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Genetic Typing of Shiga Toxin 2 Variants of *Escherichia coli* by PCR-Restriction Fragment Length Polymorphism Analysis

Liesbet De Baets,[1,2]* Imme Van der Taelen,[1,2]† Marina De Filette,[1,2]‡ Denis Piérard,[3] Lesley Allison,[4] Henri De Greve,[2]§ Jean-Pierre Hernalsteens,[2] and Hein Imberechts[1]

Dienst Algemene Bacteriologie, Centrum voor Onderzoek in Diergeneeskunde en Agrochemie,[1] Onderzoeksgroep Genetische Virologie, Vrije Universiteit Brussel,[2] and Afdeling Microbiologie, Academisch Ziekenhuis Vrije Universiteit Brussel,[3] Brussels, Belgium, and Scottish E. coli O157 Reference Laboratory, Department of Clinical Microbiology, Western General Hospital, Edinburgh, Scotland[4]

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Shiga toxins Stx1 and Stx2 play a prominent role in the pathogenesis of Shiga toxin-producing *Escherichia coli* (STEC) infections. Several variants of the stx_2 gene, encoding Stx2, have been described. In this study, we developed a PCR-restriction fragment length polymorphism system for typing stx_2 genes of STEC strains. The typing system discriminates eight described variants and allows the identification of new stx_2 variants and STEC isolates carrying multiple stx_2 genes. A phylogenetic tree, based on the nucleotide sequences of the toxin-encoding genes, demonstrates that stx_2 sequences with the same PvuII HaeIII HincII AccI type generally cluster together.

Shiga toxin-producing *Escherichia coli* (STEC) strains produce toxins with a biological activity similar to those produced by *Shigella dysenteriae* type I. Shiga toxins (Stx) are cytotoxic on cultured Vero cells and are therefore also called verocytotoxins (18). Most STEC strains induce the formation of attaching-effacing lesions in intestinal epithelial cells by means of gene products of the pathogenicity island locus of enterocyte effacement (21, 33, 39) with genes coding, for example, for intimin (EaeA) (48) and the translocated intimin receptor (Tir) (17). Furthermore, STEC may carry a 60-MDa plasmid that codes for an enterohemolysin and carries regulatory sequences (3, 11, 37, 42). Enterohemorrhagic *E. coli* (EHEC) strains are defined as a subset of STEC that cause clinical disorders in humans. A great diversity of EHEC serotypes exists, although serotype EHEC O157:H7 is the best studied and is frequently associated with hemolytic-uremic syndrome (HUS) (4, 12). Epidemiological investigations indicate that cattle are the principal reservoirs of EHEC (43, 47).

STEC strains may produce two types of Shiga toxins, i.e., those that are antigenically similar to the Shiga toxin produced by *S. dysenteriae* (Stx1) and those that differ (Stx2) (16, 25, 40). Severe clinical symptoms were more frequently associated with *E. coli* strains producing Stx2 than with those producing Stx1 (4, 12, 20, 28). Both types of Shiga toxins are encoded by *stx* genes on temperate bacteriophages (26, 40). While stx_1 is rather conserved, many variants of stx_2, showing different toxicities for cultured cells and/or animals, have been described (14, 16, 23, 29, 31, 32, 34, 38). Among them, Stx2 and Stx2c are the most prominent in human clinical isolates. Variants $Stx2_{vha}$ (14) and $Stx2_{vhb}$ (14) can be activated by intestinal mucus and are therefore classified as activatable variants, also termed Stx2d variant toxins (22). Shiga toxins are composed of a single enzymatic 32-kDa A subunit, which is the active component of the toxin, and a pentamer of 7.7-kDa B subunits (7, 25). The B subunit is responsible for binding the toxin to the host cell receptor (13, 15, 27).

Molecular typing of STEC can be performed in different ways. Pulsed-field gel electrophoresis (PFGE) is an accurate and reproducible reference method (10, 45, 46) for the molecular typing of STEC strains. DNA sequence-based methods like multilocus sequence typing are also used as epidemiological tools. Another approach for typing STEC strains is to consider polymorphisms in virulence genes. A number of multiplex PCR assays detecting virulence-associated genes have been described (5, 6, 8, 24, 30, 44). Since Stx2 is the key virulence factor (4, 35), several investigators have developed PCR-restriction fragment length polymorphism (RFLP) assays that allow the rapid identification of known and new stx_2 variants (1, 24, 34, 41, 49). However, these assays discriminate only

TABLE 1. Described stx_2 variants and their restriction profile[a]

Reference strain or plasmid	Accession no.	Stx2 variant	PHIA pattern	Reference
C600(933W)	X07865	Stx2	1-1-1-1	16
E32511	M59432	Stx2c	1-2-2-1	38
7279	X61283	$Stx2_{vhc}$	3-2-3-1	23
343	NA	$Stx2_{vha}$	2-2-2-2	14
544	NA	$Stx2_{vhb}$	3-2-3-1	14
pJCP520	X65949	$Stx2_{OX3a}$	4-3-4-1	31
pJCP521	L11079	$Stx2_{OX3b}$	1-4-2-1	32
pJCP522	L11078	$Stx2_{O111}$	4-3-4-1	29
pJCP523	Z37725	$Stx2_{O48}$	5-1-5-1	29
EH250	AF043627	$Stx2_{O118}$	4-5-4-3	34

[a] NA, not available.

* Corresponding author. Mailing address: Dienst Algemene Bacteriologie, CODA, Groeselenberg 99, B-1180 Ukkel, Belgium. Phone: 32-2-3790427. Fax: 32-2-3790670. E-mail: lideb@var.fgov.be.

† Present address: Purna Pharmaceuticals NV/SA, B-2870 Puurs, Belgium.

‡ Present address: Dienst Moleculaire Biologie, Universiteit Gent, B-9052 Zwijnaarde, Belgium.

§ Present address: Onderzoeksgroep Ultrastructuur, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, B-1050 Brussels, Belgium.

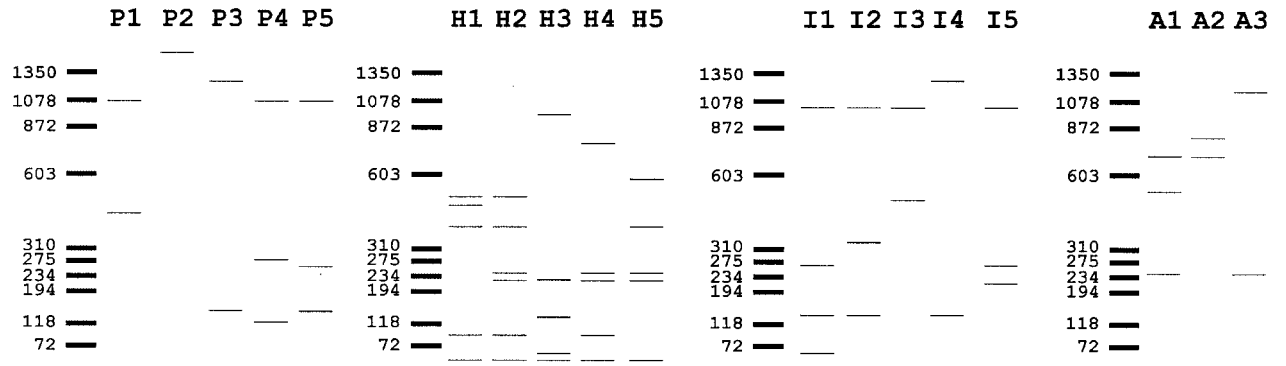

FIG. 1. Graphical representation of restriction profiles of known Stx2 variants. P1 to P5 are the profiles obtained with PvuII, H1 to H5 are the profiles obtained with HaeIII, I1 to I5 are the profiles obtained with HincII, and A1 to A3 are the profiles obtained with AccI.

a limited number of described variants. In addition, the designation of the toxin subtypes is insufficient to account for all the differences on the sequence level.

In the present study, we describe a PCR-RFLP typing system which is based on the use of four restriction enzymes (PvuII, HaeIII, HincII, and AccI), defining variants of stx_2. This PCR-RFLP typing system includes a rational classification (PHIA) that was applied to 36 bovine and 27 human STEC strains of different origins. New stx_2 variants were found, and the corresponding genes were cloned, sequenced, and analyzed. The PCR-RFLP assay was validated with Scottish *E. coli* O157 isolates from three different outbreaks.

Analysis of known stx_2 sequences. The sequences of 10 known stx_2 variants (Table 1) were compared in order to select primers in conserved regions that would amplify the complete Stx2-encoding region. Primers oli320b (5′-GGTCACTGGTTCGA ATCCAGTAC-3′; accession number X07865) and oli321 (5′-GGGATCCTGAATTGTGACACAGATTACACTTGTT AC-3′; accession number X07865) were chosen. Subsequently, the 1.4-kb stx_2 fragment was explored, by use of the VectorNTI 5.0 software, for restriction enzymes that, upon digestion, would result in profiles specific to each stx_2 variant. Our analysis suggested that the combination of the profiles obtained using enzymes PvuII, HaeIII, HincII, and AccI, also used in previous studies (1, 34), would be discriminatory for 8 out of the 10 stx_2 variants. The individual profiles thus obtained are schematically represented in Fig. 1.

PCR-RFLP analysis of the corresponding reference strains confirmed the predicted profiles. PCR amplification was performed with total bacterial DNA (genomic DNA buffer set; QIAGEN) in a 50-μl reaction mixture with 1 U of ExTaq DNA polymerase (Takara) in a Perkin-Elmer apparatus under the following conditions: an initial denaturation at 94°C for 50 s, followed by 10 cycles of 94°C for 10 s, 55°C for 30 s and 68°C for 2 min. During the next 20 cycles, the elongation time at 68°C was increased 15 s each cycle. Denaturation and annealing times remained constant. PCR products were purified with the Qiaquick PCR purification kit (QIAGEN). One microgram of the purified PCR product was digested with the enzymes PvuII, HaeIII, HincII, and AccI. Restriction fragments were separated by agarose gel electrophoresis on a 3% Nusieve 3:1 agarose gel (BioWhittaker Molecular Applications).

The typing scheme described here represents a rational nomenclature for Stx2 variants (Table 1). Three methods for the further subtyping of the stx_2 gene by PCR-RFLP were previously described (1, 34, 41). The stx_2 subtyping scheme designed by Tyler et al. (41) includes identification of Stx2 and variants $Stx2_{vha}$ and $Stx2_{vhb}$ and consists of PCR amplification of the B subunit sequence and digestion with endonucleases HaeIII, RsaI, and NciI. Piérard et al. (34) extended Tyler's typing system by including three new primers, allowing amplification of variants stx_2-O118, stx_2-OX3a, and stx_2-O111, carried by some less-virulent STEC strains. By use of the PCR-RFLP system described by Bastian et al. (1), with the primer set of Lin et al. (19) and restriction endonucleases HincII and AccI, four different restriction profiles were obtained among variants Stx2, Stx2c, $Stx2_{vhb}$, $Stx2_{vha}$, $Stx2_{OX3b}$, $Stx2_{OX3a}$, and $Stx2_{O111}$. This system did not discriminate between variants Stx2c, $Stx2_{OX3b}$, and $Stx2_{vha}$, whereas these variants showed different PHIA profiles. The PHIA typing system shows higher discrimination due to the larger PCR fragment and the combination of four enzymes (Fig. 2). In spite of the higher discrimination, variants $Stx2_{vhb}$ and $Stx2_{vhc}$ shared the same PHIA profile (3-2-3-1) but could be further distinguished by use of EcoRV that cuts only the stx_2-vhb sequence, producing fragments of 258 bp and 1,160 bp. Variants $Stx2_{OX3a}$ and $Stx2_{O111}$ both have PHIA profile 4-3-4-1, since they differ at only four positions in the nucleotide sequence. Variants Stx2e and Stx2f were not included in this study because of their rare occurrence among human cases and their considerable sequence divergence.

***stx*$_2$ subtyping of bovine STEC strains and human EHEC strains from sporadic cases.** The stx_2 genes of 36 bovine and 27 human STEC strains, randomly selected among Belgian isolates, were typed with the PHIA system. Among the bovine STEC strains, the most frequently observed variant profiles were 1-2-2-1 and 1-1-1-1 and, to lesser extents, 1-4-2-1, 3-2-3-1, and 2-2-2-2 (Table 2). For human EHEC strains of serogroup O157, PHIA profiles 1-1-1-1, 1-2-2-1, and 1-4-2-1 were found, which were also detected among the bovine isolates of this serogroup (Table 2). For the human strains of the other serogroups studied, PHIA profile 1-1-1-1 was found for all except one isolate, which showed profile 1-4-2-1. PHIA profiles of the related strains, either the bovine STEC strains EBC32, EBC34, and EBC35 isolated on the same farm at different times and from different sources (animal or environmental) or the human EHEC strains EH123 and EH125, originating from the

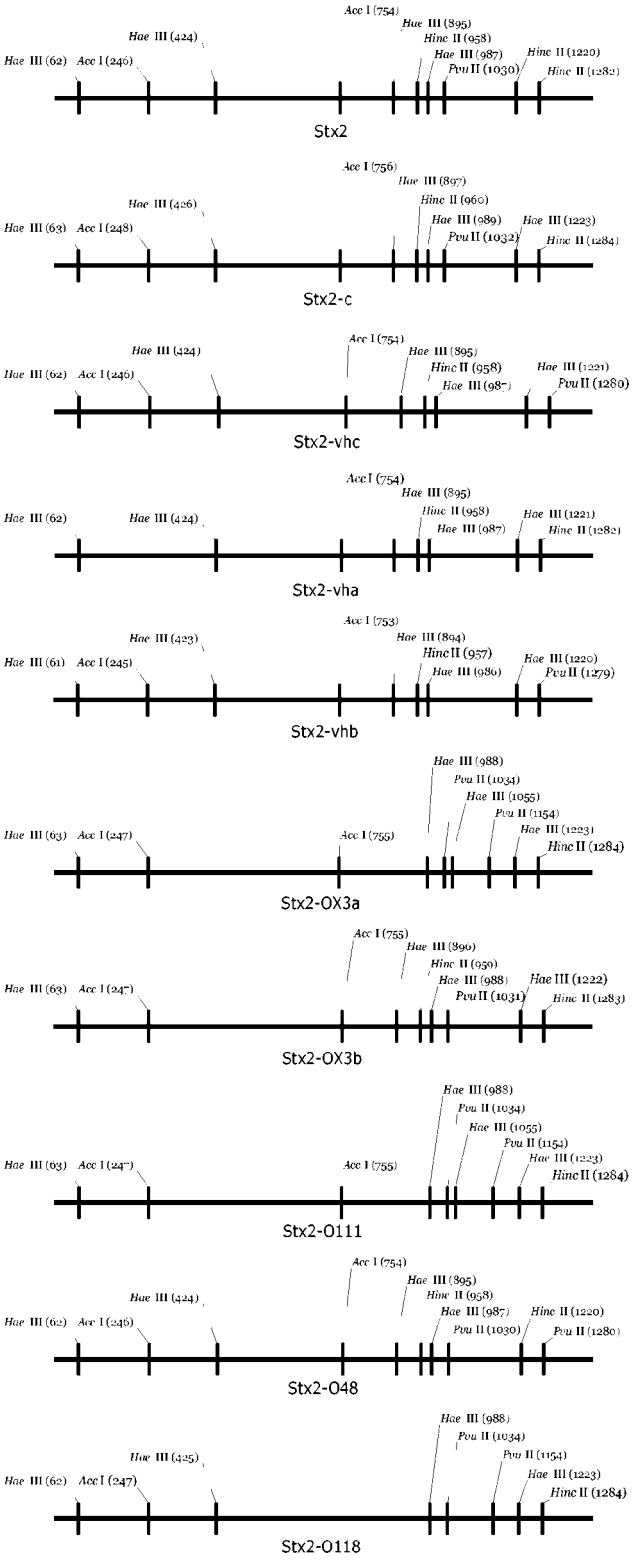

FIG. 2. Graphical representation of the 1.4-kb fragment indicating the positions (between brackets) of the relevant restriction sites for the different stx_2 variants.

TABLE 2. Distribution of PHIA variants among bovine and human STEC isolates with a single stx_2 copy[a]

PHIA pattern	Serogroup[a]	No. of isolates with indicated pattern	
		Human	Bovine
1-1-1-1	O26	1	0
	O103	1	0
	O111	1	1
	O145	1	0
	O157	10	6
	—	3	2
1-2-2-1	O103	0	1
	O157	7	9
	—	0	2
1-4-2-1	O157	2	3
	—	1	0
3-2-3-1	O113	0	1
	O157	0	1
2-2-2-2	O103	0	1
	—	0	2
2-1-1-1	O103	0	2[b]
2-1-2-1	O128	0	1[b]
3-1-5-1	O103	0	1[b]

[a] —, no agglutination with antisera to any of the following serogroups: O26, O103, O111, O113, O145, and O157.
[b] Cloned and sequenced.

same family, were the same. In none of the *E. coli* strains studied could we detect variant $Stx2_{OX3a}$, $Stx2_{O111}$, or $Stx2_{O118}$. This result confirms the findings of Piérard et al. (34), who did not detect these stx_2 variant gene products in isolates of the most-virulent *E. coli* serotypes, i.e., O157, O26, O103, O111, and O145. Also, these variants were not detected during other studies of human O157 isolates and non-O157 STEC strains (24, 36). Friedrich et al. (9) did not find these variants in isolates associated with HUS. In contrast to other studies (24, 36), there was no indication of a clear association between PHIA type and serogroup.

Among the bovine STEC strains, four showed profiles differing from those found in the reference strains, i.e., 2-1-1-1 (two strains), 2-1-2-1, and 3-1-5-1. The stx_2 genes of the corresponding STEC strains were cloned in the expression vector pBADMycHisC (Invitrogen) to facilitate future toxicity studies. After amplification with primers oli320b and oli321H (5′-GGGAAGCTTTGAATTGTGACACAGATTACACTTGTTAC-3′), the purified PCR product (Qiaquick PCR purification kit; QIAGEN) was digested with HindIII and cloned (Ready-

TABLE 3. Complex PHIA profiles for bovine STEC isolates and their corresponding stx_2 clones[a]

Strain	STEC isolate		stx_2 clone	
	PHIA pattern	Serogroup	Clone	PHIA pattern
EBC229	1+2+3-1-1+5-1	O103	pVTEC2	1-1-1-1
			pVTEC3	3-1-5-1
			pVTEC16	2-1-1-1
EBC258	1+2-1+6-1+6-1+3	—[a]	pVTEC1	1-1-1-1
			pVTEC15	2-6-6-3
EBC281	1+2-2-2-1	O103	pVTEC7	2-2-2-1
			pVTEC9	1-2-2-1

[a] —, no agglutination with antisera to any of the following serogroups: O26, O103, O111, O113, O145, and O157.

TABLE 4. PHIA pattern of related Scottish *E. coli* O157 isolates from outbreaks

Outbreak[a]	PHIA pattern	Clone	PHIA pattern
1 (n = 9)	1-1+2-1+2-1	pEHEC398	1-1-1-1
		pEHEC399	1-2-2-1
2 (n = 3)	1-1+2-1+2-1	pEHEC410	1-1-1-1
		pEHEC414	1-2-2-1
3 (n = 11)	1-1-1-1	pEHEC412	1-1-1-1

[a] n, number of isolates.

To-Go T4 DNA ligase kit; Amersham Biosciences) between the filled-in NcoI site and the HindIII site of pBADMycHisC. Clones were given a pEHEC number and sequenced. The PHIA patterns obtained for the clones after PCR-RFLP and sequence analysis confirmed the PHIA patterns of the corresponding STEC strains (Table 2).

STEC strains may contain more than one copy of stx_2 or stx_2 variants (1, 2, 14, 38). Among the selection of bovine STEC isolates, we identified three STEC strains showing a complex restriction profile (Table 3). The stx_2 genes of these isolates were cloned in the SmaI site of pUC18 or pUC19 (Sureclone ligation kit; Amersham Biosciences). Transformants were given a pVTEC number and sequenced. After PCR-RFLP analysis, different PHIA patterns were found among the respective clones. If these profiles were superimposed, the result corresponded with the PHIA pattern obtained for the STEC strain (Table 3). Among the clones, two new stx_2 variants were identified, i.e., 2-2-2-1 and 2-6-6-3. The latter variant has a restriction profile for HaeIII and HincII that did not correspond with any of the reference strains. Consequently, in addition to new combinations of existing profiles, new restriction profiles for one or more endonucleases were identified.

In our study, more variation in PHIA types was observed among the bovine isolates than among the human isolates for which no complex or new restriction profiles were found. PHIA types from human EHEC isolates were a subset of those found in the bovine STEC population. In this study, variants $Stx2_{vha}$ (2-2-2-2) and $Stx2_{vhb}$ (3-2-3-1) were found only in bovine STEC, although they are associated with HUS according to the literature.

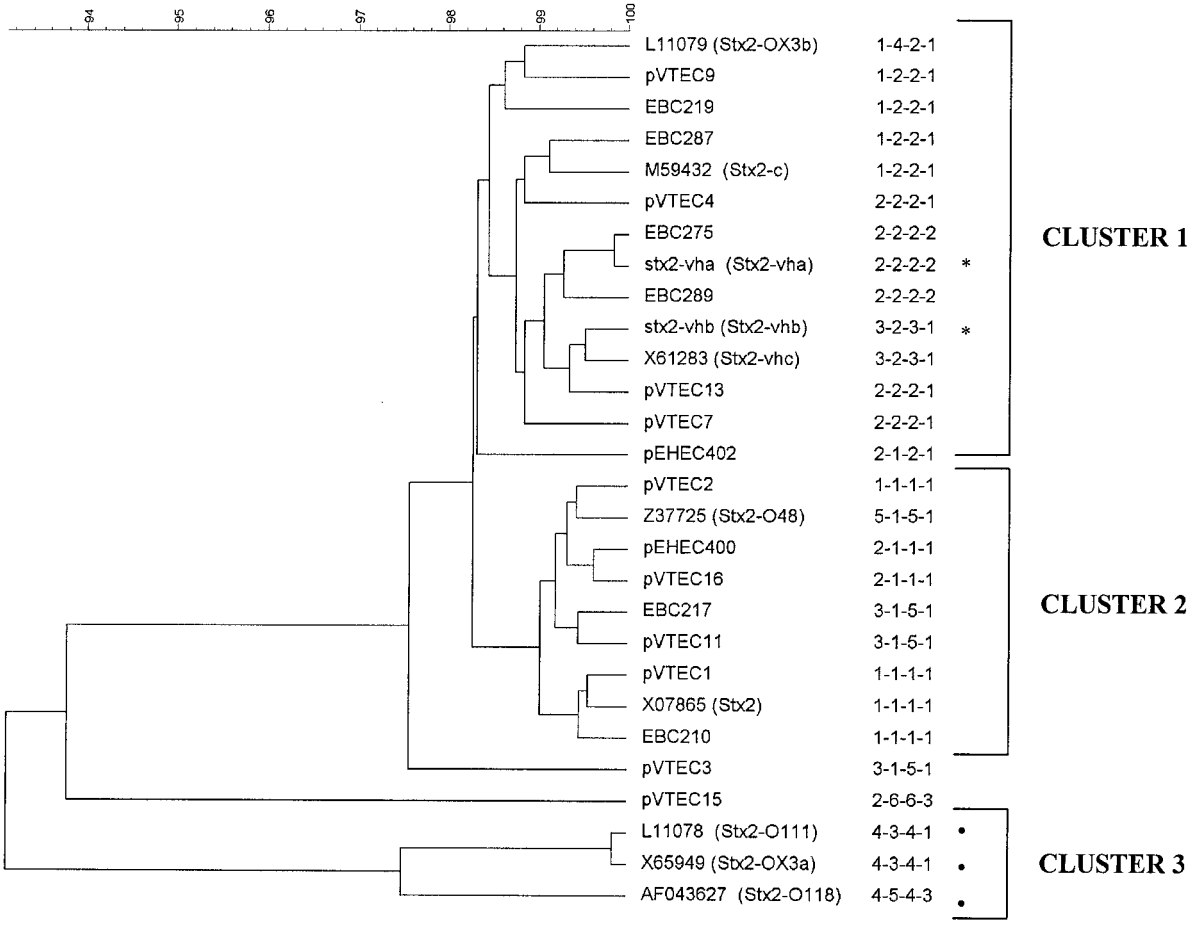

FIG. 3. Phylogenetic tree (unweighted pair group method with arithmetic mean) of stx_2 sequences analyzed in this study. The sequences start at the ATG start codon of the A-subunit gene and end at the stop codon of the B-subunit gene. Accession numbers AY443043, AY443044, AY443045, AY443046, AY443047, AY443048, AY443049, AY443050, AY443051, AY443052, AY443053, AY443054, AY443055, AY443056, AY443057, AY443058, AY443059, AY443060, respectively, were given to the stx_2 sequences of the following isolates or clones: EBC219, pVTEC9, EBC287, pVTEC4, EBC275, pVTEC7, EBC289, pVTEC13, pVTEC2, pEHEC400, pVTEC16, EBC217, pVTEC11, pVTEC1, EBC210, pEHEC402, pVTEC3, and pVTEC15. *, activatable by intestinal mucus; •, low cytotoxicity for cultured Vero cells.

These preliminary results may indicate that not all bovine STEC strains are pathogenic for humans. This hypothesis is supported by the data of an ongoing study on Belgian STEC and EHEC isolates which were typed with the PHIA system (L. De Baets et al., unpublished data).

***stx*$_2$ subtyping of STEC strains from outbreaks.** To validate our PCR-RFLP analysis, we analyzed Scottish isolates associated with three different *E. coli* O157 outbreaks (Table 4). In addition, three random Scottish STEC isolates were typed. Isolates were from a clinical, animal, or environmental origin. The PHIA type was the same for all strains within one outbreak, as was their PFGE pattern (results not shown). As shown in Table 4, the PHIA profiles of strains from outbreaks one and two (1-1+2-1+2-1) were combinations of two simple profiles. The *stx*$_2$ genes of one strain from both outbreaks were cloned in pBADMycHisC and subsequently analyzed by means of PCR-RFLP. Two different PHIA patterns (1-1-1-1 and 1-2-2-1) were found among these clones. When the two profiles were superimposed, the result corresponded to the PHIA pattern obtained for the STEC isolate. Within outbreak three, variant 1-1-1-1 was found for all strains. Random isolates showed the PHIA profiles 1-2-2-1 (two times) and 1-1-1-1. Since related strains show the same PHIA type, we conclude that the PHIA system is an efficient tool for epidemiological research on STEC infections. The typing method can be used to compare STEC strains and to identify the source of infection.

DNA sequence analysis and phylogenetic comparison of *stx*$_2$ sequences. Ten described *stx*$_2$ sequences and 18 sequences determined in this study were selected to construct a phylogenetic tree by use of the Bionumerics 3.0 (Applied Maths) software. For bovine STEC strains EBC210, EBC217, EBC219, EBC275, EBC287, and EBC289, the *stx*$_2$ PCR fragments were directly sequenced. pVTEC and pEHEC clones were sequenced as described above. Nucleotide sequences were aligned from the start codon of *stx*$_{2a}$ to the stop codon of *stx*$_{2b}$ (Fig. 3). None of the *stx*$_2$ sequences are identical at the nucleotide level. Nevertheless, in the phylogenetic tree, *stx*$_2$ sequences of the same PHIA type generally clustered together. Two main clusters that shared 98% similarity were identified. Moreover, Stx2 variants with a specific phenotypic characteristic were located in the same cluster. The activatable variants (Stx2$_{vha}$ and Stx2$_{vhb}$) are located in the first cluster. The amino acid sequences allowing activation by intestinal mucus (22) are also present in four other variants (EBC275, Stx2$_{vhc}$, pVTEC 13, and pEHEC 402), which were grouped in the first cluster. Stx2, which cannot be activated (22), is located in the second cluster. A third cluster showing less than 94% sequence similarity contained the Stx2 variant toxins Stx2$_{OX3a}$, Stx2$_{O111}$, and Stx2$_{O118}$, which showed low cytotoxicity for Vero cells (29). Together with the indications for a reduced toxicity, these data support the classification of these variants as a different Stx2 subtype. The new variant designated by PHIA profile 2-6-6-3 (pVTEC 15) also showed more sequence divergence. Our results suggest that *stx*$_2$ sequences and PHIA types with the same phenotypic characteristics are grouped together in the phylogenetic tree. Future toxicity experiments using cell cultures with members of different clusters (PHIA types) of the phylogenetic tree may further elucidate the association with their cytotoxicity.

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