (Budapest, Hungary). By using the 16 typing phages all together, 90 PTs could be differentiated.

Colicin production was tested as described previously (1) using an *E. coli* K-12 strain sensitive to a wide range of colicins.

The resistance of *E. coli* strains to the following antimicrobials was investigated by the disk diffusion method using antibiotic disks (Oxoid Ltd.) on Mueller-Hinton agar: ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfonamide, sulfamethoxazole-trimethoprim, and tetracycline. The zones of growth inhibition were evaluated according to the 2005 recommendations of the Clinical and Laboratory Standards Institute.

(i) Shiga toxin production. Vero cell assays were used to detect Shiga toxin production by *E. coli* O157 strains as described previously (49). Briefly, doubling dilutions of *E. coli* O157 culture supernatants were examined for the presence of Stx on Vero cell monolayers (10^4 cells per well). The degree of cytotoxicity was determined after 2 days of incubation at 37°C in 5% CO_2 by microscopic examination. The wells showing at least 50% cytotoxicity were considered positive.

CDT production was investigated with bacterial lysates as described previously (54). The morphological changes in HeLa cells characteristic of the presence of CDT were investigated after staining of the cells with Giemsa stain. CDT-V-producing *E. coli* strain 493/89 (27) was used as a positive control, and *E. coli* C600 was used as a negative control.

(ii) Prophage induction. For phage induction, mitomycin C at a final concentration of 0.5 μg/ml and a subinhibitory concentration of norfloxacin (34) were used, and plaques were isolated as described previously using *E. coli* K-12 derivative C600 as the indicator strain (55). Single plaques were picked up and investigated by stx_2- and stx_1-specific PCR analyses.

DNA methods. (i) Genotypic characterization by PCR. *E. coli* O157 strains were first examined for O157:H7 *rfbE* ($rfbE_{O157:H7}$) by diagnostic PCR analysis as described by Paton and Paton (47) and for the *fliC* gene as described by Fields et al. (16). The presence of *stx* genes was evaluated with Lin up/Lin down *stx* universal primers (36), and *stx* genes were typed using stx_1-specific B54/B55 and stx_2-specific B56/B57 PCR primers (13). B52/B53 primers were used for *eae* (13). EAF1/EAF2 PCR primers were used for *eaf* (17) and BFP1/BFP2 were used for *bfp* (22) according to the published protocols. The *eae* genes were typed as described by Oswald et al. (45), and *tir* genes were typed as described by Ogura et al. (42). Ehly1/Ehly5 primers were used for *ehxA* (57). The presence of *efa1* 5′ and *efa1* 3′ regions was investigated with primers Efa1-upper/Efa1-lower (R. La Ragione, unpublished data) and Efa1 3′fwd/Efa1 3′rev (39), respectively. The tccP-F1/tccP-R1 primers (21) were used for *tccP* genes, urea-F/urea-R primers (18) were used for *ureA*, and terB1/terB2 primers (52) were used for *terB*. Z4321-a/Z4321-b, Z4326-a/Z4326b, Z4332-a/Z4332-b, and Z4333-a/Z4333-b PCR primers were used for detecting O island 122 (OI-122)-specific marker genes *pagC*, *sen*, and *efa1* as described by Karmali et al. (32). Detection and typing of *cdt* genes were performed with previously described PCR primers (54), as well as *cdt-VA*-specific (c338f/c2135r) and *cdt-VC*-specific (P105/c2767r) primers (8). Q antiterminator genes were typed with primers 595, Q 933, and Q 21 as described by Lejeune et al. (35). The presence of *fuyA* was detected with FyuA-F/FyuA-R primers (51), and lpfA-F/lpfA-R primers (15) were used for detecting $lpfA_{O113}$. The *sodC*-specific O157-2For/O157-2Rev primers were used as described previously (14), and *icf*-specific primers were kindly provided by R. La Ragione (Veterinary Laboratories Agency, Weybridge, United Kingdom). Modified versions of PCR primers described previously were used for detecting the non-LEE effector genes *espJ*, *espK*, *espM1*, *espM2*, and *espT* (Table 1).

(ii) MLST. In this study, the sequences of the housekeeping genes *adk*, *arcA*, *fumC*, *mdh*, and *mtlD* were determined and compared as described previously (7). Briefly, fresh single colonies, which were grown overnight on Luria-Bertani agar plates, were suspended in 50 μl of a 0.9% NaCl solution by using sterile toothpicks and subsequently diluted 1:6 in 0.9% NaCl solution. PCR samples were prepared in a total volume of 50 μl containing 5 μl of this bacterial suspension, 5 μl of 10-fold-concentrated polymerase reaction buffer with 15 mM $MgCl_2$ (Promega, Mannheim, Germany), 200 μM (each) deoxynucleoside triphosphates, 30 pmol of each primer, and 1.5 U of *Taq* DNA polymerase (Promega). PCR was performed in a GeneAmp PCR system 2700 (Applied Biosystems Applera, Weiterstadt, Germany). Samples of 8 μl of the PCR products were analyzed for purity on 0.7 to 1% Tris-borate-EDTA agarose gels using a maximum voltage of 5 V/cm. The primers used are listed in Table 2.

(iii) Purification of PCR products. For the removal of PCR primers, 5-μl samples of the PCR products were combined with 2 μl of a reaction mixture containing 10 U of exonuclease I (New England Biolabs) and 2 U of shrimp alkaline phosphatase (USB Biochemicals). Exonuclease I was diluted to 1 U/μl in 50 mM Tris-HCl, pH 7.5, and incubated at 37°C for 30 min and subsequently at 80°C for 15 min to inhibit further enzymatic activity.

TABLE 2. Primers used for MLST analysis

Primer	Sequence (5′–3′)	Target gene	PCR product size (bp)	Length (bp) of sequence used	Reference
adk3	GGGGAAAGGGACTCAGGCTCAG	Adenylate kinase gene (*adk*)	556	462	7
adk4	AACTTTCGCGTATTTGGTATT				
arcA-p1	GAAGACGAGTTGGTAACACG	ArcA respiration control protein gene (*arcA*)	645	544	7
arcA-p2NW	CTTCCAGATCACCGCAGAAGC				
arcA-p3	CCATGAATCTGGGTACTGTC				This study
arcA-p4	TGCCTGGGTTTTCACAGAAG				This study
mlp-p3	CGTTGATCTGATTGCTCAGG	Mannitol-1-phosphate dehydrogenase gene (*mtlD*)	580	410	7
mlp-p4	TGATCAGTACCGCACCACTT				
mlp-3A	CAAAGCGTGGGTAGAAGAAC				This study
mlp-4A	CCAGCGGATCGTTAGTTGC				This study
fumC-1	CGGCTCCGGCACGCAAAGTAA	Fumarate dehydrogenase gene (*fumC*)	753	514	7
fumC-2	ATCGCCACGTCGTTCCCCATCA				
mdh+55	CTGTTAAAAACCCAACTGCC	Malate dehydrogenase gene (*mdh*)	817	496	7
mdh-872	GCGTTCTGTTCAAATGCG				
mdh+307	CAGCAAGTTGCGAAAACCTG				This study
mdh+443	CGCTGGATATCATTCGTTC				This study
mdh-441	GGTAACGCCGAACAGTTTG				This study
mdh-544	AGCGGCAGAATGGTAACAC				This study

(iv) Sequencing. The treated PCR products were sequenced with a CEQTM 8000 genetic analysis system (Beckmann Coulter, Krefeld, Germany). The applied DNA concentration was 50 fmol. Precipitation of DNA with ethanol and sequence analysis were conducted with the GenomeLab dye terminator cycle sequencing with quick start kit according to the instructions of the manufacturer (Beckmann Coulter). The program for amplification consisted of 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 360 s. In general, the reading lengths were between 400 and 650 bp. The single fragments were assembled with BioEdit sequence alignment software (version 7.0.7.0.; Ibis Biosciences, Carlsbad, CA).

(v) Software for DNA analysis. Following double-strand sequencing, the sequences were edited and aligned. DNA fragments between 410 and 725 bp long from each sequence were used for sequence analysis (Table 2). Raw DNA sequence data were analyzed with ABI 377 software. DNA sequences were edited and aligned with BioEdit, version 4.8.10 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (23), converted into FASTA files, and loaded into S.T.A.R.T. (Sequence Type Analysis and Recombinational Tests; http://outbreak.ceid.ox.ac.uk/software.htm). The dendrograms were compiled with S.T.A.R.T. using the unweighted-pair group method with arithmetic mean (UPGMA). This software constructs a phylogenetic tree on the basis of allele numbers. Split decomposition analysis (5, 25) was performed with SplitsTree, version 2.0 (http://bibiserv.techfak.uni-bielefeld.de/splits/).

(vi) PFGE. PFGE was performed by following the PulseNet protocol for EHEC with XbaI (Fermentas) as the restriction enzyme. The same plugs were also digested with 20 U/150 μl NotI (Fermentas) in the buffer provided by the manufacturer. Fingerprints were analyzed using the software Fingerprinting II (Bio-Rad). Similarity was assessed using the Dice coefficient (optimization, 1%; position tolerance, 1 to 1.5%), and clustering was performed using UPGMA. The threshold for relatedness was chosen at 90%.

Nucleotide sequence accession numbers. The nucleotide sequences obtained by sequencing of the PCR products from all alleles of five *E. coli* housekeeping genes have been entered into the EMBL nucleotide database under continuous accession numbers from FN257307 to FN257337 for *adk*, FN257338 to FN257368 for *arcA*, FN257369 to FN257399 for *fumC*, FN257400 to FN257430 for *mdh*, and FN257431 to FN257461 for *mtlD*.

RESULTS

Isolation of *E. coli* O157 and STEC. Using the verotoxigenic *E. coli* screening kit, the *E. coli* O157-specific IMS technique, and a latex agglutination kit, we were able to isolate a total of 42 *E. coli* O157 and 13 non-O157 STEC strains. In these isolation trials, 756 samples originating from 542 healthy cattle were processed. The detailed investigation was conducted with strains that proved to be *E. coli* O157.

Virulence gene typing of *E. coli* O157 strains. *E. coli* O157 isolates were subjected to numerous PCR assays, but first, the presence of *stx* and *eae* genes was established and the strains were grouped. We defined an *E. coli* O157 strain as EHEC if it carried both *eae* and *stx* genes; EPEC was identified if the strain carried only the *eae* gene. Finally, in the absence of these key virulence genes, the *E. coli* O157 strains were designated atypical O157. Of the 42 *E. coli* O157 strains, 11 proved to be EHEC, 22 proved to be EPEC, and 9 strains were classified as atypical O157. Ten EHEC strains carried stx_1 and stx_2 genes, and in one strain, only stx_2 was detected (Table 3).

The EPEC and EHEC O157 strains carried *eae*-gamma and *tir*-gamma genes and the *tccP* gene. The other investigated virulence genes also characterized the EPEC and EHEC O157 strains at similar frequencies (Fig. 1). Like the EHEC EDL933 and Sakai strains, none of our *E. coli* O157 strains carried the complete *efa* (EHEC factor for adherence) gene. Nine EHEC and 13 EPEC O157 strains carried the 5′ fragment of the *efa* gene. Interestingly, in one EPEC O157 strain the terminal fragment of the *efa1* gene was detected, but this strain did not carry the initial fragment of *efa1*. The genes *icf* (synonymous with *paa*) and *sodC* were present in all EHEC strains and in 21 and 20 EPEC strains, respectively. Five EHEC and 11 EPEC O157 strains harbored the *ureA* gene. The *tenB* gene was detected in 8 EHEC and 19 EPEC strains. Two EHEC and two EPEC O157 strains carried *fyuA*. The lpf_{O113} gene occurred in one EHEC and six EPEC O157 strains. The OI-122-specific genes were detected in all the EHEC O157 strains, while *sen* was detected in 18, *pagC* was detected in 21, *efa1* open reading frame (ORF) Z4332 was found in 21, and *efa1* ORF Z4333 occurred in 21 EPEC O157 strains (Fig. 1).

In addition to *tccP*, the strains were tested for other non-LEE effector protein genes, including *espJ*, *espK*, *espM1*, *espM2*, and *espT*. The Sakai-specific *espJ* gene was present in 10 EHEC O157 strains (90.9%) and in 19 EPEC O157 strains (86.4%). The *espK* gene was found in four EHEC O157 strains (36.4%). The *espM1* gene was present in 8 EHEC

TABLE 3. Origins, serotypes, pathotypes, and PTs of *E. coli* O157 bovine strains isolated in Hungary

Strain[a]	Origin	Serotype	Pathotype[b]	*stx* gene(s) present	*eae*-gamma	*tir*-gamma	PT[c]
34	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT21
52	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT33
254	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT21
R4	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT21
R67	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT21
F67	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT21
318	Slaughterhouse	O157:NM	EHEC	stx_1, stx_2	+	+	PT8
319	Slaughterhouse	O157:NM	EHEC	stx_1, stx_2	+	+	PT8
320	Slaughterhouse	O157:NM	EHEC	stx_1, stx_2	+	+	PT8
321	Slaughterhouse	O157:H7	EHEC	stx_1, stx_2	+	+	PT8
4979	Dairy farm	O157:H7	EHEC	stx_2	+	+	PT8
64	Slaughterhouse	O157:H7	EPEC		+	+	PT8
65	Slaughterhouse	O157:H7	EPEC		+	+	PT33
67	Slaughterhouse	O157:H7	EPEC		+	+	PT33
68	Slaughterhouse	O157:H7	EPEC		+	+	PT8
103	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
121	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
122	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
127	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
129	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
137	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
138	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
144	Slaughterhouse	O157:H7	EPEC		+	+	NT-R
165	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
168	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
169	Slaughterhouse	O157:H7	EPEC		+	+	NT-R
174	Slaughterhouse	O157:H7	EPEC		+	+	NT-R
177	Slaughterhouse	O157:H7	EPEC		+	+	NT-R
178	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
179	Slaughterhouse	O157:H7	EPEC		+	+	NT-R
R6	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
R30	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
F30	Slaughterhouse	O157:H7	EPEC		+	+	PT50d
B20	Dairy farm	O157:H12	AT		−	−	NT-R
B47	Dairy farm	O157:NM	AT		−	−	NT-R
B54	Dairy farm	O157(rough):H12[d]	AT		−	−	NT-R
T4	Dairy farm	O157(rough):H12[d]	AT		−	−	NT-R
T22	Dairy farm	O157:H43	AT		−	−	NC
T16	Dairy farm	O157:H43	AT		−	−	NC
T50	Dairy farm	O157:H43	AT		−	−	NC
T34	Dairy farm	O157(rough):H9[d]	AT		−	−	PT21
T49	Dairy farm	O157(rough):H37r[d]	AT		−	−	NC
EDL933		O157:H7	EHEC	stx_1, stx_2	+	+	PT21
Sakai		O157:H7	EHEC	stx_1, stx_2	+	+	PT14

[a] Bovine O157 isolates with designations highlighted in bold were used for MLST analysis.
[b] EHEC, *stx* and *eae* positive; EPEC, *eae* positive; AT, atypical (*eae* and *stx* negative).
[c] NT, nontypeable; R, phage resistant; NC, noncharacteristic PT; d, derivative.
[d] Identified as O157 and *rfbE* positive by PCR and by latex agglutination and as O rough by tube agglutination.

O157 strains (72.7%) and in 15 EPEC O157 strains (68.2%), and *espM2* was found in 10 EHEC O157 strains (90.9%) and in 17 EPEC O157 strains (77.3%). The *espT* gene was absent from all O157 strains. None these effector genes were detected in the atypical O157 strains (Fig. 1).

In one atypical *E. coli* O157 strain, the *ehxA* gene was present, while three strains carried the *ureA* gene. However, in five atypical O157 strains, *cdt-VABC* genes were detected, and CDT production was demonstrated in tissue cultures (data not shown). Interestingly, these CDT-V-producing strains also carried the $lpfA_{O113}$ gene, encoding the major fimbrial subunit of long polar fimbriae (Fig. 1).

Phage induction experiments were performed with 11 EHEC O157 strains. Lytic phages induced from eight strains and the purified plaques were lysed and investigated by PCR for the presence of *stx* genes. The results revealed that all eight functioning lytic phages carried stx_2 genes (Table 4). Interestingly, in the three strains with noninducible stx_2 phages, the Q21-like antiterminator gene sequences were identified, while in seven of eight inducible stx_2 phages, the EDL99-like Q antiterminator gene was detected. Stx production by EHEC strains was proven in Vero tissue cultures (data not shown).

Serotypes, PTs, and colicin production. *E. coli* O157 strains all carried the *rfbE* gene (specific for O157 antigen), detected by PCR. Four strains positive for O157 antigen by PCR and latex agglutination proved to be O rough by O tube agglutination. Three *E. coli* O157 EHEC strains did not produce detectable H antigen. However, PCR-restriction fragment length polymorphism analysis revealed that the RsaI-digested *fliC* PCR products from these strains yielded the same patterns as

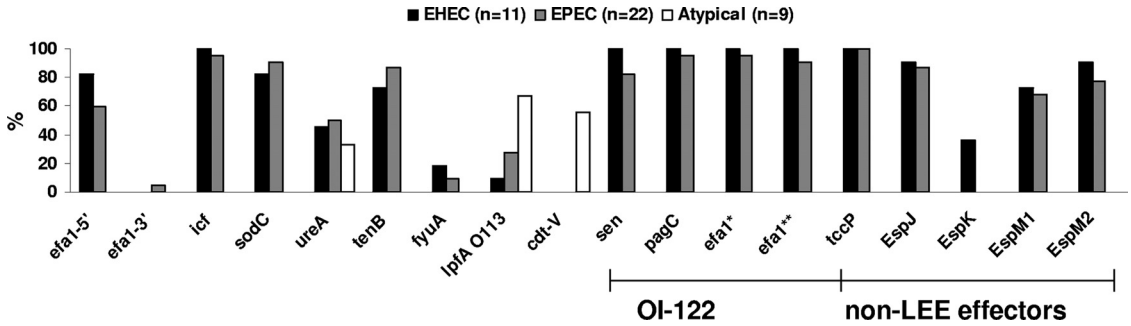

FIG. 1. Distribution of virulence genes in *E. coli* O157 bovine strains isolated in Hungary. None of the O157:H7 strains carried *eaf*, *bfp*, and *espT* genes. *efa1**, ORF Z4332, and *efa1***, ORF Z4333 (32).

those from H7-producing *E. coli* O157 strains (data not shown). Three atypical O157 strains produced H43, one strain produced H12, and one was NM. The O rough but PCR-positive O157 strains carried flagella associated with H12 (n = 2), H9 (n = 1), and H37 (n = 1) (Table 3).

None of the 42 *E. coli* O157 strains fermented sorbitol, produced colicin, or showed multidrug resistance. Only some strains were resistant to one or two antibiotics (data not shown).

The EHEC and EPEC O157 strains proved to belong to PTs typical for O157 EHEC, including PT8, PT21, and PT33. The atypical O157 strains could not be classified by the typing phages used: all these strains either proved to be phage resistant or belonged to noncharacteristic PTs. Results of serotyping and phage typing are shown in Table 3.

MLST. The virulence gene profiles and the subtyping of *eae* and *tir* genes clearly indicate a strong relationship between the EHEC and EPEC O157 strains and substantial diversity between these strains and the *E. coli* O157 atypical strains. To explore further the phylogenetic relationship among O157 strains, MLST and PFGE were conducted.

Altogether, 31 *E. coli* O157 strains, including 11 EHEC, 11 EPEC, and 9 atypical O157 strains, were compared by investigating the sequences of five housekeeping genes. The genes chosen for MLST were distributed evenly throughout the chromosome. DNA sequences of corresponding housekeeping genes from the *E. coli* K-12 strain MG1655 (accession no. NC_000913) and the O157:H7 strains EDL933 (accession no. NC_002655) and Sakai (accession no. NC_002695) were compiled from the respective published genome sequences.

DNA sequences were obtained from all 31 *E. coli* O157 bovine strains studied, and the sequences of all five housekeeping genes could be aligned without gaps. The corresponding DNA sequences from *E. coli* K-12 strains (MG1655 and C600) and O157:H7 strains (EDL933 and Sakai) were included in our scheme. The alleles of the five genes analyzed were numbered in ascending order. Numbers and types of base substitutions were not taken into account for allele designation (7). The allele frequencies and combinations of allele numbers for all isolates are shown in Table 5. Each unique combination of allele numbers represents one sequence type (profile). As a result, seven MLST profiles (I to VII) were established; six MLST profiles (I to VI) were attributed to the *E. coli* O157 strains, while MLST profile VII characterized the K-12 strains. The 11 EPEC and 11 EHEC O157 strains showed MLST profile I, and the 9 atypical strains showed five different MLST profiles: II to VI (Table 6). One of the five dendrograms is shown in Fig. 2 as an example.

PFGE. PFGE was performed on 42 bovine O157 isolates, 1 human (C81) O157:NM strain (53), and 2 EHEC reference strains: O157:H7 EDL933 (48) and Sakai (24). Macro-restriction fragment analysis using the enzyme XbaI yielded 45 patterns (Fig. 3). Two human EHEC type strains (Sakai and EDL933) and two epidemiologically unrelated Hungarian EHEC strains (a bovine isolate, 4979, and a human isolate, C81) were also tested for comparison.

PFGE typing was very useful for the identification of genetically closely related *stx*-positive and *stx*-negative MLST profile I strains. Four main clusters (A, B, C, and D) were outlined by enzyme XbaI (Table 5). XbaI cluster A included 17 EPEC strains from different slaughterhouses in Hungary; cluster B comprised 7 EHEC isolates, interestingly including the single human Hungarian O157:NM EHEC isolate; cluster C comprised four EHEC strains isolated from cattle on the same farm; and cluster D consisted of four atypical CDT-V-producing strains, all derived from milk samples from that dairy farm (Fig. 3. and Table 5).

The 22 MLST profile I *E. coli* O157 bovine strains were subdivided by PFGE into three XbaI clusters, A to C. Interestingly, EDL933 and Sakai strains gave very similar XbaI patterns and belonged to none of the four clusters.

TABLE 4. Genotypic and phenotypic characteristics of EHEC O157 bovine strains[a]

Strain	Serotype	Q_{EDL933} antiterminator gene	Q_{21} antiterminator gene	stx_2 phage induction	Stx production
34	O157:H7	+	−	+	+
52	O157:H7	+	−	+	+
254	O157:H7	+	−	+	+
R4	O157:H7	+	−	+	+
R67	O157:H7	+	−	+	+
F67	O157:H7	+	−	+	+
321	O157:H7	+	−	+	+
320	O157:NM	−	+	+	+
318	O157:NM	−	+	−	+
319	O157:NM	−	+	−	+
4979	O157:H7	−	+	−	+

[a] +, present; −, absent.

TABLE 5. Pathotypes, serotypes, and allelic and MLST profiles of *E. coli* O157 and *E. coli* K-12 strains

Pathotype or group	Serotype	No. of strains	MLST profile	MLST result for:					XbaI PFGE group[a]
				adk	*arcA*	*fumC*	*mdh*	*mtlD*	
EHEC	O157:H7[b]	2	I	1	1	1	1	1	
	O157:H7	6	I	1	1	1	1	1	B
	O157:H7	1	I	1	1	1	1	1	C
	O157:NM	3	I	1	1	1	1	1	C
	O157:H7	1	I	1	1	1	1	1	
EPEC	O157:H7	11	I	1	1	1	1	1	A
Atypical	O157:H12	1	II	2	3	2	2	3	
	O157(rough):H12[c]	1	III	2	3	2	2	4	
	O157:H43	3	IV	3	4	3	3	5	D
	O157(rough):H37r[c]	1	V	3	4	3	3	6	D
	O157(rough):H9[c]	1	V	3	4	3	3	6	
	O157:NM	1	V	3	4	3	3	6	
	O157(rough):H12[c]	1	VI	4	4	4	2	1	
Nonpathogenic strains	K-12	2	VII	2	2	2	2	2	

[a] PFGE groups A to D are listed. Five atypical (*eae*- and *stx*-negative) O157 strains and the epidemiologically unrelated bovine *E. coli* O157 strain 4979 did not establish a further PFGE group.
[b] Two EHEC prototype strains (EDL933 and Sakai) and two epidemiologically unrelated strains (4979 of bovine origin and C81 [53] of human origin) were used in PFGE studies (Fig. 3). The allelic profile of C81 was not investigated.
[c] Identified as O157 and *rfbE* positive by PCR and by latex agglutination and as O rough by tube agglutination.

DISCUSSION

In the present study, 42 *E. coli* O157 strains isolated from dairy and slaughterhouse cattle in Hungary were characterized genetically and phenotypically. This process entailed determining the PT, serotype, and antibiotic resistance profile; detecting the presence of key virulence genes and a wide range of putative virulence genes; and identifying genomic DNA macro-restriction fragment patterns and MLST profiles. Stx production by EHEC O157:H7 strains and CDT production by atypical O157 strains were also examined, and phage induction experiments were conducted.

Our results show that *E. coli* O157 strains are relatively common in the Hungarian cattle population: indeed, *E. coli* O157 strains were isolated from more than 7% of the cattle investigated.

In our non-sorbitol-fermenting EPEC and EHEC O157 strains, as in the sequenced prototype EHEC O157:H7 strains EDL933 and Sakai, only initial or terminal fragments of the

TABLE 6. Allelic and MLST profiles of *E. coli* O157 and *E. coli* K-12 strains

Profile	Serotype(s) (pathotype[s])[c]	Profile designation	Frequency[d] (%)
1,1,1,1,1[a]	O157:H7/NM (EHEC, EPEC)	I	24/35 (68.6)
2,3,2,2,3	O157:H12 (AT)	II	1/35 (2.8)
2,3,2,2,4	O157(rough):H12 (AT)	III	1/35 (2.8)
3,4,3,3,5	O157:H43 (AT)	IV	3/35 (8.6)
3,4,3,3,6	O157:NM (AT), O157(rough):H9 (AT), O157(rough):H37 (AT)	V	3/35 (8.6)
4,4,4,2,1	O157(rough):H12 (AT)	VI	1/35 (2.8)
2,2,2,2,2[b]	K-12	VII	2/35 (5.7)

[a] Profile of bovine and *E. coli* O157:H7 EDL933 and Sakai strains.
[b] Profile of *E. coli* K-12 strains C600 and MG1655.
[c] AT, atypical.
[d] Number of strains with indicated profile/number of strains analyzed.

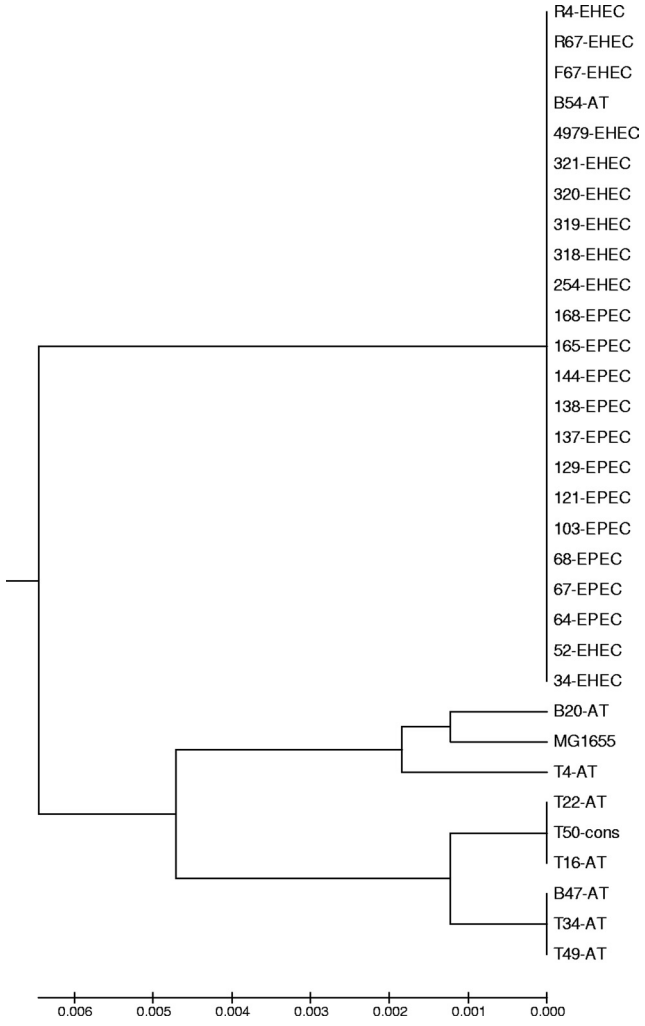

FIG. 2. UPGMA-based dendrogram of *mtlD* sequences in the example of bovine O157 *E. coli* strains. AT, atypical.

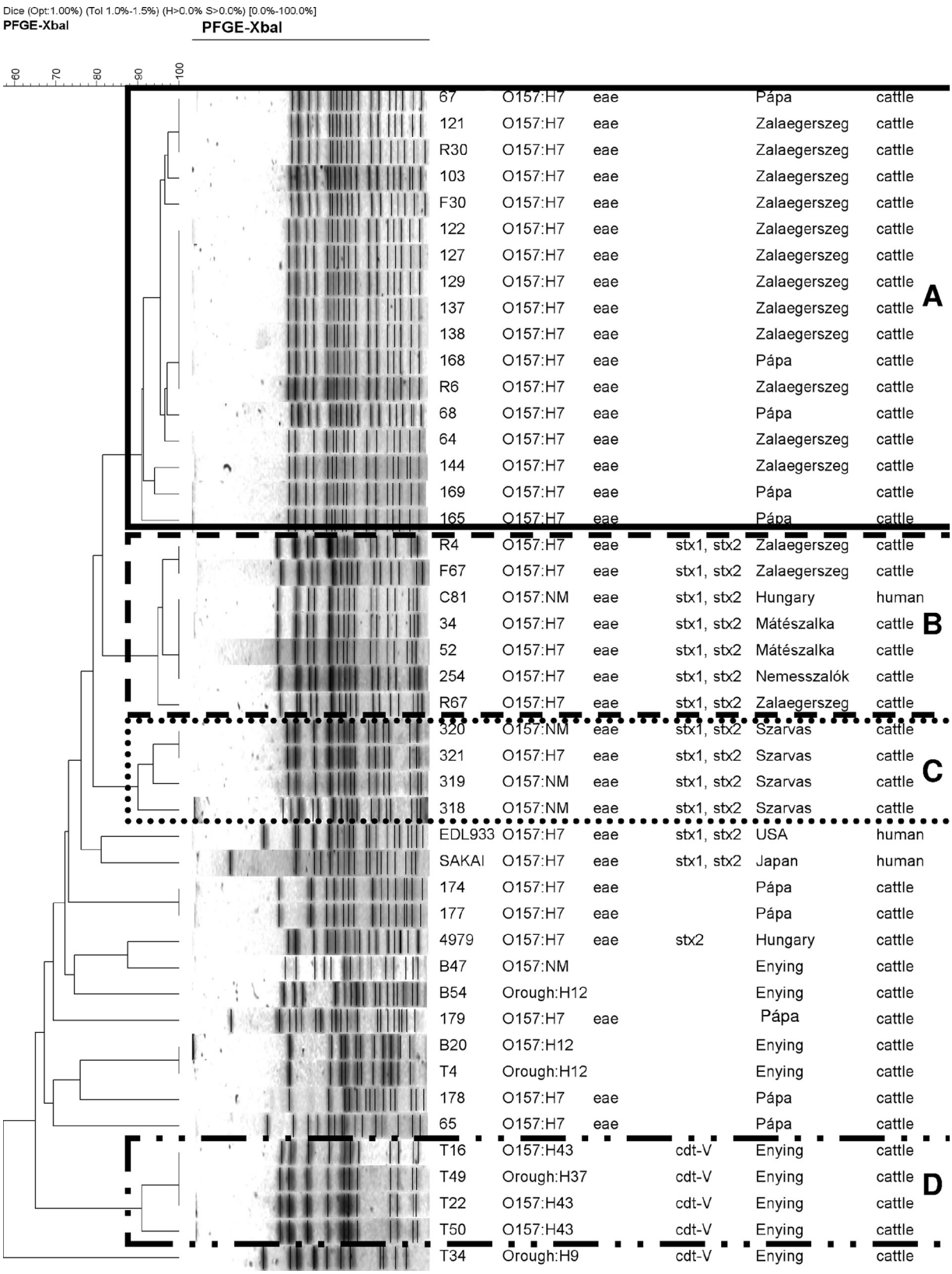

FIG. 3. Dendrogram of PFGE patterns of *E. coli* O157 bovine strains. DNA samples were digested with XbaI, and the patterns were calculated with the Dice coefficient as described in Materials and Methods. A, B, C, and D are the main PFGE clusters.

efa1 gene (also called *lifA*, for lymphocyte inhibitory factor) were detected (24, 48). These results are in accord with the data reported in the literature since the whole *efa1* gene has never been found in non-sorbitol-fermenting *E. coli* strains. However, the large, 9,669-bp gene was detected previously in sorbitol-fermenting EHEC O157:NM strains (28). The *efa1* gene in these strains showed 99.9% sequence homology to *efa1* from EHEC O111:NM and *lifA* from EPEC strain E2348/69 of the O127:H6 serotype (28).

In harmony with the findings described in the literature, non-LEE effector genes *tccP* and *espJ* (21), as well as *espM* (3), were detected frequently in EHEC and EPEC O157 strains, while *espT* was absent from all O157 strains (3). In contrast, none of these effector genes were present in atypical O157 strains.

The urease gene cluster is located within OI-43 and OI-48 (48) in EDL933. Urease genes were detected in the major EHEC groups O26, O111, and O157, as well as in sorbitol-fermenting EHEC O157:NM strains, but were absent from diarrheagenic *E. coli* strains of several other pathogroups, including enteroaggregative *E. coli*, enteroinvasive *E. coli*, and EPEC strains (18, 40). In enterotoxigenic *E. coli*, there is a homologue of the O157 urease-encoding OI-48 (46). These investigations indicate the virulence potential of urease in EHEC. Not only do the tellurite resistance (52) and the periplasmic or membrane-associated superoxide dismutase (6) characterize the prototype EHEC strains, but these virulence factors also were identified in EHEC clinical isolates. The fact that the marker genes for urease (*ureA*), potassium tellurite resistance (*terB*), and superoxide dismutase (*sodC*) frequently characterized our EPEC and EHEC O157 bovine strains is suggestive of their pathogenic and zoonotic potential.

MLST demonstrates that our non-sorbitol-fermenting O157 EHEC and EPEC strains represent a single clone complex that may belong to the non-sorbitol-fermenting O157:H7 worldwide clone and that the atypical *E. coli* O157 strains are independent. Our genotyping results indicate that *stx* genes may be part of the gain and loss of Stx-converting bacteriophages among *E. coli* O157 strains isolated from healthy bovines. Evidence in support of either possibility exists. First, Schmidt et al. (50) lysogenized laboratory strains as well as a broad range of enteric *E. coli* strains, including two EPEC strains, with a derivative of a Shiga toxin 2-encoding phage originating from an *E. coli* O157 strain. James et al. (26) also lysogenized several wild-type *E. coli* and *Shigella* strains in vitro by using an *aph3* gene-labeled Stx2-encoding phage. Acheson et al. (2) were able to transduce a laboratory strain in the murine gastrointestinal tract with a derivative of phage H-19B encoding Stx1, while Tóth et al. (55) were able to lysogenize a porcine EPEC O45 strain with a derivative of an Stx2-encoding phage in porcine ligated ileal loops. The results of these transduction experiments are consistent with the idea that EHEC emerged from EPEC by the acquisition of *stx* genes. Recently, Mellmann et al. (38) reported the loss of Stx phages from some EHEC strains in HUS patients within a short interval. Comparative molecular analyses of the *E. coli* strains isolated from original and sequential stool samples from 210 HUS patients revealed that of the 137 *stx*- and *eae*-positive *E. coli* strains originally isolated, 6 strains lost their Stx phages. Five of these six strains were serotype O26:H11, and one was O157:NM. Bielaszewska et al. (10) also verified the gain and loss of the *stx* gene in *E. coli* O26 in vitro. We observed a similar spontaneous loss of the stx_2 gene during the storage of one of our EHEC O157 strains (data not shown).

Although our atypical *E. coli* O157 strains did not carry *stx* and/or *eae* genes, five atypical O157 strains carried *cdt-VABC* genes and produced CDT. It was reported previously that the *cdt-V* allele is present in the majority of sorbitol-fermenting *E. coli* O157:NM strains (27) and in 5% of non-O157 EHEC clinical isolates (8). CDT-V occurs frequently in EHEC serotypes O113:H21 and O91:H21, the rare *eae*-negative EHEC types that cause HUS (31). These data suggest that CDT may contribute to the pathogenicity of *eae*-negative EHEC. Previously, Friedrich et al. (19) identified *cdt-V* in 4.9% of "classical" non-sorbitol-fermenting EHEC O157:H7 strains. The *cdt-V* EHEC O157:H7 strains belonged to five different PTs, including PT2, PT4, PT8, PT14, and PT34. Here, we report CDT-V production by non-sorbitol-fermenting O157 strains. The fact that these atypical O157 strains lack not only *eae* but also all the known key virulence genes underlines the virulence potential of CDT-V in *E. coli* O157:H37/H9. Furthermore, the CDT-V-producing atypical *E. coli* O157 strains harbored the major fimbrial subunit gene *lpfA* as well. The presence of polar fimbria genes in the CDT-V-producing strains may also be an important virulence characteristic since lpf_{O113} was reported to function as an adhesin in LEE-negative isolates of O113:H21 EHEC (15). These CDT-producing strains may represent a potentially novel clone, as demonstrated by PFGE and MLST.

E. coli O157 strains that lack Shiga toxin genes (*stx*) and the EPEC adherence factor plasmid are classified as atypical EPEC and cause diarrhea worldwide, especially in children. In our case, the bovine EPEC O157 strains isolated from healthy cattle resembled EHEC O157 strains on the basis of their serotypes and virulence gene profiles, and their multilocus sequence types were identical to those of the investigated *stx*- and *eae*-positive O157 (EHEC) strains, suggesting the zoonotic potential of these bovine EPEC O157 strains. Thus, our results confirm the recently published results of Bielaszewska et al. (9), who gave epidemiological and clinical evidence for the pathogenic significance of *stx*-negative atypical EPEC O157 strains in human patients with bloody diarrhea. Furthermore, they also stated that these *stx*-negative, EPEC adherence factor-negative attaching and effacing O157 strains are most likely former EHEC strains that lost Shiga toxin genes during infection, and they termed them EHEC-LST. Similarly, Friedrich et al. (20) were able to isolate strains of *stx*-negative *E. coli* O157 from stool samples obtained from patients with uncomplicated diarrhea and from HUS patients.

The bovine *E. coli* O157 strains described in our study represent different pathotypes, including EHEC, EPEC O157:H7, and atypical O157, and most of them possess virulence genes with zoonotic potential. These results indicate that in addition to the typical EHEC strains, the bovine O157 EPEC and CDT-V-producing atypical O157 strains deserve attention. Based on these observations, we conclude that calves and cattle represent important reservoirs of *eae*-positive *E. coli* O157 and that CDT-producing lpf_{O113}-positive O157 strains may represent a novel pathogroup of *E. coli* O157 with virulence potential for animals and humans.

ACKNOWLEDGMENTS

This study was supported by grants from NKTH (4/040/2001), EU NoEs EPG, and MedVetNET. Short-term mission support by MedVetNet for I.T. is also acknowledged.

We thank Márta Puruczki (Budapest) and Marcus Kranz (Stuttgart) for skillful technical assistance and Roberto La Ragione for the PCR primers. We also thank Noémi Nógrády for testing antimicrobial resistance and Vic Norris for critical reading of the manuscript.

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