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Analysis of the Clonal Relationship of Shiga Toxin-Producing *Escherichia coli* Serogroup O165:H25 Isolated from Cattle

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Variations in time and space of a clonal group of *Escherichia coli* O165:H25 on a cattle farm were monitored. The virulence marker pattern (*stx* genes, *eae* gene, hly_{EHEC} gene, *katP* gene, *espP* gene, *efa* gene) suggests that *E. coli* O165:H25 of bovine origin may represent a risk for human infection.

Shiga toxin-producing *Escherichia coli* (STEC) is a group of zoonotic enteric pathogens (29). Human infections with some STEC serotypes, also designated enterohemorrhagic *Escherichia coli* (EHEC), result in hemorrhagic or nonhemorrhagic diarrhea, which may be complicated by hemorrhagic colitis (HC) and several renal sequelae, including the hemolytic-uremic syndrome (HUS) (20, 34, 41, 52). The mechanisms by which EHEC strains cause disease are not completely understood. The virulence factors include production of two major phage-encoded toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which can be produced alone or in combination. Stx1 and Stx2 are thought to cause the vascular endothelial damage observed in patients with HC and HUS (53). In addition to Stx, EHEC strains possess other virulence factors, such as the ability to cause attaching and effacing (*eae*) lesions in the large intestine (26). They often contain a plasmid that carries other potential virulence genes, such as an enterohemolysin gene (hly_{EHEC}), a catalase peroxidase gene (*katP*), and an extracellular serine protease gene (*espP*) (9, 10, 11, 45). The *efa1* (*E. coli* factor for adherence) gene represents another intestinal colonization factor in an EHEC O111:H- strain (30). The *efa1* gene of O111:H- is 99.9% homologous to the *lifA* gene in enteropathogenic *E. coli*. The *efa1* locus is not physically linked to the locus for enterocyte effacement pathogenicity island (51).

Ruminants, especially cattle, are considered the primary reservoir for human EHEC infections (23). In this study, 38 bovine *E. coli* O165:H25 isolates were characterized to assess their potential to cause EHEC disease in humans. These isolates were detected over a 4-month period in eight different animals (Table 1) from a single group of beef cattle during a long-term study (19). Sporadic cases of human infections with O165:H25 and O165:H- in Europe and Canada have been described previously (6, 15, 17, 24). The four German O165:H25 and O165:H- strains of human origin (kindly supplied by H. Tschäpe, Robert Koch-Institute, Wernigerode, Germany) used in our study for comparison were associated with diarrhea in patients (54). Human HUS cases caused by O165 isolates have been reported from Denmark (15) and Germany (17).

Typing and subtyping of genes (stx_1 and/or stx_2, *eae*, hly_{EHEC}, *katP*, and *espP*) associated with STEC were performed by LightCycler fluorescence PCR (40) and different Block cycler PCRs (Tables 2 and 3). A complete pattern of virulence markers was detected in most bovine isolates examined. An stx_2 gene, but not an stx_1 gene, was present in all O165 strains (Table 1). EHEC strains with stx_2 genes are significantly more frequently associated with HUS and other severe diseases than isolates with an stx_1 gene, which are more often associated with uncomplicated diarrhea or healthy individuals (6, 36). Stx2 is closely related to a family of Stx2 variants or alleles, which includes Stx2c (48), Stx2d (36), Stx2e (56), and Stx2f (47), although Stx2c and Stx2d are produced by STEC strains isolated from humans (36, 38, 43, 44, 48, 50). Additional genetic variants of the stx_2 gene have been described (5, 14, 28, 55). In contrast to STEC strains harboring stx_2 gene variants, however, STEC strains with the stx_2 genotype were significantly associated with HUS (17). An stx_2 gene with the $stx_{2\text{-EDL933}}$ genotype was found in all O165 isolates tested (Table 1). The nucleotide sequences of the A and B subunits of the stx_2 gene of the bovine O165:25 strain 02/09/010-1 (GenBank accession number AY652745) were identical to the sequences of the stx_2 gene of EHEC type strain EDL933 (35), a typical O157:H7 strain isolated from a HUS patient, with the sequences of the gene of bacteriophage 933W (37), and with the sequences of stx_2 genes of other *E. coli* O157:H7 strains of human origin isolated from EHEC outbreaks (25, 27). All bovine O165:H25 strains produced an Stx2 with high cytotoxicity for Vero cells as determined by an Stx enzyme-linked immunosorbent assay and by a Vero cell neutralization assay (49).

Not only factors that influence basal and inducible Stx production are important in STEC pathogenesis. In previous studies, it has been suggested that the *eae* and hly_{EHEC} genes likely contribute to STEC pathogenicity (3, 6, 42). Ritchie et al. (42) found both of these genes in all HUS-associated STEC isolates that they analyzed. The stx_2 genes were present in combination with *eae* genes in all O165 isolates that we obtained (Table 1). To date, 10 distinct variants of *eae* have been described (13, 21, 31, 39). Some serotypes were closely associated with a particular intimin variant (4, 12, 13, 55). Our study confirmed these associations. Like the O103 isolates, all bovine and human *E. coli* O165 strains were grouped into the ε-*eae* subgroup. Also, nucleotide sequencing of the bovine O165:H25 strain 02/09/010-1

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TABLE 1. Strains examined

Sampling day or year[a]	Source	Strain	Serotype	Virulence profile											
				stx_1 gene	stx_2 gene	Stx1 (toxin)	Stx2 (toxin)	stx_2 Subtype	*eae* Subtype	*efa1*/*lifA* gene	hly_{EHEC} gene	*katP* gene	*espP* gene	Plasmid(s) (bp)	Genetic subcluster
Day 1	Cattle 17	02/17/009-9	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	7
	Cattle 24	02/24/007-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-2	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-3	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-4	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-5	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-6	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	3
		02/24/007-7	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-9	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/24/007-10	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
	Cattle 26	02/26/006-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/26/006-2	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/26/006-3	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/26/006-4	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	9
		02/26/006-5	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	9
		02/26/006-6	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	−	+	67, 55	9
		02/26/006-7	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	−	−	−	55	4
		02/26/006-8	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	5
		02/26/006-9	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	−	−	−	55	6
		02/26/006-10	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
Day 28	Cattle 25	02/25/007-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-4	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-5	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	−	−	−	55	1
		02/25/007-6	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-7	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-8	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-9	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-10	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
Day 56	Cattle 9	02/09/010-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	8
		02/09/010-2	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	8
	Cattle 18	02/18/011-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70	10
		02/18/011-5	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	10
		02/18/011-6	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	10
	Cattle 25	02/25/008-1	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	1
		02/25/007-3	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	2
Day 119	Cattle 19	02/19/013-7	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	−	+	+	+	70, 55	11
		02/19/013-8	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	8
		02/19/013-10	O165:H25	−	+	−	+	$stx_{2\text{-EDL933}}$	ε	+	+	+	+	70, 55	8
1998	Human, Germany, diarrhea	98-4982[b]	O165:H-	−	+	−	+	$stx_{2/2c}$	ε	+	+	+	+	75	
1998	Human, Germany, diarrhea	98-8419-1[b]	O165:H-	−	+	−	+	$stx_{2/2c}$	ε	+	+	+	+	95, 75	
1999	Human, Germany, diarrhea	99-2258[b]	O165:H25	−	+	−	+	$stx_{2/2c}$	ε	+	+	+	+	95, 75	
2002	Human, Germany, diarrhea	02-11228[b]	O165:H25	−	+	−	+	$stx_{2/2c}$	ε	+	+	+	+	95, 75	

[a] On days −120 to 0 O165:H25 was not detected on eight occassions, on day 91 O165:H25 was not detected, and on days 147 to 511 O165:H25 was not detected by 17 investigations.
[b] Kindly provided by H. Tschäpe.

(GenBank accession number AF479581) revealed a high level of sequence homology (99.7%) to the *eae* gene of an O103:H2 strain (31). *E. coli* O103:H2 strains have frequently been associated with human HUS cases in Europe. Like the *eae* gene, the hly_{EHEC} gene was found in association with severe disease in humans (45, 46). In our study, the hly_{EHEC} gene was detected in the O165 strains in which a 70-kb plasmid was also found (Table 1). The presence of a 70-kb plasmid was associated with the occurrence of additional virulence markers, such as the *espP* gene, and all but one isolate contained the *katP* gene (10, 11). The reason for the slightly smaller size (67 kb) of the plasmid in this isolate may be linked to fact that the *katP* gene was not present in this isolate (Table 1). The *efa1* genes were detected in 37 of 38 bovine O165:H25 isolates with two DNA probes by colony hybridization, and the results were confirmed by Southern hybridization after pulsed-field gel electrophoresis (PFGE); in this analysis the DNA probes were labeled with digoxigenin (DIG), primers *lifA*1 and *lifA*2 and primers *lifA*3 and *lifA*4 (Table 3) were used with a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany), and DIG Easy Hyb solution (Roche) was used for prehybridization and hybridization. The *efa-1* gene was located on an approximately 240-kb XbaI fragment or on an approximately 440-kb NotI fragment. These fragments were missing in the isolate that was negative as determined by colony hybridization (Fig. 1). To determine this, slices of the plugs were digested for 4 h with XbaI, NotI (New England Biolabs GmbH, Frankfurt am Main, Germany), BlnI (AvrII), or SpeI (Amersham Biosciences Inc., Buckinghamshire, United Kingdom). The resulting fragments were separated in a 1.0% agarose gel (SeaKem Gold agarose; Cambrex) in 0.5× Tris-borate-EDTA at 10°C with a CHEF Mapper XA system. The pulse times for XbaI and NotI digests were increased from 5 to 50 s (gradient, 6 V/cm) during 25 h at a constant angel of 120°. The switch time

TABLE 2. Oligonucleotide primers and LightCycler hybridization probes used for LightCycler PCR

Target(s)	Primer	Sequence (5′–3′)[a]	Reference
stx_1 and stx_2	STEC-1	GA(AG) C(AG)A AAT AAT TTA TAT GTG	40
	STEC-2	TGA TGA TG(AG) CAA TTC AGT AT	33
stx_1	STEC-I HP-1	TTT ACG TTT TCG GCA AAT ACA GAG GGG AT-(FL)	40
	STEC-I HP-2	(Red 640)-TCG TAC AAC ACT GGA TGA TCT CAG TGG G-Ph	
stx_2	STEC-II HP-1	TCA GGC ACT GTC TGA AAC TGC TCC TGT GTA-(FL)	40
	STEC-II HP-2	(Red 705)-ACC ATG ACG CCG GGA GAC GTG GAC CT-Ph	
eae	eaeAF	GAC CCG GCA CAA GCA TAA GC	32
	eaeAR	CC ACCT GCA GCA ACA AGA GG	
eae	eae HP1	ACA GTT CTG AAA GCG AAA TGA TGA AGG c-(FL)	40
	eae HP2	(Red 640)-CCT GGT CAG CAG ATC ATT TTG CCA CT-Ph	
hly_{EHEC}	hlyAF	GCA TCA TCA AGC GTA CGT TCC	32
	hlyAR	AAT GAG CCA AGC TGG TTA AGC T	
hly_{EHEC}	hlyA HP1	GCA TGG CTC TTG ATG AAT TGC T-(FL)	40
	hlyA HP2	(Red 705)-CAA CGG GAA GGA GAG GAT ATA AGT CAG-Ph	

[a] FL, fluorescein; Red 640, LC Red 640-N-hydroxy-succinimide ester; Red 705, LC Red 705-phosphoramidite; Ph, 3-phosphate.

values for BlnI and SpeI were set using the Auto Algorithm function of the CHEF Mapper XA to separate fragments in the range from 50 to 450 kb (BlnI) or from 30 to 350 kb (SpeI). All fragments larger than 45 kb (up to 27 fragments with XbaI, up to 24 fragments with NotI, up to 25 fragments with BlnI, and up to 29 fragments with SpeI) were included in the clonal analysis of the isolates.

We also analyzed the spatial and temporal behavior of the clonal group of O165:H25 strains in the herd by genomic typing with PFGE (Fig. 1). During a 3-year monitoring program on four cattle farms (19), the O165:H25 clone was detected on only one farm for 4 months. This serotype was not detected before or after this period, although many other potential EHEC strains belonging to other serotypes were found in this herd (19). Twenty O165:H25 isolates were found in four different cattle on the first date of detection. Different subclusters were already present and were even isolated from a single animal (Table 1). These results suggest that the O165 clone either had been introduced into the farm shortly before the first detection or was the result of recombination due to horizontal gene transfer (8, 18). The occurrence of different subclusters at the same time could have been the result of interactions between different bacteria or between bacteria and the host in the bovine intestine. At later sampling dates the num-

TABLE 3. PCR primers used for detection and characterization of STEC by conventional PCR

Target	Primer	Sequence (5′–3′)	PCR conditions							Product length (bp)	Reference
			Denaturation		Annealing		Extension		No. of cycles		
			Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)			
$stxB_{2/2c}$	GK3	ATG AAG AAG ATG TTT ATG	94	30	52	60	72	40	35	260	22
	GK4	TCA GTC ATT ATT AAA CTG									
$stxB_{2d}$	VT2-cm	AAG AAG ATA TTT GTA GCG G	94	30	52	60	72	60	35	256	36
	VT2-f	TAA ACT GCA CTT CAG CAA AT									
$stxB_{2e}$	FK1	CCG GAT CCA AGA AGA TGT TTA TAG	94	30	55	60	72	40	35	280	16
	FK2	CCC GAA TTC TCA GTT AAA CTT CAC C									
$stxB_{2f}$	128-1	AGA TTG GGC GTC ATT CAC TGG TTG	94	30	57	60	72	60	35	428	47
	128-2	TAC TTT AAT GGC CGC CCT GTC TCC									
α-*eae*	SK1	CCC GAA TTC GGC ACA AGC ATA AGC	94	30	55	60	72	120	30	2,807	31
	LP2	CCC GAA TTC GGC ACA AGC ATA AGC									
β-*eae*	SK1	CCC GAA TTC GGC ACA AGC ATA AGC	94	30	55	60	72	120	30	2,287	31
	LP4	CCC GTG AT ACCA GTA CCA ATT ACG GTC									
γ-*eae*	SK1	CCC GAA TTC GGC ACA AGC ATA AGC	94	30	45	60	72	120	3	2,792	31
	LP3	CCC GAA TTC TTA TTC TAC ACA AAC CGC	94	30	52	60	72	120	28		
δ-*eae*	Int-d	TAC GGA TTT TGG GGC AT	95	20	45	60	72	60	30	544	1
	Int-Ru	TTT ATT TGC AGC CCC CCA T									
ε-*eae*	SK1	CCC GAA TTC GGC ACA AGC ATA AGC	94	30	55	60	72	120	30	2,608	31
	LP5	AGC TCA CTC GTA GAT GAC GGC AAG CG									
katP	wkat-B	CTT CCT GTT CTG ATT CTT CTG G	94	30	56	60	72	150	30	2,125	10
	wkat-F	AAC TTA TTT CTC GCA TCA TCC									
espP	EspA	AAA CAG CAG GCA CTT GAA CG	94	30	56	60	72	150	30	1,830	11
	EspB	GGA GTC GTC AGT CAG TAG AT									
lifA/*efa1*	lifA1	AGC CAT TCC ATC AAT CCG AT	95	30	50	60	72	60	30	532	7
	lifA2	TCC CTG CCA AAC TAC CGA CAC									
lifA/*efa1*	lifA3	CAG CTA CAG GAG ACC GTT TTT	95	30	50	60	72	60	30	560	7
	lifA4	CAA TAT CAG GCC AAT CAA									

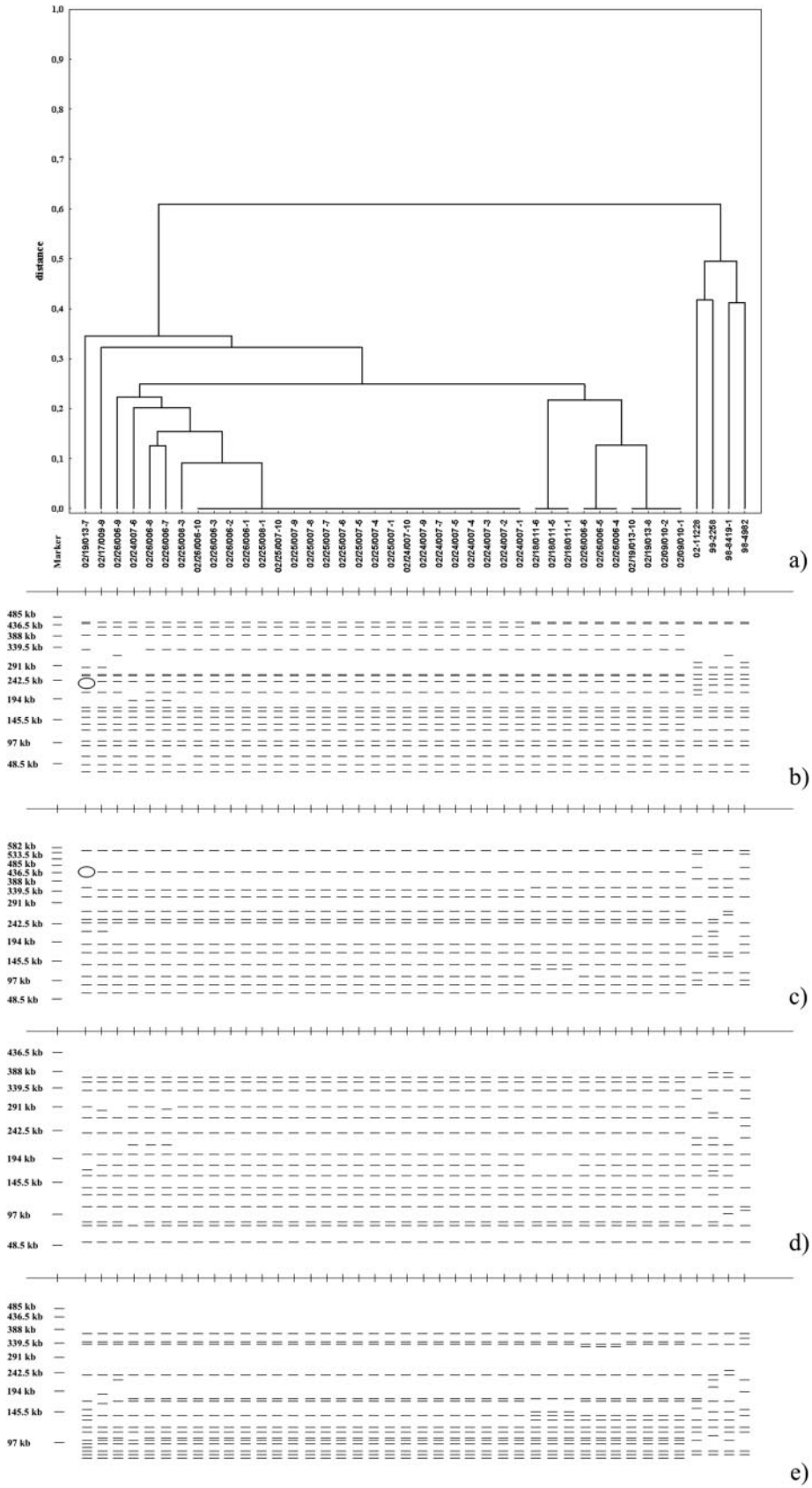

FIG. 1. (a) Dendrogram of DNA divergence (distance) generated by using the Dice coefficient for pairwise comparisons of banding patterns for XbaI, NotI, BlnI, and SpeI restriction of potential EHEC O165:H25 and O165:H- isolates. (b to e) Schematic representations of restriction patterns of bovine and human *E. coli* O165:H25 and O165:H- strains after digestion with XbaI (b), NotI (c), BlnI (d), and SpeI (e). Ellipses indicate the missing XbaI and NotI fragments in strain 02/19/013-7. The *efa1* gene is located at this position in the other bovine strains.

ber of O165:H25 isolates was reduced. The dominant subcluster, subcluster 1, could still be found, but many other subclusters were detected at the same time. The genetic distance between the O165:H25 isolates increased, and in some isolates plasmid-encoded virulence markers or complete virulence plasmids were missing. Variations in the O165:H25 clonal group might have been caused by increasing competition between the bacterial populations of various subtypes in the bovine intestine or by potential interactions between the O165:H25 EHEC and the host. The O165:H25 clonal group finally disappeared from the herd after it had persisted for 4 months. Perhaps the loss of the *efa-1* gene in one isolate obtained on the last date when O165:H25 was isolated from the herd can help us understand why the clone disappeared. Efa1 is considered an *E. coli* factor for colonization of the bovine intestine by non-O157 STEC (51). Also, this O165:H25 strain exhibited the greatest genetic distance compared to the remaining strains in the clonal group (Fig. 1). The distances were calculated from the fragmentation patterns produced by each of the four PFGE enzymes by using the RAPDistance program, version 1.04 (2). The Euclidean distance in three dimensions was not calculated because all O165:H25 strains were isolated from the same farm (i.e., the geographic distance was zero for all isolates). A cluster analysis, using the unweighted pair group method with arithmetic averages, was performed for PFGE by using Statistica 6.1 for Windows (StatSoft Inc., Tulsa, OK). In any case, our results suggest that the persistence of a distinct clone of EHEC may be limited in time and space (i.e., in a cattle herd). This is apparently not a unique property of O165:H25, since we obtained similar results for clonal groups of other EHEC serotypes (O26:H11 and O157:H7) in cattle. The four human O165H25/H- isolates belonged to different other clonal groups (Fig. 1).

In conclusion, bovine O165:H25 isolates can carry virulence factors of EHEC that are associated with EHEC-related disease in humans, particularly HC and HUS. Therefore, strains of bovine origin may represent a considerable potential risk for human infection.

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