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JOURNAL OF CLINICAL MICROBIOLOGY, June 2003, p. 2716–2722 Vol. 41, No. 6
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.6.2716–2722.2003
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Bovine Non-O157 Shiga Toxin 2-Containing *Escherichia coli* Isolates Commonly Possess $stx_{2\text{-EDL933}}$ and/or stx_{2vhb} Subtypes

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Received 28 May 2002/Accepted 18 December 2002

stx_2 genes from 138 Shiga toxin-producing *Escherichia coli* (STEC) isolates, of which 127 were of bovine origin (58 serotypes) and 11 of human origin (one serotype; O113:H21), were subtyped. The bovine STEC isolates from Australian cattle carried *ehxA* and/or *eaeA* and predominantly possessed $stx_{2\text{-EDL933}}$ (103 of 127; 81.1%) either in combination with stx_{2vhb} (32 of 127; 25.2%) or on its own (52 of 127; 40.4%). Of 22 (90.9%) bovine isolates of serotype O113:H21, a serotype increasingly recovered from patients with hemolytic uremic syndrome (HUS) or hemorrhagic colitis, 20 contained both $stx_{2\text{-EDL933}}$ and stx_{2vhb}; 2 isolates contained stx_{2vhb} only. Although 7 of 11 (63.6%) human O113:H21 isolates associated with diarrhea possessed $stx_{2\text{-EDL933}}$, the remaining 4 isolates possessed a combination of $stx_{2\text{-EDL933}}$ and stx_{2vhb}. Three of the four were from separate sporadic cases of HUS, and one was from an unknown source.

Shiga toxin-producing *Escherichia coli* (STEC) is commonly carried by ruminants (especially cattle) and is an important enteropathogen, causing human diseases ranging from mild diarrhea to more severe conditions such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (7, 15, 22a, 25, 28, 47). Although over 150 serotypes of STEC have been associated with human disease (12, 20), serotype O157:H7 is believed to be responsible for the majority of outbreaks and sporadic cases of HUS in the United States, Japan, and Europe. However, non-O157 STEC is of greater significance in Australia (16) and Argentina (35), and its role in HUS and HC in the United States and Europe is gaining recognition (24).

The most critical factors of STEC associated with severe human disease are the Shiga toxins. STEC can produce one or both of these groups of toxins which comprise two immunologically non-cross-reactive groups (designated Stx1 and Stx2), although other virulence factors such as intimin and enterohemolysin either directly contribute to (or are implicated by association with) the pathogenicity of STEC (11, 21).

stx_2 comprises 11 distinct variants (5, 19, 23, 31, 38, 39, 42, 45, 46, 50) and is considered the most important STEC virulence factor associated with human disease (11, 21, 34, 41). More importantly, differences in the degree of pathogenicity of STEC serotypes have been associated with variations in the stx_2 subtype (27, 29, 30, 42). The most frequently reported stx_2 subtypes are represented by stx_{2c}, stx_{2d}, and stx_{2e} (42, 46, 50).

Although more than 200 different O:H serotypes of STEC have been isolated from cattle (1, 7, 12, 20, 24, 49, 51), there is a paucity of information regarding associations between serotype, stx_2 subtype, and virulence factor profiles among STEC isolates from meat-producing animals (9, 10, 22a, 26, 33, 43). stx_2 subtypes $stx_{2\text{-EDL933}}$ and stx_{2c} (stx_{2vha} and stx_{2vhb}) have been described in European studies of bovine STEC; however, their association with serotype has not been reported previously (8, 42, 46). Ramachandran et al. (44) demonstrated that stx_2-containing STEC from ovine feces usually belonged to the stx_{2d} subtypes ($stx_{2d\text{-Ount/O111/OX3a}}$).

The purpose of this study was to subtype stx_2 genes among a serologically diverse collection of 138 stx_2-containing STEC isolates primarily derived from bovine feces collected from the eastern states of Australia and to compare stx_2 subtypes of STEC isolates of serotype O113:H21 (an important serotype increasingly associated with HUS and HC in humans) of bovine and human origin.

A total of 138 STEC isolates, of which 134 were non-O157, were used in this study. These consisted of 127 isolates from cattle and 11 human isolates from individual cases of clinical infections (Table 1). All isolates were prepared and subjected to multiplex PCR for the detection of STEC virulence factors stx_1, stx_2, *ehxA*, and *eaeA* as described previously (14). Amplified PCR products were then resolved by gel electrophoresis through agarose (2% wt/vol) and stained with ethidium bromide (5 μl/ml). Visualization was undertaken using UV illumination, and images were captured using a GelDoc 1000 image analysis station (Bio-Rad).

Bovine STEC isolates with stx_2 were subjected to subtyping using typing schemes previously reported (4, 42, 48). For this study, stx_{2d} subtypes are defined as nucleotide sequence variants of stx_2 ($stx_{2d\text{-Ount}}$, $stx_{2d\text{-O111}}$, and $stx_{2d\text{-OX3a}}$), as described previously (42), and are not the potentially mucin-activatable stx_{2d} variants described by Melton-Celsa et al. (30) (defined as stx_{2vha} and stx_{2vhb} in this study). stx_2 genes amplified with VT2-e and VT2-f primers and Lin F and Lin R primers were subjected to restriction fragment length polymorphism (RFLP) analysis with the enzymes *Hae*III and *Pvu*II (42) and

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TABLE 1. Virulence factor profiles and stx_2 subtypes of STEC of bovine and human origin

Source	Serotype	No. of isolates of animal or human origin	Virulence factor				No. of isolates containing stx_2 variant(s)							
			stx_1	stx_2	*ehxA*	*eaeA*	$stx_{2\text{-EDL933}}$	$stx_{2\text{vhb}}$	$stx_{2\text{d-Ount}}$	$stx_{2\text{-EDL933}}/stx_{2\text{vhb}}$	$stx_{2\text{-EDL933}}/stx_{2\text{vhb}}/stx_1$	$stx_{2\text{vhb}}/stx_1$	$stx_{2\text{-EDL933}}/stx_1$	$stx_{2\text{d-OX3a}}$
B[a]	O2:H8	1	−	+	+	−		1						
B	O3:H7	2	+	+	+	−					1	1		
B	O5:H−	2	+	+	+	−								2
B	O5:H7	2	+	+	+	−						1	1	
B	O6:H8	1	−	+	+	−				1				
B	O6:H28	1	−	+	+	−				1				
B	O6:H34	3	−	+	+	−	3							
B	O8(KA):H51	1	−	+	+	−	1							
B	O8:H16	1	+	+	+	−					1			
B	O8:H19	6	−	+	+	−	6							
BD[b]	O21:H21	1	−	+	+	−	1							
B	O28:H8	3	−	+	+	−	3							
B, BD	O28:H40	2	−	+	+	−	2							
B	O49:H−	1	−	+	+	+				1				
B	O53:H2	1	−	+	+	−	1							
B	O76:H7	1	−	+	+	+	1							
B	O81:H21	1	−	+	+	−	1							
B	O82:H8	5	+	+	+	−					1		4	
B	O82:H40	2	+	+	+	−							2	
B	O91:H−	1	+	+	+	−			1					
B	O91:H21	1	−	+	+	+								1
B	O93:H19	1	−	+	+	−	1							
B	O104:H7	1	−	+	+	−	1							
B	O108:H7	2	+	+	+	−						2		
B	O110:H40	1	+	+	+	−							1	
B	O111:H−	1	−	+	+	+							1	
B	O111:H−	1	+	+	+	+							1	
BD	O111:H8	1	+	+	+	+							1	
BD	O113:H−	1	−	+	+	−				1				
B BD	O113:H21	20	−	+	+	−				20				
B	O113:H21	2	+	+	−	−		2						
H[c] (HUS, HA[d])	O113:H21	3	−	+	+	−				3				
H (Diarrhea, HUS)	O113:H21	6	−	+	+	−	6							
H (?)	O113:H21	1	−	+	−	−	1							
H (?)	O113:H21	1	−	+	−	−				1				
B	O116:H21	1	−	+	+	−	1							
B	O130:H11	1	−	+	+	−							1	
B	O130:H11	6	−	+	+	−	6							
B	O130:H38	2	+	+	+	−						2		
B	O141:H49	1	−	+	+	−	1							
B	O153:H8	1	+	+	+	−			1					
B	O153:HR	2	+	+	+	−							2	
B, BD	O157:H8	2	−	+	+	−	2							
B	O157:H−	1	+	+	+	+						1		
B	O157:H7	1	−	+	+	+		1						
B	O163:H−	1	+	+	+	−					1			
B	O163:H19	1	−	+	+	−	1							
B	O163:H19	1	+	+	+	−						1		
B	Ont:H2	2	−	+	+	−	2							
B	Ont:H5	1	−	+	+	−	1							
B	Ont:H7	2	−	+	+	−	1			1				
B	Ont:H8	1	+	+	+	−						1		
B	Ont:H8	3	−	+	+	−	3							
B	Ont:H11	2	+	+	+	−						1	1	
B	Ont:H11	2	−	+	+	−		2						
BD	Ont:H11	1	−	+	+	−		1						
B	Ont:H16	1	+	+	+	−					1			
B	Ont:H21	1	+	+	+	−							1	
B	Ont:H28	1	−	+	+	−	1							
B	Ont:H28	1	+	+	+	−							1	
B	Ont:H30	1	+	+	+	−							1	
B	Ont:H41	1	+	+	+	−							1	
B	Ont:H49	3	−	+	+	−	2			1				
B	Ont:H49	1	+	+	+	−	1							
B	Ont:HR	1	−	+	+	−	1							

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TABLE 1—*Continued*

Source	Serotype	No. of isolates of animal or human origin	Virulence factor				No. of isolates containing stx_2 variant(s)							
			stx_1	stx_2	*ehxA*	*eaeA*	$stx_{2\text{-EDL933}}$	$stx_{2\text{vhb}}$	$stx_{2\text{d-Ount}}$	$stx_{2\text{-EDL933}}$/$stx_{2\text{vhb}}$	$stx_{2\text{-EDL933}}$/$stx_{2\text{vhb}}$/stx_1	$stx_{2\text{vhb}}$/stx_1	$stx_{2\text{-EDL933}}$/stx_1	$stx_{2\text{d-OX3a}}$
B	OR:H−	1	−	+	+	−		1						
B	OR:H3	1	−	+	+	−	1							
B	OR:H8	1	−	+	+	−				1				
B	OX3:H8	5	−	+	+	−	5							
B	OX3:H8	1	+	+	+	−						1		
B	OX3:H21	1	−	+	−	+	1							
B	OX3:H40	1	−	+	+	−	1							
Total		138					59	8	2	31	5	11	19	3

[a] B, feces from healthy cattle unless otherwise specified.
[b] BD, diagnostic fecal samples from cattle.
[c] H, human feces.
[d] HA, hemolytic anemia.

the enzymes *Hinc*II and *Acc*I (28), respectively. In circumstances in which a stx_2 gene could not be reliably subtyped using the above-described approach, the gene was amplified with Tyler F and Tyler R primers and subjected to RFLP analysis with the enzymes *Msp*I, *Nci*I, and *Rsa*I (Table 2) (48). PCR products were digested for a minimum of 4 h at 37°C. Fragments were separated by electrophoresis through agarose gels (2% wt/vol), and subtypes were determined according to comparison with profiles reported previously (4, 42, 48).

DNA sequence analysis of both the A and B subunits of two stx_2-containing STEC isolates with serotype O110:H40 and Ont:H11 were undertaken, since RFLP analyses confirmed these isolates possessed stx_2 subtypes representative of the majority of the collection. stx_2 was amplified using 10 pmol of each of the oligonucleotide primers Stx2F and Stx2R (Table 3). PCRs (50 μl) were carried out containing 2 μl of nucleic acid of a crude whole-cell DNA template prepared using Instagene matrix as described previously (17), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase.

Thermocycler steps involved an initial denaturation step (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30 s), annealing (60°C for 45 s), and extension (72°C for 90 s). A final extension step of 72°C for 5 min completed the PCR. The amplified PCR product was separated by 2% agarose gel electrophoresis. For DNA sequencing, PCR amplification products were purified using a QIAquick DNA purification kit (Qiagen, Hilden, Germany). Sequencing reactions were performed using the Big Dye terminator cycle sequencing ready reaction DNA sequencing kit and electrophoresed on an ABI prism 377 DNA sequencer (Perkin Elmer, Foster City, Calif.) as described previously (44). Auto Assembler software (Perkin Elmer) was used to compile and analyze the DNA sequences. Nucleotide and amino acid analysis was performed using programs accessed via the Australian National Genomic Information Service (www.angis.org.au). Sequences were compared with those deposited in public databases using the BlastN and BlastP algorithms (2).

All 138 isolates possessed stx_2 and comprised 58 serotypes. STEC serotypes and their virulence factor profiles are listed in Table 1. A significant proportion of bovine STEC isolates (32 of 127; 25.2%) possessed more than one stx_2 variant or a combination of an stx_2 variant(s) together with stx_1 (35 of 127; 27.6%). Isolates with two copies of stx_2 were shown to possess $stx_{2\text{-EDL933}}$ and $stx_{2\text{vhb}}$ (31 of 127; 24.4%) (Table 1). STEC isolates possessing $stx_{2\text{-EDL933}}$ alone were also prevalent (59 of 138; 42.8%). These 59 isolates belonged to 28 serotypes (Table 4). Isolates with $stx_{2\text{-EDL933}}$ and stx_1 comprised the third most common group (19 of 138; 13.7%), representing 13 serotypes, followed by $stx_{2\text{vhb}}$ and stx_1 (11 of 138; 7.9%), comprising 9 serotypes. STEC possessing the three Shiga toxin factors $stx_{2\text{-EDL933}}$, $stx_{2\text{vhb}}$, and stx_1 was identified with a frequency of 5 of 138 (3.6%) and was represented by 5 serotypes (Table 4). STEC containing $stx_{2\text{vhb}}$ alone (8 of 138; 5.8%), $stx_{2\text{d-Ount}}$ (2 of 138; 1.4%), or $stx_{2\text{d-OX3a}}$ (3 of 138; 2.2%) was isolated infrequently (Table 4). Two bovine isolates of the serotype O5:H−, a serotype commonly recovered from ovine feces (14), possessed an $stx_{2\text{d-OX3a}}$ subtype; this subtype is commonly associ-

TABLE 2. Restriction fragment sizes used for analysis of stx_2

Primers used to amplify fragment	Restriction enzyme	Expected fragment size(s) for:				Reference
		$stx_{2\text{-EDL933}}$	$stx_{2\text{vhb}}$	$stx_{2\text{d-Ount}}$	$stx_{2\text{d-OX3}}$	
VT2-e, VT2-f	*Hae*III	348	216, 132	216, 132	167, 132, 49[a]	42
	*Pvu*II	323, 25[a]	250, 73, 25[a]	200, 120, 28[a]	200, 120, 28[a]	
Lin F, Lin R	*Hinc*II	555, 262, 62	555, 340	880, 15[a]	880, 15	28
	*Acc*I	544, 351	544, 351	906	544, 351	
Tyler F, Tyler R	*Msp*I	232, 48[a]	108, 73, 51[a]		155, 125	48
	*Nci*I	385	159, 126			
	*Rsa*I	216, 69	216, 69			

[a] Fragment too small to visualize under the electrophoresis conditions used.

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TABLE 3. Primer pairs used to amplify stx_2

Primer	Sequence (5′ to 3′)	Size of fragment (bp)	Reference
stx$_2$ typing			
VT2-e VT2-f	AATACATTATGGGAAAGTAATA TAAACTGCACTTCAGCAAAT	348	42
Lin F Lin R	GAACGAAATAATTTATATGT TTTGATTGTTACAGTCAT	900[a]	28
Tyler F Tyler R	AAGAAGATGTTTATGGCGGT CACGAATCAGGTTATGCCTC	285	48
stx$_2$ sequencing			
StxF StxR	TATCTGCGCCGGGTCT CAAAKCCKGARCCTGA	1,280	44
Gannon F Gannon R	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	18
Paton F Paton R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	37
ST F Stx2R	AATGCAATGGCGG CAAATCCGGAGCCTGC	200	This study
Stx2F2 ST R	AATCCAGTACAACGCGCCA AACGCAGAACTGCTCT	600	This study
Tyler F Tyler R	AAGAAGATGTTTATGGCGGT CACGAATCAGGTTATGCCTC	285	48
KBStx2F KBStx2R	AATCCAGTACAACGCGCC TTGCTGAATAATCAGACG	395	This study

[a] Amplicons differ by a few nucleotides depending on the variant.

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ated with ovine STEC (44). Representative RFLP profiles produced by the digestion of PCR amplification products spanning different regions of stx_2 are depicted in Fig. 1 and 2. DNA sequence analysis of stx_2 derived from STEC serotype O110:H40 showed 99.8% sequence identity with sequences of $stx_{2\text{-EDL933}}$ (accession number Z37725.1) (36) and the *p* gene derived from phage 933W (13). The two nucleotide polymorphisms that differentiated the stx_2 sequence of O110:H40 with $stx_{2\text{-EDL933}}$ did not alter the predicted amino acid sequence. The stx_2 gene from an O110:H40 isolate was selected for DNA sequence analysis, because PCR-RFLP analysis indicated that it was indicative of stx_2 genes representative of the majority of bovine isolates used in this study (Fig. 1, lane 6). DNA sequence analysis of the stx_2 gene amplified from the Ont:H11 isolate showed 99.5% (5 nucleotide polymorphisms in the A subunit) sequence identity with $stx_{2\text{vhb}}$, a sequence variant of $stx_{2\text{c}}$ (accession number X61283.1) (31). These data concur with the results of PCR-RFLP analyses used to type stx_2 variants in this study. Of the five nucleotide polymorphisms identified in the A-subunit sequence, only two polymorphisms resulted in a change in the amino acid sequence. The B subunit of $stx_{2\text{vhb}}$ in Ont:H11 showed nucleotide and predicted amino

TABLE 4. stx_2 variant(s) and association with serotype

stx_2 variant(s)	No. (%) of isolates[a]	Serotypes
$stx_{2\text{-EDL933}}$	59 (42.8)	O6:H34, O8:(KA):H51, O8:H19, O21:H21, O28:H8, O28:H40, O53:H2, O76:H7, O81:H21, O93:H19, O104:H7, O113:H21, O116:H21, O130:H11, O141:H49, O157:H8, O163:H19, Ont:H2, Ont:H5, Ont:H7, Ont:H8, Ont:H28, Ont:H49, Ont:HR, OR:H3, OX3:H8, OX3:H21, OX3:H40
$stx_{2\text{-EDL933}}/stx_{2\text{vhb}}$	31 (32.6)	O6:H8, O6:H28, O49:H$^-$, O113:H$^-$, O113:H21, Ont:H7, Ont:H49, OR:H8
$stx_{2\text{-EDL933}}/stx_1$	19 (10.4)	O5:H7, O82:H8, O82:H40, O110:H40, O111:H$^-$, O111:H8, O103:H11, O153:HR, Ont:H11, ONT:H21, Ont:H28, Ont:H30, Ont:H41
$stx_{2\text{vhb}}/stx_1$	11 (8.0)	O3:H7, O5:H7, O108:H7, O130:H38, O157:H$^-$, O163:H19, Ont:H8, Ont:H11, OX3:8
$stx_{2\text{vhb}}$	8 (5.8)	O2:H8, O113:H21, O157:H7, Ont:H11, OR:H$^-$
$stx_{2\text{-EDL933}}/stx_{2\text{vhb}}/stx_1$	5 (3.6)	O3:H7, O8:H16, O82:H8, O163:H$^-$, Ont:H16
$stx_{2\text{d-OX3a}}$	3 (2.2)	O5:H$^-$, O91:H21
$stx_{2\text{d-Ount}}$	2 (1.4)	O91:H$^-$, O153:H8

[a] Values are numbers and percentages compared to a total of 138 isolates.

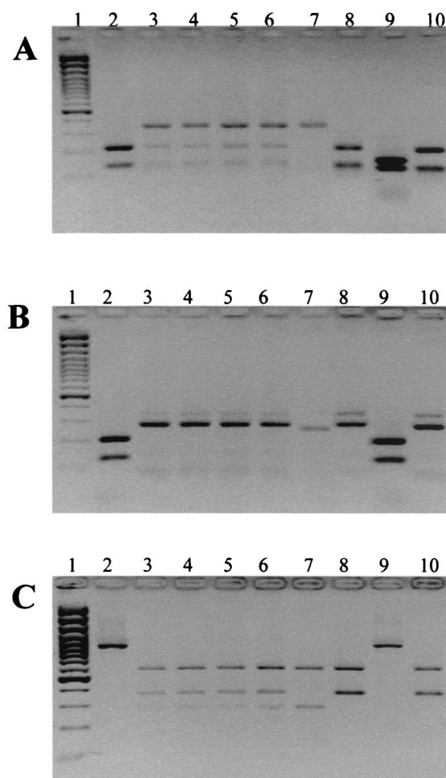

FIG. 1. *Hae*III (A) and *Pvu*II (B) digests of PCR products obtained with VT2-e and VT2-f primers and *Hinc*II (C) digests of PCR products obtained with Lin F and Lin R primers. Lanes: 1, 100-bp Plus marker; 2, $stx_{2d\text{-Ount}}$ variant of serotype O91:H$^-$; 3, $stx_{2\text{-EDL933}}/stx_{2vhb}$ variant of serotype O113:H21; 4, $stx_{2\text{-EDL933}}/stx_{2vhb}$ variant of serotype O113:H21; 5, $stx_{2\text{-EDL933}}/stx_{2vhb}$ variant of serotype O113:H21; 6, $stx_{2\text{-EDL933}}/stx_{2vhb}$ variant of serotype O110:H40; 7, $stx_{2\text{-EDL933}}$ variant of serotype Ont:H5; 8, stx_{2vhb} variant of serotype Ont:HR; 9, $stx_{2\text{-OX3a}}$ variant of serotype O5:H$^-$; 10, stx_{2vhb} variant of serotype Ont:H11.

acid sequences identical to those of stx_{2vhb} (accession number X61283.1) (31).

In this study, we determined the stx_2 subtypes of 127 bovine STEC isolates belonging to 58 serotypes and showed that Australian bovine STEC which also possesses *ehxA* and/or *eaeA* predominantly possesses either $stx_{2\text{-EDL933}}$ or stx_{2vhb} subtypes (alone or in combination with one another). We also demonstrated that several serotypes, particularly O113:H21, simultaneously possess both these subtypes.

In a recent study of 167 stx_2-containing STEC isolates from healthy cattle in France, stx_{2vhb}, $stx_{2\text{-EDL933}}$, and stx_{2vha} subtypes were commonly identified (5). Our study identified $stx_{2\text{-EDL933}}$ and stx_{2vhb} as the predominant subtypes (122 of 127; 96.1%) among Australian bovine STEC isolates; stx_{2vha}-positive STEC isolates were not observed among any of STEC isolates in our study. Although the serotypes belonging to the stx_2-containing STEC isolates from France were not reported (5), a recent study of 186 STEC isolates from cattle during a 1-year study in France (43) showed that few isolates possessed serotypes in common with those identified in this study. Our data suggest that stx_{2vha}-positive STEC belongs to serotypes not commonly found in Australian cattle. Alternatively, stx_{2vha} may only be found in STEC isolates that lack the accessory virulence factors *ehxA* and/or *eaeA* (termed complex STEC [22]), since all but 4 of the 138 STEC isolates in this study possessed one or both of these factors. In support of the latter hypothesis, the study by Bertin et al. (5) identified enterohemolysin among STEC isolates harboring $stx_{2\text{-EDL933}}$ (78%) or a combination of $stx_{2\text{-EDL933}}$ and stx_{2vhb} (85%); only 6 of 43 (14%) stx_{2vha}-containing isolates also possessed *ehxA*, and the presence of *eaeA* was not reported. Furthermore, recent studies in our laboratories have identified stx_{2vha}-containing STEC in isolates that do not possess *ehxA* and/or *eaeA* (our unpublished data). STEC isolates simultaneously containing three stx_2 variants were not observed among the 138 Australian STEC isolates, unlike the results reported by Bertin et al. (5). Furthermore, the untypeable stx_2 variant (identified as $stx_{2\text{-NV206}}$) which comprised 24 of 167 (14.4%) of isolates in the study by Bertin et al. (5) was not observed in our Australian collection; 25% of STEC isolates possessing the $stx_{2\text{-NV206}}$ possessed *ehxA* (5). Collectively, stx_2-subtyping data suggest that different non-O157 STEC populations predominate in Australia and France. However, the fact that 134 of 138 (97.1%) of the Australian stx_2-containing STEC isolates and 54 of 130 (41.5%) of the stx_2-containing French isolates possessed *ehxA* and/or *eaeA* may bias comparisons between these two STEC populations. Further studies are required to validate these hypotheses.

Serotype O113:H21 is increasingly being isolated from patients with HUS (6, 17, 40) and was the most predominant serotype in stx_2-containing STEC isolates from healthy cattle in Australia (22a). Studies conducted in France (43), Japan (26), and Spain (9, 10, 33) all indicated that O113:H21 is among the most prevalent STEC serotypes recovered from cattle feces. Furthermore, O113:H21 was identified in 10 of 41 (24.4%) STEC isolates recovered from a Canadian study of naturally contaminated beef (3). All of the O113:H21 isolates recovered in our study (22 bovine and 11 human) were *eaeA* negative; 29 of 33 (87.9%) possessed the *ehxA* gene, and only 2 of 33 (6.1%) possessed stx_1. Of 22 (90.9%) bovine isolates of serotype O113:H21, 20 concomitantly possessed both $stx_{2\text{-EDL933}}$ and stx_{2vhb} while 2 (9.1%) only possessed stx_{2vhb}. Two O113:H21 isolates

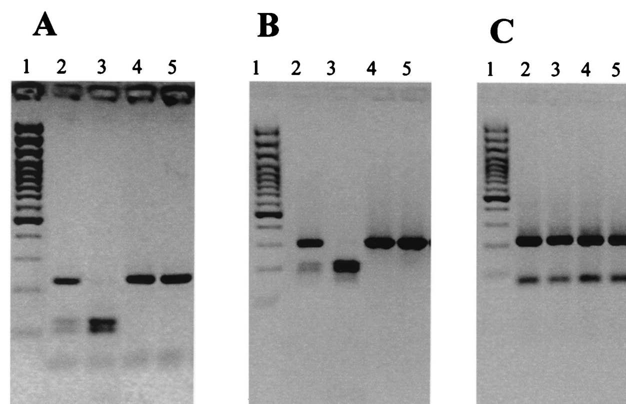

FIG. 2. *Msp*I (A), *Nci*I (B), and *Rsa*I (C) digests of PCR products obtained with Tyler F and Tyler R primers. Lanes: 1, 100-bp Plus marker; 2, $stx_{2\text{-EDL933}}/stx_{2vhb}$ variant of serotype O113:H21; 3, stx_{2vhb} variant of serotype Ont:HR; 4, $stx_{2\text{-EDL933}}$ variant of serotype Ont:H5; 5, $stx_{2\text{-EDL933}}$ variant of serotype Ont:H5.

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recovered from HUS patients and a third isolate from a patient with anemia also simultaneously possessed $stx_{2\text{-EDL933}}$ and stx_{2vhb}. However, four isolates of O113:H21 recovered from human cases of diarrhea, two from patients with HUS, and one isolate from an asymptomatic human individual possessed $stx_{2\text{-EDL933}}$ only. Of the 58 different serotypes identified in our study, 7 serotypes have been reported to cause HUS (O5:H$^-$, O91:H$^-$, O91:H21, O111:H$^-$, O111:H8, O157:H7, and O163:H19).

Ramachandran et al. (44) reported that ovine STEC isolates that also contain *ehxA* and/or *eaeA* predominantly possess stx_{2d} ($stx_{2d\text{-Ount}}$, $stx_{2d\text{-OX3a}}$, and stx_{2dO111}) subtypes. In contrast, the cattle STEC isolates described in this study rarely possessed stx_{2d} subtypes (5 of 138; 3.6%) and three of these isolates possessed STEC serotypes (O5:H$^-$ and O91:H$^-$) typically found in sheep. Furthermore, none of the ovine isolates examined in the study of Ramachandran et al. (44) contained stx_{2vhb} and only a single isolate possessed $stx_{2EDL933}$, highlighting a dramatic contrast with results reported in the present study of bovine STEC. Bertin et al. (5) showed that 167 bovine stx_2-positive STEC isolates from healthy cattle rarely possessed an stx_{2d} subtype (8.5% of 167), but their serotype(s) was not reported. Beutin et al. (8) also found that with the exception of a single serotype (O90:H24), cattle and sheep differed with respect to the O:H types of their STEC floras. Together, these and other studies (14, 22, 22a, 32) suggest that genetically and serologically different populations of STEC inhabit the gastrointestinal tracts of cattle and sheep.

This work was supported by funds from the Australian Cooperative Research Centre for Cattle and Beef Quality.

We thank Christine Gillen for technical assistance with sequencing and Kari Gobius for the supply of control DNA preparations containing STEC DNA with the stx_{2vha} Shiga toxin gene.

REFERENCES

1. **Aleksic, S.** 1995. W. H. O. report on Shiga-like toxin producing *Escherichia coli* (SLTEC), with emphasis on zoonotic aspects. World Health Organization, Geneva, Switzerland.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Atalla, H. N., R. Johnson, S. McEwen, R. W. Usborne, and C. L. Gyles.** 2000. Use of a Shiga toxin (Stx)-enzyme-linked immunosorbent assay and immunoblot for detection and isolation of Stx-producing *Escherichia coli* from naturally contaminated beef. J. Food Prot. **63:**1167–1172.
4. **Bastian, S. N., T. Carle, and F. Grimont.** 1998. Comparison of 14 PCR systems for the detection and subtyping of *stx* genes in Shiga-toxin-producing *Escherichia coli*. Res. Microbiol. **149:**457–472.
5. **Bertin, Y., K. Boukhors, N. Pradel, V. Livrelli, and C. Martin.** 2001. Stx2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: detection of a new stx_2 subtype and correlation with additional virulence factors. J. Clin. Microbiol. **39:**3060–3065.
6. **Bettelheim, K. A.** 2001. Enterohaemorrhagic *Escherichia coli* O157:H7: a red herring? J. Med. Microbiol. **50:**201–202.
7. **Beutin, L., D. Grier, H. Steinruck, S. Zimmermann, and F. Scheutz.** 1993. Prevalence and some properties of verotoxin (Shiga-like-toxin)-producing *Escherichia coli* in seven different species of healthy animals. J. Clin. Microbiol. **31:**2483–2488.
8. **Beutin, L., D. Geier, S. Zimmermann, S. Aleksic, H. A. Gillespie, and T. S. Whittam.** 1997. 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.
9. **Blanco, M., J. E. Blanco, J. Blanco, E. A. Gonzalez, M. P. Alonso, H. Maas, and W. H. Jansen.** 1996. Prevalence and characteristics of human and bovine verotoxigenic *Escherichia coli* strains isolated in Galicia (north-western Spain). Eur. J. Epidemiol. **12:**13–19.
10. **Blanco, M., J. E. Blanco, J. Blanco, A. Mora, C. Prado, M. P. Alonso, M. Mourino, C. Madrid, C. Balsalobre, and A. Juarez.** 1997. Distribution and characterization of faecal verotoxin-producing *Escherichia coli* (VTEC) isolated from healthy cattle. Vet. Microbiol. **54:**309–319.
11. **Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles.** 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. **37:**497–503.
12. **Clarke, R. C., J. B. Wilson, S. C. Read, S. Renwick, K. Rahn, R. P. Johnson, D. Alves, M. A. Karmali, H. Loir, S. A. McEwen, J. Spika, and C. L. Gyles.** 1994. Verocytotoxin-producing *Escherichia coli* (VTEC) in the food chain: preharvest and processing perspectives, p. 17–24. *In* M. A. Karmali and A. G. Goglio (ed.), Recent advances in verocytotoxin-producing *Escherichia coli* infections. Elsevier Science B.V., Amsterdam, The Netherlands.
13. **Datz, M., C. Janetzki-Mittmann, S. Franke, F. Gunzer, H. Schmidt, and H. Karch.** 1996. Analysis of the enterohemorrhagic *Escherichia coli* O157 DNA region containing lamboid phage gene *p* and Shiga-like toxin structural genes. Appl. Environ. Microbiol. **62:**791–797.
14. **Djordjevic, S. P., M. A. Hornitzky, G. Bailey, P. Gill, B. Vanselow, K. Walker, and K. Bettelheim.** 2001. Virulence properties and serotypes of Shiga toxin-producing *Escherichia coli* from healthy Australian slaughter-age sheep. J. Clin. Mirobiol. **39:**2017–2021.
15. **Edelman, R., M. A. Karmali, and P. A. Fleming.** 1988. Summary of the international symposium and workshop on infections due to verocytotoxin (Shiga-like toxin)-producing *Escherichia coli*. J. Infect. Dis. **157:**1102–1104.
16. **Elliott, E. J., R. M. Robbins-Browne, E. V. O'Loughlin, V. Bennet-Wood, J. Bourke, P. Henning, G. G. Hogg, J. Knight, H. Powell, and D. Redmond.** 2001. Nationwide study of haemolytic uremic syndrome: clinical, microbiological, and epidemiological features. Arch. Dis. Child. **85:**125–131.
17. **Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic.** 1999. Detection of Shiga-like toxin (stx_1 and stx_2), intimin (*eaeA*), and enterohaemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol. **65:**868–872.
18. **Gannon, V. P., R. K. King, J. Y. Kim, and E. J. Golsteyn Thomas.** 1992. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**3809–3815.
19. **Gannon, V. P. J., C. Teerling, S. A. Masri, and C. L. Gyles.** 1990. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. J. Gen. Microbiol. **136:**1125–1135.
20. **Griffin, P. M.** 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739–758. *In* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract. Raven Press, New York, N.Y.
21. **Gyles, C., R. Johnson, A. Gao, K. Ziebell, D. Pierard, S. Aleksic, and P. Boerlin.** 1998. Association of enterohaemorrhagic *Escherichia coli* hemolysin with serotypes of Shiga-like-toxin-producing *Escherichia coli* of human and bovine origins. Appl. Environ. Microbiol. **64:**4134–4141.
22. **Hornitzky, M. A., K. A. Bettelheim, and S. P. Djordjevic.** 2001. The detection of Shiga toxin-producing *Escherichia coli* in diagnostic bovine faecal samples using vancomycin-cefimine-cefsulodin blood agar and PCR. FEMS Microbiol. Lett. **198:**17–22.

22a. **Hornitzky, M. A., B. A. Vanselow, K. Walker, B. Corney, P. Gill, G. Bailey, and S. J. Djordjevic.** 2002. Virulence properties and serotypes of Shiga toxin-producing *Escherichia coli* from healthy Australian cattle. Appl. Environ. Microbiol. **68:**6439–6445.

23. **Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi.** 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with hemolytic uremic syndrome. Microb. Pathog. **8:**47–60.
24. **Johnson, R. P., R. C. Clarke, J. B. Wilson, S. C. Read, K. Rahn, S. A. Renwick, K. A. Sandhu, D. Alves, M. A. Karmali, H. Loir, S. A. McEwen, J. S. Spika, and C. L. Gyles.** 1996. Growing concerns and recent outbreaks involving non-O157:H7 serotypes of verotoxigenic *Escherichia coli*. J. Food Prot. **59:**1112–1122.
25. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
26. **Kobayashi, H., J. Shimada, M. Nakazawa, T. Morozumi, T. Pohjanvitra, S. Pelkonnen, and K. Yamamoto.** 2001. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl. Environ. Microbiol. **67:**484–489.
27. **Kokai-Kun, J. F., A. R. Melton-Celsa, and A. D. O'Brien.** 2000. Elastase in intestinal mucus enhances the cytotoxicity of Shiga toxin type 2d. J. Biol. Chem. **275:**3713–3721.
28. **Lin, Z., S. Yamasaki, H. Kurazono, M. Ohmura, T. Karasawa, T. Inoue, S. Sakamoto, T. Suganami, T. Takeoka, Y. Taniguchi, and Y. Takeda.** 1993. Cloning and sequencing of two new verotoxin 2 variant genes of *Escherichia coli* isolated from cases of human and bovine diarrhea. Microbiol. Immunol. **37:**451–459.
29. **Lindgren, S. W., A. R. Melton, and A. D. O'Brien.** 1993. Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model. Infect. Immun. **61:**3832–3842.
30. **Melton-Celsa, A. R., S. C. Darnell, and A. D. O'Brien.** 1996. Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with

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virulence of enterohemorrhagic *Escherichia coli* O91:H21 isolates in orally infected, streptomycin-treated mice. Infect. Immun. **64:**1569–1576.
31. **Meyer, T., H. Karch, J. Hacker, H. Bocklage, and J. Heesemann.** 1992. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. Zentbl. Bacteriol. **276:**176–188.
32. **Montenegro, M. A., M. Bulte, T. Trumpf, S. Aleksic, G. Reuter, E. Bulling, and R. Helmuth.** 1990. Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. J. Clin. Microbiol. **28:**1417–1421.
33. **Orden, J. A., J. A. Ruiz-Santa-Quiteria, D. Cid, S. Garcia, R. Sanz, and R. De la Fuente.** 1998. Verotoxin-producing *Escherichia coli* (VTEC) and *eae*-positive non-VTEC in 1–30-days-old diarrhoeic dairy calves. Vet. Microbiol. **63:**239–248.
34. **Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi.** 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994–998.
35. **Parma, A. E., M. E. Sanz, J. E. Blanco, J. Blanco, M. R. Viñas, M. Blanco, N. L. Padola, and A. I. Etcheverria.** 2000. Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Eur. J. Epidemiol. **16:**757–762.
36. **Paton, A. W., A. J. Bourne, P. A. Manning, and J. C. Paton.** 1995. Comparative toxicity and virulence of *Escherichia coli* clones expressing variant and chimeric Shiga-like toxin type II operons. Infect. Immun. **63:**2450–2458.
37. **Paton, A. W., and J. C. Paton.** 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. J. Clin. Microbiol. **36:**598–602.
38. **Paton, A. W., J. C. Paton, P. N. Goldwater, and P. A. Manning.** 1993. Direct detection of *Escherichia coli* Shiga-like toxin genes in primary fecal cultures by polymerase chain reaction. J. Clin. Microbiol. **31:**3063–3067.
39. **Paton, A. W., J. C. Paton, M. W. Heuzenroeder, P. N. Goldwater, and P. A. Manning.** 1992. Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. Microb. Pathog. **13:**225–236.
40. **Paton, A. W., M. Woodrow, R. M. Doyle, J. A. Lanser, and J. C. Paton.** 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. J. Clin. Microbiol. **37:**3357–3361.
41. **Pierard, D., G. Cornu, W. Proesmans, A. Dediste, F. Jacobs, J. Vande Walle, A. Mertens, J. Ramet, S. Lauwers, and the Belgian Society for Infectology and Clinical Microbiology HUS Study Group.** 1999. Hemolytic uremic syndrome in Belgium: incidence and association with verocytotoxin-producing *Escherichia coli* infection. Clin. Microbiol. Infect. **5:**16–22.
42. **Pierard, D., G. Muyldermans, L. Moriau, D. Stevens, and S. Lauwers.** 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J. Clin. Microbiol. **36:**3317–3322.
43. **Pradel, N., V. Livrelli, C. De Champs, J.-B. Palcoux, A. Reynaud, F. Scheutz, J. Sirot, B. Joly, and C. Forestier.** 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. J. Clin. Microbiol. **38:**1023–1031.
44. **Ramachandran, V., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic.** 2001. The common ovine Shiga toxin 2-containing *Escherichia coli* serotypes and human isolates of the same serotypes possess a Stx2d toxin type. J. Clin. Microbiol. **39:**1932–1937.
45. **Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. H. Wieler, and H. Karch.** 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl. Environ. Microbiol. **66:**1205–1208.
46. **Schmitt, C. K., M. L. McKee, and A. D. O'Brien.** 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H^- strain E32511. Infect. Immun. **59:**1065–1073.
47. **Smith, H. R., and S. M. Scotland.** 1988. Vero cytotoxin-producing strains of *Escherichia coli*. J. Med. Microbiol. **26:**77–85.
48. **Tyler, S. D., W. M. Johnson, H. Loir, G. Wang, and K. R. Rozee.** 1991. Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. **29:**1339–1343.
49. **Weiler, L. H., E. Vieler, C. Erpenstein, T. Schlapp, H. Steinruck, R. Bauerfeind, A. Byomi, and G. Baljer.** 1996. Shiga toxin-producing *Escherichia coli* strains from bovines: association of adhesion with carriage of *eae* and other genes. J. Clin. Microbiol. **34:**2980–2984.
50. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J. Bacteriol. **170:**4223–4230.
51. **Willshaw, G. A., S. M. Scotland, H. R. Smith, and B. Rowe.** 1992. Properties of vero cytotoxin-producing *Escherichia coli* of human origin of O serogroups other than O157. J. Infect. Dis. **166:**797–802.

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