SUPPLEMENT

Description of a Novel Intimin Variant (Type ζ) in the Bovine O84:NM Verotoxin-Producing *Escherichia coli* Strain 537/89 and the Diagnostic Value of Intimin Typing

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Infections with verotoxin-producing *Escherichia coli* (VTEC) has resulted in increasing numbers of human illnesses annually. These illnesses usually result from the ability of VTEC to cause the attaching and effacing lesions (AE lesion). The AE phenotype is encoded by the locus of enterocyte effacement (LEE) pathogenicity island. A key adhesion factor involved is the outer membrane protein intimin, encoded by the *eae* gene within the LEE. Intimin types α, β, γ, δ, and ε have been described previously. Each intimin represents distinct phylogenetic lineages of LEE-positive strains. A new intimin type ζ was identified in a VTEC strain of the serotype O84:NM (nonmotile) that was isolated from a calf with diarrhea. ζ intimin showed the highest similarity (88%) of its amino acid sequence to the α intimin. For diagnostic purposes, we established a polymerase chain reaction (PCR) method for diagnosis of the key virulence traits of VTEC (i.e., verotoxins and intimins). This method also distinguishes between the toxins (VT1 and VT2) and the six intimin types. By applying the PCR method, intimin ζ in strains of other VTEC serotypes O84:H2, O92:NM, O119:H25, and O150:NM was identified. Because the intimin types represent distinctive phylogenetic *E. coli* lineages, application of the intimin subtyping PCR offers significant benefits. These include improving diagnosis of VTEC infection and increasing the understanding of evolution of attaching and effacing VTEC and other LEE-positive bacteria. Exp Biol Med 228:370–376, 2003

Key words: *Escherichia coli*; intimin types; diagnosis; food-borne pathogens; verotoxins

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Verotoxin-producing *Escherichia coli* (VTEC) are significant enteric pathogens responsible for hemorrhagic colitis and the hemolytic uremic syndrome in humans. It is well established that verotoxins (VT1 and VT2), encoded on lambdoid phages, are key virulence factors involved in VTEC infections (1–3). Recent epidemiological evidence suggests that the possession of additional virulence factors, namely the locus of enterocyte effacement (LEE) and the EHEC hemolysin (Hly_{EHEC}), renders VTEC strains more virulent (2, 4). Although Hly_{EHEC} can easily be monitored on washed blood agar dishes (5), identification of the LEE pathogenicity island requires more fastidious techniques. The LEE enables VTEC strains to cause the attaching and effacing (AE) lesions (6). The LEE-specific genes also are found in other bacteria such as enteropathogenic *E. coli* (EPEC), *Citrobacter rodentium*, and *Escherichia alvei* (formerly known as *Hafnia alvei*). The LEE encodes a type III secretion system, an adhesin (intimin) factor, the translocated intimin receptor (Tir), and at least five secreted proteins (7–9). The key adhesion factor intimin, an outer membrane protein, is encoded by a mosaic gene and consists of a conserved N-terminal region and a variable C-terminal region (9, 10). The C-terminal region of the protein was shown to be responsible for binding to the translocated intimin receptor (Tir) and the intimate binding activity of the outer membrane protein to eukaryotic enterocytes (11–13).

Five different intimin types have so far been identified, designated α, β, γ, δ, and ε intimin. Types α, β, γ, and δ have been distinguished by antibodies, whereas type ε was differentiated by polymerase chain reaction (PCR). Recent analysis by Oswald *et al.* (14) suggested that intimin δ is a

subtype of intimin β. Interestingly, the different intimin types are associated with the phylogeny of attaching and effacing *E. coli* strains (10, 14–17). Intimin α is specifically expressed by strains belonging to one evolutionary lineage of EPEC known as cluster EPEC 1. In contrast, intimin β is mainly associated with strains of clusters EPEC 2 and STEC 2. Intimin γ is associated with VTEC O157:H7 and EPEC O55:H7 (16, 18, 19). In addition to this important phylogenetic aspect, intimin types seem to be responsible for different host- and tissue-targeting specificities of the respective VTEC and EPEC strains (20, 21).

During the analysis of a larger set of LEE-positive bovine VTEC strains, we identified strains of O-types O4, O80, O84, O92, O119, and O150 with *eae* genes that were not typeable by PCR for differentiation of intimin types (14, 19). We hypothesized a novel intimin type, possibly being associated with a distinctive phylogenetic VTEC lineage. The objective was to describe the nucleotide sequence of the new intimin type, referred to as ζ intimin, in the bovine VTEC strain 537/89 of serotype O84:NM. Additionally, a diagnostic PCR for detection of intimin ζ was described.

Materials and Methods

Bacterial Strains. Out of 122 bovine intimin-positive VTEC strains isolated from a total of 5438 *E. coli* strains (22), 36 representative strains were analyzed. Reference *E. coli* strains used for PCR and cell culture assays were previously reported (14, 23–28) and included PMK5 (EHEC O103:H2), MG1655 (*E. coli* K12), RW1374 (VTEC O103:H2), E2348/69 (EPEC O127:H6), EDL933 (EHEC O157:H7), 413/89-1 (VTEC O26:NM), and 537/89 (VTEC O84:NM), respectively.

DNA and PCR Analysis. Total genomic DNA was isolated by using standard procedures (29). A total of 10 ng of template DNA was used for each PCR. Reactions were carried out in a total volume of 50 μl with Herkulase Enhanced Polymerase Blend (Stratagene, Amsterdam, The Netherlands) as recommended by the manufacturer. All PCR reactions were performed on a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Langen, Germany). Intimin α gene was detected by using a primer pair SK1-LP2, intimin β by primer pair SK1-LP4, intimin γ by primer pair SK1-LP3, and intimin ε by primer pairs SK1-LP5 as described previously (14). The *eae* gene was tested by primers ECW1-ECW2 (30) as well as SK1-SK2 (14). The genes for VT1 and VT2 were identified (31) using primer pairs SK1 (VT) SK2 (VT) and SK3 (VT) SK4 (VT). The *eae* gene containing region in strain 537/89 was amplified (via PCR) by using primer pair eaef-escD1 (described herein). All primer sequences are given in Table I. DNA sequence analysis was performed by the chain termination sequencing technique (32). Phylogenetic analysis of *eae* sequences was performed by using the CLUSTAL W program (33). The ζ intimin gene sequence was deposited in GenBank (accession no. AJ298279).

Cell Culture Assays. The ability of strain 537/89 to cause AE lesions was detected in HEp-2 cells and in fetal calf lung (FCL) cells by the fluorescence actin staining (FAS) test (34, 35).

Results

Types of *eae* Genes in Bovine VTEC Strains. Out of the 122 *eae*-positive bovine VTEC strains described by Wieler *et al.* (22), the intimin types of 36 strains representing 26 serotypes were analyzed. By using the different *eae* primer pairs (Table I), four different intimin types in these 36 VTEC strains were identified. Intimin β strains represented the largest group of strains (n = 17), and strains with this intimin type displayed the highest number (n = 14) of different serotypes. Intimin γ was only identified in strains of serotypes O111:NM, O111:H2, O145:H28, and O157:H7, whereas the recently described intimin ε was only found in strains of serotype O103:H2. Eight strains displaying different serotypes were not typeable by PCR identify-

Table I. Sequence of Primer Pairs Used for Detection of Verotoxins and Intimins

Name	Sequence in 5′–3′ direction	Target region	Reference
eaef	GAG CAC AAT CGC TGT TGT TAG C	LEE	
escD1	TAT CAA CAT CTC CCG CCC AG	LEE	
ECW1	TGC GGC ACA ACA GGC GGC GA	*eae*	30
ECW2	CGG TCG CCG CAC CAG GAT TC	*eae*	30
SK1	CCC GAA TTC GGC ACA AGC ATA AGC	*eae*	14
SK2	CCC GGA TCC GTC TCG CCA GTA TTC G	*eae*	14
LP2	CCC GAA TTC TTA TTT TAC ACA AGT GGC	*eae* type α	14
LP4	CCC GTG ATA CCA GTA CCA ATT ACG GTC	*eae* type β	14
LP3	CCC GAA TTC TTA TTC TAC ACA AAC CGC	*eae* type γ	14
LP5	AGC TCA CTC GTA GAT GAC GGC AAG CG	*eae* type ε	14
LP6	TAA CTT GAC CAG TGG AAT CC	*eae* type ζ	
Ehly1	GAG CGA GCT AAG CAG CTT G	hly_{EHEC}	27
Ehly5	CCT GCT CCA GAA TAA ACC ACA	hly_{EHEC}	27
SK1 (VT)	GAC TAC TTC TTA TCT GGA TTT	*vt1*	31
SK2 (VT)	AAC GAA AAA TAA CTT CGC TG	*vt1*	31
SK3 (VT)	CCG GGC GTT TAC GAT AGA CTT	*vt2*	31
SK4 (VT)	TGC AGC TGT ATT ACT TTC CC	*vt2*	31

ing intimins of types α, β, γ, or ε. These strains were O84:NM, O84:H2, O92:NM, O119:H25, O150:NM, O untypeable (OUT):NM, O4:NM, and O80:NM (Table II).

Sequencing of the *eae* Gene of the Bovine VTEC Strain 537/89. Because these eight strains harbored an *eae* gene (as tested with primer pairs SK1-SK2 and ECW1-ECW2) but were negative for other intimin types (14), the occurrence of a novel type of intimin was suspected. Therefore, the *eae* gene of strain 537/89 (O84:NM) was analyzed. This strain (isolated from a calf with diarrhea) contained the LEE pathogenicity island (data not shown) as indicated by hybridization experiments of genomic DNA with the LEE-specific probes A, B, C, and D (6). This strain also was able to induce a response in the FAS test using FCL cells, which indicated a functional intimin. However, it should be noted that no reaction with HEp-2 cells was detected. In addition, this strain harbored the VT1 gene as indicated by the PCR experiments (data not shown).

A PCR with primers located upstream and downstream of the *eae* gene was performed. The PCR with the primer pair eaef and escD revealed a product of 4 kb. Sequence analysis of the amplicon revealed an open reading frame of 2817 bp, which encodes a protein of 938 amino acids and has similarity to other intimin gene sequences. Two cysteine residues that have been shown to be necessary for the formation of a disulfide bond and the binding activity in other intimin types (9) were also found in the new intimin sequence (Fig. 1). The intimin of strain E2348/69 (α intimin) was shown to have the highest overall similarity (88%) to the intimin of 537/89 (data not shown). The C-terminal sequence (last 280 amino acids) was different from that of other intimin type sequences described so far and it had only 70% similarity to α intimin (Fig. 1). This sequence divergence indicated a new intimin type, referred to as intimin ζ. This finding prompted us to further investigate the set of bovine VTEC strains for the presence of this new intimin type.

Table II. Distribution of Intimin Types Among Bovine VTEC Strains

Serotype	Intimin type	Verotoxin gene	No. of strains
O5:NM	β	*vt1*	1
O15:H11	β	*vt1*	1
O17,77:H18	β	*vt1*	1
O26:NM	β	*vt1*	1
O26:H+	β	*vt1*	3
O26:H11	β	*vt1*	2
O118:NM	β	*vt1*	1
O118:H16	β	*vt1* and *vt2*	2
O145:H+	β	*vt1*	1
O153:H+	β	*vt1*	1
OUT:H11	β	*vt1*	1
OUT:H6	β	*vt2*	1
OUT:H+	β	*vt1*	1
O111:NM	γ	*vt1*	4
O111:H2	γ	*vt1*	1
O145:H28	γ	*vt2*	2
O157:H7	γ	*vt1* and *vt2*	1
O157:H7	γ	*vt2*	1
O103:H2	ε	*vt1*	2
O84:NM	ζ	*vt1*	1
O84:H2	ζ	*vt1*	1
O92:NM	ζ	*vt1*	1
O119:H25	ζ	*vt1*	1
O150:NM	ζ	*vt1* and *vt2*	1
ONT:NM	?	*vt1*	1
O4:NM	?	*vt2*	1
O80:NM	?	*vt1*	1

Nonmotile (NM).
O Untypeable (OUT).

Construction of a Specific Intimin ζ Primer and Occurrence of the New Type Intimin in Other Bovine VTEC Strains. On the basis of the ζ intimin gene sequence from strain 537/89, a specific reverse primer of the variable region of the 3′ end (LP6), which could be used in combination with primer SK1, was generated. A PCR using primers SK1-LP6 yielded a product of the expected size of 2411 bp using chromosomal DNA from strain 537/89. In addition, the untypeable strains of serotypes O84:H2, O92:NM, O119:H25, and O150:NM were also positive in this PCR, revealing that they also harbored intimin ζ (Fig. 2). However, the same PCR did not generate amplicons with DNA from VTEC strains of serotypes OUT:NM, O4:NM, and O80:NM.

Phylogenetic Analysis of Intimin. Based on the amino acid sequence of the new intimin variant, we constructed a phylogenetic tree to accumulate more information on the evolution of the intimin variants. Therefore, we performed a CLUSTAL W alignment by using the last 280 C-terminal amino acids of intimin (starting with alanine 656). In this analysis, intimin ζ was found to establish its own distinct branch, sharing highest phylogenetic relatedness with intimin α (Fig. 3).

Discussion

In addition to the five intimin types identified so far (14, 15, 36), we were able to characterize a novel type, termed intimin ζ. The biological significance of this finding has yet to be investigated, but it has previously been shown that the intimin type influences tissue and host tropism of attaching and effacing *E. coli* strains (20, 21). To our knowledge, VTEC strains of O-type O92 have so far only been isolated from cattle (22, 37–39). Clearly, VTEC strains of the three O-types O84, O92, and O150 are of low prevalence. Only strains of VTEC O84, O150, and O119:H25 have been isolated from cattle, sheep, or humans in Europe and in North America (22, 38, 40–42). Strains of O-type O119 traditionally represent classical EPEC strains (43, 44). Intimins of strains belonging to O-types O84, O92, O119, and O150 have not been characterized with regard to their respective intimin types α, β, γ, δ, or ε (14, 19, 45). Moreover, the ζ intimin has been detected in strains of O-types O98, O111, and O156 (42, 46).

Although different intimin types are associated with distinctive phylogenetic lineages of LEE-positive *E. coli*

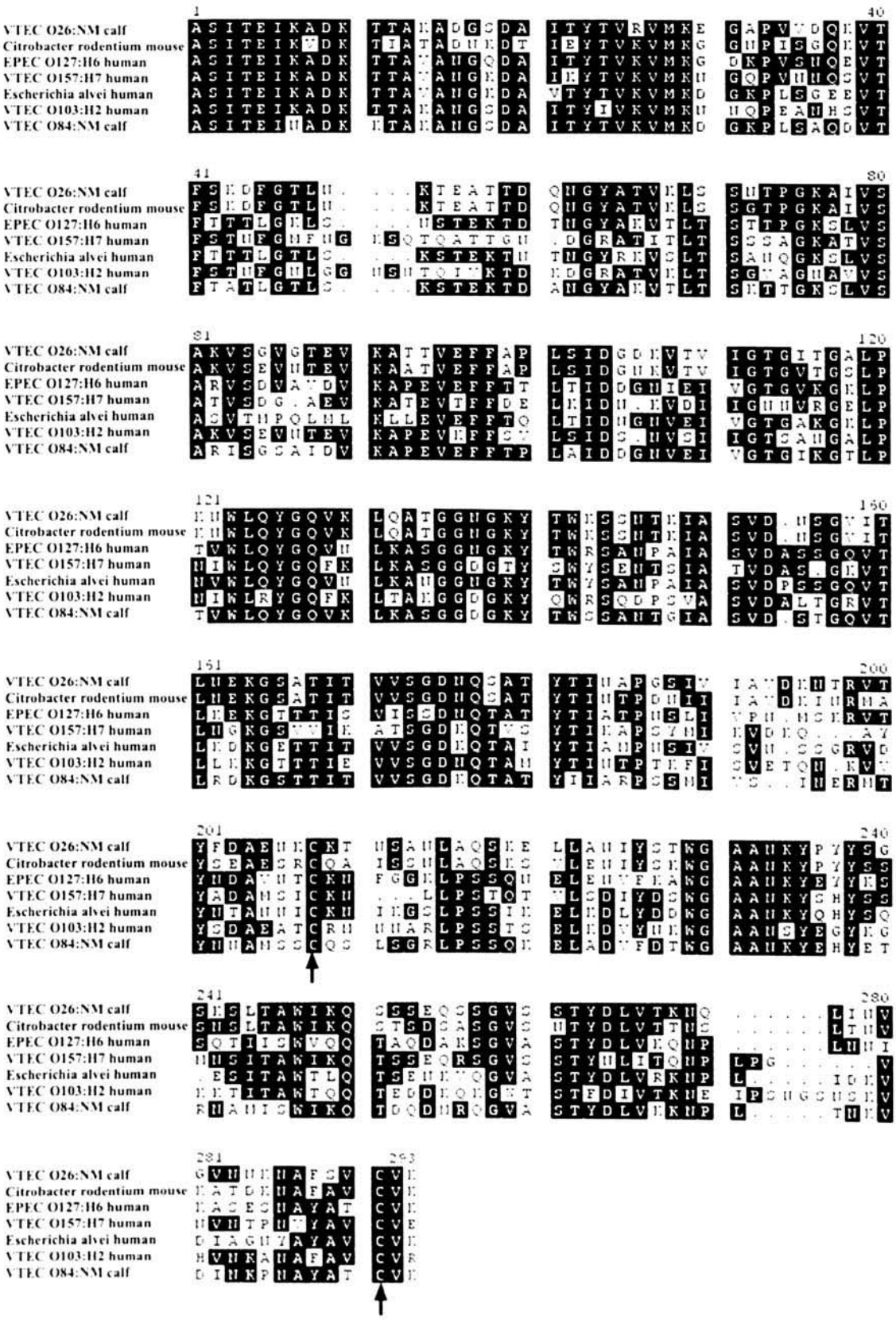

Figure 1. CLUSTAL W alignment of ξ intimin from bovine VTEC strain 537/89 (O84:NM) with different published intimin types. This multiple alignment is based on the C-terminal amino acids of the displayed intimins starting with alanine 658. Different intimin sequences were retrieved from GenBank (VTEC 26:NM; AJ223063, *Citrobacter rodentium*; L11691, EPEC O127:H6; M58154, VTEC O157:H7; Z11541, *E. alvei*; L29509, VTEC O103:H2; AF116899, and VTEC O84:NM, AJ298279). Black shaded boxes indicate identity, gray shaded boxes indicate similarity of amino acids, and arrows indicate cysteine residues responsible for formation of disulfide bonds.

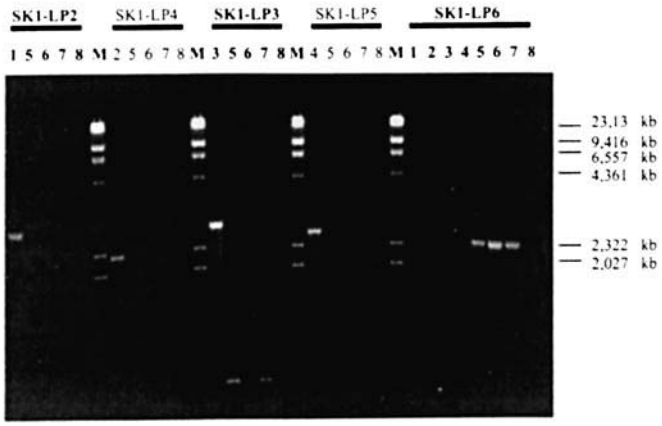

Figure 2. PCR amplicons of different strains with primer pairs specific for intimin α (SK1-LP2), intimin β (SK1-LP4), intimin γ (SK1-LP3), intimin ε (SK1-LP5), and intimin ζ (SK1-LP6). M, molecular weight marker (λ *Hind*III); Lane 1 strain E2348/69 (O127:H6), Lane 2 strain 413/89-1 (O26:NM), Lane 3 strain EDL933 (O157:H2), Lane 4 strain PMK5 (O103:H2), Lane 5 strain 537/89 (O84:NM), Lane 6 strain IHIT3669 (O84:H2), Lane 7 strain IHIT3000 (O150:NM), and Lane 8 strain MG1655.

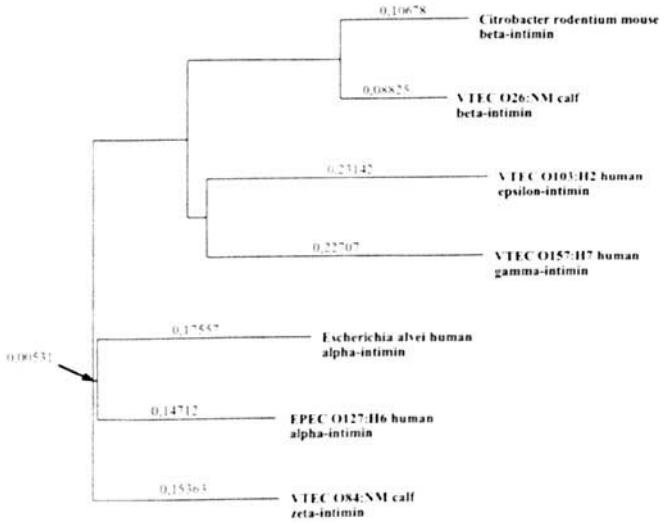

Figure 3. Phylogenetic tree based on five intimin types was generated using the C-terminal amino acids of the displayed intimins and starts with alanine 658.

strains (10, 15–17), our results do not suggest a ζ intimin adaptation to the bovine gut despite the finding that strain 537/89 reacted FAS positive when tested in bovine FCL cells and negative in HEp-2 cells of humans. However, the distribution of the ζ intimin in other LEE-positive *E. coli* strains remains to be elucidated.

The wide spectrum of different intimin types (β, γ, ε, and ζ) identified in bovine VTEC disputes a species specific role of this adhesion factor. The finding that the intimins of serotype strains OUT:NM, O4:NM, and O80:NM could not be typed by PCR suggests a larger variety of intimins. These strains also may belong to subtypes of the distinctive intimins as described previously (14). Intimin α strains have exclusively been identified in EPEC (10). The identified variety of bovine intimin VTEC strains further substantiates the reservoir function of cattle for infections of humans with LEE-positive VTEC strains. It is not known why many phylogenetic different VTEC strains can be isolated from cattle. There is substantial knowledge of the transfer possibility of phages in the rumen (47), and it has been hypothesized that VT genes, encoded on phages, are spread in the ruminants intestines. This spread leads to the exchange of VT-encoding phages between *E. coli* in the intestines of ruminants (48). However, it should be noted that the natural transfer mechanisms of the LEE pathogenicity island is still unknown. Although we and others have identified remnants of phage sequences flanking different LEE pathogenicity islands, there is no indication of their functionality (7, 24).

It is possible that certain phylogenetic lineages are highly host adapted, and it has been suggested that the diversity within intimin is driven by natural selection (15). We were recently able to show that sera from calves reacted with different intensity when tested against recombinant intimin α and intimin β proteins (49). It has also been reported that rabbit and human antisera react with differential intensity with intimin from different VTEC and EPEC strains (15, 50–52). Whether this difference has any protective value remains to be determined.

We are currently investigating the phylogeny of these ζ intimin strains in association with the clusters described by others (18). The phylogenetic investigation in Figure 3 is based on the intimin sequence only. It revealed a distinct phylogenetic branch for this novel intimin type. The N-terminal region of the intimin ζ type showed the highest similarity of amino acids to type α intimin. Further research is needed to identify antigenic differences that may explain the biological properties of intimin ζ and that could be used to differentiate intimin types by the use of antibodies. Despite this missing antibody tool, we developed a PCR method to differentiate the new intimin in other LEE-harboring strains with specific primer pairs (SK1-LP6). With this approach, diagnostic laboratories should be able to screen LEE-positive *E. coli* strains and even strains of *E. alvei* or *C. rodentium* for their respective intimin types. Thus, by the application of PCR, investigators can better understand the virulence potential of these pathogens and can gain direct insight into the worldwide distribution and phylogeny of such VTEC strains.

1. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201, 1998.
2. Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 11:450–479, 1998.
3. O'Loughlin EVO, Robins-Browne RM. Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. Microbes Infect 3:493–507, 2001.
4. Bockemühl J, Karch H. Zur aktuellen Bedeutung der enterohämorrhagischen *Escherichia coli* (EHEC) in Deutschland (1994–1995). Bundesgesundheitsblatt 39:290–296, 1996.
5. Beutin L, Prada J, Zimmermann S, Stephan R, Ørskov I, Ørskov F,

Enterohemolysin, a new type of hemolysin produced by some strains of enteropathogenic *E. coli* (EPEC). Zblt Bakteriol **267**:576–588, 1988.
6. McDaniel TK, Jarvis KJ, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A **92**:1664–1668, 1995.
7. Perna NT, Mayhew GF, Posfai G, Elliott S, Donnenberg MS, Kaper JB, Blattner FR. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun **66**:3810–3817, 1998.
8. Elliott SJ, Krejany EO, Mellies JL, Robins-Browne RM, Sasakawa C, Kaper JB. EspG, a novel type III system-secreted protein from enteropathogenic *Escherichia coli* with similarities to VirA of *Shigella flexneri*. Infect Immun **69**:4027–4033, 2001.
9. Frankel G, Phillips AD, Trabulsi LR, Knutton S, Dougan G, Matthews S. Intimin and the host cell: is it bound to end in Tir(s)? Trends Microbiol **9**:214–218, 2001.
10. McGraw EA, Li J, Selander RK, Whittam TS. Molecular evolution and mosaic structure of α, β, and γ intimins of pathogenic *Escherichia coli*. Mol Biol Evol **16**:12–22, 1999.
11. Frankel G, Candy DCA, Everest P, Dougan G. Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. Infect Immun **62**:1835–1842, 1994.
12. Hartland EL, Batchelor M, Delahay RM, Hale C, Matthews S, Dougan G, Knutton S, Connerton I, Frankel G. Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. Mol Microbiol **32**:151–158, 1999.
13. Reece S, Simmons CP, Fitzhenry RJ, Matthews S, Phillips AD, Dougan G, Frankel G. Site-directed mutagenesis of intimin α modulates intimin-mediated tissue tropism and host specificity. Mol Microbiol **40**:86–98, 2001.
14. Oswald E, Schmidt H, Morabito S, Karch H, Marches O, Caprioli A. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect Immun **68**:64–71, 2000.
15. Abu-Bobie J, Frankel G, Bain C, Goncalves AG, Trabulsi L, Douce G, Knutton S, Dougan G. Detection of intimins α, β, γ, δ, four intimin derivatives expressed by attaching and effacing microbial pathogens. J Clin Microbiol **36**:662–668, 1998.
16. Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature **406**:64–67, 2000.
17. Zehmke K, Jores J, Roumer L, Kießling S, Lautenschläger S, Wieler LH. Evolution of bovine non-O157-Shigatoxin-producing *E. coli* (STEC) strains with respect to acquisition of the Locus of Enterocyte Effacement (LEE). In: 4th International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections; 2000; Kyoto, Japan. p127. Abstract nr 265.
18. Whittam TS, McGraw AE. Clonal analysis of EPEC serogroups. Rev Microbiol **27**(Suppl 1):7–16, 1996.
19. Reid SD, Betting DJ, Whittam TS. Molecular detection and identification of intimin alleles in pathogenic *Escherichia coli* by multiplex PCR. J Clin Microbiol **37**:2719–2722, 1999.
20. Tzipori S, Gunzer F, Donnenberg MS, de Montigny L, Kaper JB, Donohue-Rolfe A. The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. Infect Immun **63**:3621–3627, 1995.
21. Phillips AD, Frankel G. Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. J Infect Dis **181**:1496–1500, 2000.
22. Wieler LH, Vieler E, Erpenstein C, Schlapp T, Steinrück H, Bauerfeind H, Byomi A, Baljer G. Shiga toxin-producing *Escherichia coli* (STEC) from bovines: association of adhesion with the carriage of *eae* and other genes. J Clin Microbiol **34**:2980–2984, 1996.
23. Blattner FR, Plunkett GR, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453–1474, 1997.
24. Jores J, Rumer L, Kiessling S, Kaper JB, Wieler LH. A novel locus of enterocyte effacement (LEE) pathogenicity island inserted at *pheV* in bovine shiga toxin producing *E. coli* strain O103:H2. FEMS Microbiol Lett **204**:75–79, 2001.
25. Jerse AE, Yu J, Tall BD, Kaper JB. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A **87**:7839–7843, 1990.
26. Yu JC, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157:H7. Mol Microbiol **6**:411–417, 1992.
27. Wieler LH, Tigges M, Schäferkordt S, Ebel F, Djafari S, Schlapp T, Chakraborty T. The enterohemolysin phenotype of bovine Shiga-like toxin-producing *Escherichia coli* (SLTEC) is encoded by the EHEC-hemolysin gene. Vet Microbiol **52**:153–164, 1996.
28. Wieler LH, Bauerfeind R, Baljer G. Characterization of Shiga-like toxin producing *Escherichia coli* (SLTEC) isolated from calves with and without diarrhoea. Zblt Bakteriol **276**:243–253, 1992.
29. Ausubel FM. Current Protocols in Molecular Biology. New York: John Wiley & Sons, Inc., 2001.
30. Heuvelink AE, van de Kar NC, Meis JF, Monnens LA, Melchers WJ. Characterization of verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uraemic syndrome in Western Europe. Epidemiol Infect **115**:1–14, 1995.
31. Wieler LH, Busse B, Steinruck H, Beutin L, Weber A, Karch H, Baljer G. Enterohemorrhagic *Escherichia coli* (EHEC) strains of serogroup O118 display three distinctive clonal groups of EHEC pathogens. J Clin Microbiol **38**:2162–2169, 2000.
32. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A **74**:5463–5467, 1977.
33. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22**:4673–4680, 1994.
34. Knutton S, Baldwin T, Williams PH, McNeish AS. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect Immun **57**:1290–1298, 1989.
35. Wieler LH, Schwanitz A, Vieler E, Busse B, Steinruck H, Kaper JB, Baljer G. Virulence properties of Shiga toxin-producing *Escherichia coli* (STEC) strains of serogroup O118, a major group of STEC pathogens in calves. J Clin Microbiol **36**:1604–1607, 1998.
36. Agin TS, Wolf MK. Identification of a family of intimins common to *Escherichia coli* causing attaching-and-effacing lesions in rabbits, humans and swine. Infect Immun **65**:320–326, 1997.
37. Wells JG, Shipman LD, Greene KD, Sowers EG, Green JH, Cameron DN, Downes FP, Martin ML, Griffin PM, Ostroff SM, Potter ME. Isolation of *Escherichia coli* serotype O157:H7 and other Shiga-like toxin-producing *E. coli* from dairy cattle. J Clin Microbiol **29**:985–989, 1991.
38. Sandhu KS, Clarke RC, McFadden K, Brouwer A, Louie M, Wilson J, Lior H, Gyles CL. Prevalence of the *eaeA* gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario. Epidemiol Infect **116**:1–7, 1996.
39. Leung PH, Yam WC, Ng WW, Peiris JS. The prevalence and characterization of verotoxin-producing *Escherichia coli* isolated from cattle and pigs in an abattoir in Hong Kong. Epidemiol Infect **126**:173–179, 2001.
40. Bitzan M, Karch H, Maas MG, Meyer T, Rüssmann H, Aleksic S, Bockemühl J. Clinical and genetic aspects of Shiga-like toxin production in traditional enteropathogenic *Escherichia coli*. Zblt Bakteriol **274**:496–506, 1991.
41. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing

Escherichia coli in seven different species of healthy domestic animals. J Clin Microbiol **31**:2483–2488, 1993.
42. Blanco M, Blanco JE, Mora A, Alonso MP, Gonzáles EA, Bernádez MI. Epidemiology of verocytotoxigenic *Escherichia coli* (VTEC) in ruminants. In: Duffy G, Garvey P, McDowell DA, Eds. Verocytotoxigenic *E. coli*. Trumbull, CT: Food and Nutrition Press, 2001.
43. Goncalves AG, Campos LC, Gomes TA, Rodrigues J, Sperandio V, Whittam TS, Trabulsi LR. Virulence properties and clonal structures of strains of *Escherichia coli* O119 serotypes. Infect Immun **65**:2034–2040, 1997.
44. Saridakis HO, el Gared SA, Vidotto MC, Guth BE. Virulence properties of *Escherichia coli* strains belonging to enteropathogenic (EPEC) serogroups isolated from calves with diarrhea. Vet Microbiol **54**:145–153, 1997.
45. Vieira MA, Andrade JR, Trabulsi LR, Rosa AC, Dias AM, Ramos SR, Frankel G, Gomes TA. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry *eae* and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. J Infect Dis **183**:762–772, 2001.
46. Tarr CL, Whittam TS. Molecular evolution of the intimin gene in O111 clones of pathogenic *Escherichia coli*. J Bacteriol **184**:479–487, 2002.
47. Swain RA, Nolan JV, Klieve AV. Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. Appl Environ Microbiol **62**:994–997, 1996.
48. Beutin L, Geier D, Zimmermann S, Aleksic S, Gillespie HA, Whittam TS. Epidemiological relatedness and clonal types of natural populations of *Escherichia coli* strains producing shiga toxins in separate populations of cattle and sheep. Appl Environ Microbiol **63**:2175–2180, 1997.
49. Wagner C. Detection of antibodies against secreted proteins of the locus of enterocyte effacement (LEE) from Shiga toxin-producing *E. coli* in sera from calves. Institute of Microbiology and Animal Epidemic Disaeses. Free University Berlin, Germany, p141, 2001.
50. Paton AW, Voss E, Manning PA, Paton JC. Shiga toxin-producing *Escherichia coli* isolates from cases of human disease show enhanced adherence to intestinal epithelial (Henle 407) cells. Infect Immun **65**:3799–3805, 1997.
51. Paton AW, Voss E, Manning PA, Paton JC. Antibodies to lipopolysaccharide block adherence of Shiga toxin- producing *Escherichia coli* to human intestinal epithelial (Henle 407) cells. Microb Pathog **24**:57–63, 1998.
52. Voss E, Paton AW, Manning PA, Paton JC. Molecular analysis of Shiga toxigenic *Escherichia coli* O111:H$^-$ proteins which react with sera from patients with hemolytic-uremic syndrome. Infect Immun **66**:1467–1472, 1998.