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Bovine feces from animals with gastrointestinal infections are a source of serologically diverse atypical enteropathogenic Escherichia coli and Shiga toxin-producing E. coli strains that commonly possess intimin

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) cells were isolated from 191 fecal samples from cattle with gastrointestinal infections (diagnostic samples) collected in New South Wales, Australia. By using a multiplex PCR, *E. coli* cells possessing combinations of stx_1, stx_2, *eae*, and *ehxA* were detected by a combination of direct culture and enrichment in *E. coli* (EC) (modified) broth followed by plating on vancomycin-cefixime-cefsulodin blood (BVCC) agar for the presence of enterohemolytic colonies and on sorbitol MacConkey agar for the presence of non-sorbitol-fermenting colonies. The high prevalence of the intimin gene *eae* was a feature of the STEC (35 [29.2%] of 120 isolates) and contrasted with the low prevalence (9 [0.5%] of 1,692 fecal samples possessed STEC with *eae*) of this gene among STEC recovered during extensive sampling of feces from healthy slaughter-age cattle in Australia (M. Hornitzky, B. A. Vanselow, K. Walker, K. A. Bettelheim, B. Corney, P. Gill, G. Bailey, and S. P. Djordjevic, Appl. Environ. Microbiol. 68:6439-6445, 2002). Forty-seven STEC serotypes were identified, including O5:H−, O8:H19, O26:H−, O26:H11, O113:H21, O157:H7, O157:H− and Ont:H− which are known to cause severe disease in humans and 23 previously unreported STEC serotypes. Serotypes Ont:H− and O113:H21 represented the two most frequently isolated STEC isolates and were cultured from nine (4.7%) and seven (3.7%) animals, respectively. Fifteen *eae*-positive *E. coli* serotypes, considered to represent atypical EPEC, were identified, with O111:H− representing the most prevalent. Using both techniques, STEC cells were cultured from 69 (36.1%) samples and EPEC cells were cultured from 30 (15.7%) samples, including 9 (4.7%) samples which yielded both STEC and EPEC. Culture on BVCC agar following enrichment in EC (modified) broth was the most successful method for the isolation of STEC (24.1% of samples), and direct culture on BVCC agar was the most successful method for the isolation of EPEC (14.1% samples). These studies show that diarrheagenic calves and cattle represent important reservoirs of *eae*-positive *E. coli*.

Keywords

animals, infections, source, bovine, intimin, diverse, atypical, coli, shiga, producing, e, that, commonly, gastrointestinal, serologically, enteropathogenic, toxin, strains, possess, feces, escherichia

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Bovine Feces from Animals with Gastrointestinal Infections Are a Source of Serologically Diverse Atypical Enteropathogenic *Escherichia coli* and Shiga Toxin-Producing *E. coli* Strains That Commonly Possess Intimin

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Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) cells were isolated from 191 fecal samples from cattle with gastrointestinal infections (diagnostic samples) collected in New South Wales, Australia. By using a multiplex PCR, *E. coli* cells possessing combinations of stx_1, stx_2, *eae*, and *ehxA* were detected by a combination of direct culture and enrichment in *E. coli* (EC) (modified) broth followed by plating on vancomycin-cefixime-cefsulodin blood (BVCC) agar for the presence of enterohemolytic colonies and on sorbitol MacConkey agar for the presence of non-sorbitol-fermenting colonies. The high prevalence of the intimin gene *eae* was a feature of the STEC (35 [29.2%] of 120 isolates) and contrasted with the low prevalence (9 [0.5%] of 1,692 fecal samples possessed STEC with *eae*) of this gene among STEC recovered during extensive sampling of feces from healthy slaughter-age cattle in Australia (M. Hornitzky, B. A. Vanselow, K. Walker, K. A. Bettelheim, B. Corney, P. Gill, G. Bailey, and S. P. Djordjevic, Appl. Environ. Microbiol. 68:6439–6445, 2002). Forty-seven STEC serotypes were identified, including O5:H−, O8:H19, O26:H−, O26:H11, O113:H21, O157:H7, O157:H− and Ont:H− which are known to cause severe disease in humans and 23 previously unreported STEC serotypes. Serotypes Ont:H− and O113:H21 represented the two most frequently isolated STEC isolates and were cultured from nine (4.7%) and seven (3.7%) animals, respectively. Fifteen *eae*-positive *E. coli* serotypes, considered to represent atypical EPEC, were identified, with O111:H− representing the most prevalent. Using both techniques, STEC cells were cultured from 69 (36.1%) samples and EPEC cells were cultured from 30 (15.7%) samples, including 9 (4.7%) samples which yielded both STEC and EPEC. Culture on BVCC agar following enrichment in EC (modified) broth was the most successful method for the isolation of STEC (24.1% of samples), and direct culture on BVCC agar was the most successful method for the isolation of EPEC (14.1% samples). These studies show that diarrheagenic calves and cattle represent important reservoirs of *eae*-positive *E. coli*.

Enteropathogenic *Escherichia coli* (EPEC) and a subset of the Shiga toxin-producing *E. coli* (STEC) known as enterohemorrhagic *E. coli* (EHEC) represent two of the five pathotypes of the diarrheagenic *E. coli* recognized at present (32, 43). The genetic diversity of diarrheagenic *E. coli* isolated from both healthy and clinically affected humans (diarrhea, bloody diarrhea [BD], and hemolytic uremic syndrome [HUS]) continues to increase as more epidemiological studies of humans rely on the use of molecular tools (e.g., PCR) to detect virulence genes. This is exemplified in a recent study of 677 STEC strains isolated from humans in Germany over a 3-year period where 55 *E. coli* O groups and 24 different H types were identified (4). Although 11 serotypes accounted for 69% of all STEC strains isolated (4), 31 serotypes had previously not been described as human STEC. Importantly, of these 677 isolates, 392 (58%) possessed the intimin gene (*eae*) and a significant proportion of these 334 (85.2%) ($P < 0.01$) were from patients with diarrhea, BD, or HUS. Of 108 isolates from patients with BD or HUS, 94 (87%) possessed *eae*. These and other studies underscore the importance of the association between (i) the presence of intimin in STEC cells that cause disease in humans (8, 14) and (ii) using molecular approaches to screen for clinically important *E. coli* (4, 7, 12, 20, 45).

Unlike other diarrheagenic *E. coli* isolates, EPEC and many EHEC isolates share the ability to form attaching and effacing (A/E) lesions on the surfaces of epithelial cells in the gastrointestinal tract (17). Genes encoding proteins required for the induction of A/E lesions reside on a chromosomally located pathogenicity island known as the locus of enterocyte effacement (LEE). The LEE encodes genes for a type III secretion system, various translocated proteins including Tir and Esp proteins, and intimin. The intimin gene, *eae*, was the first gene to be associated with A/E activity, and its presence is often used as a marker for the presence of the entire LEE island since many *eae*-positive EPEC and EHEC strains are positive when examined with the fluorescent-actin staining (FAS) assay

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(5, 26, 27, 39) and many *eae*-positive EPEC that are negative by the FAS assay become FAS positive when the EAF plasmid pMAR is introduced by conjugation (4). Thus, *eae* is a key marker for infections caused by most EHEC and EPEC strains.

EPEC cells have been broadly defined as diarrheagenic *E. coli* cells that do not possess Shiga toxin genes and possess the ability to form A/E lesions on intestinal cells (24). EPEC strains remain a leading cause of endemic infantile diarrhea in developing countries, and although the role of classic EPEC serotypes as diarrheagenic agents in developing countries is important, their frequency of appearance in infants less than 1 year old is very rare (32). However, recent studies suggest that EPEC strains may comprise a genetically more complex group of *E. coli* (5, 45). Multilocus enzyme electrophoresis studies identified two distinct genetic groups of EPEC known as EPEC 1 and EPEC 2 (47). Furthermore, two types of EPEC, known as the typical and atypical EPEC, have been described. Typical EPEC cells possess a large EPEC adherence factor (EAF) plasmid, generate a characteristic adherence pattern (localized adherence pattern) within 3 h of incubation with tissue culture cells (42), and belong to a defined group of serotypes including O55:H6, O86:H34, O111:H2, O114:H2, O119:H6, O127:H6, and O142:H6/H34 (45). Atypical EPEC cells do not possess the EAF plasmid and can be subdivided into two groups: those that possess the LEE island and those that possess LEE and other virulence attributes including genes for enterohemolysin (*ehxA*) and the enteroaggregative heat stable toxin (EAST1) (45). Unlike typical EPEC, atypical EPEC has been isolated from the feces of meat-producing animals (45).

Ruminants, particularly cattle and sheep, are a recognized source of STEC and atypical EPEC and represent a major reservoir for entry of these pathogens into the human population via the food chain (3, 7, 12, 20, 40, 45). Extensive studies of healthy, Australian slaughter-age cattle and sheep indicate that these meat-producing animals harbor a wide variety of serologically diverse STEC strains, several of which (O157:H−/H7, O26:H1/H11, O113:H21; O5:H−; O103:H2) have been commonly associated with both sporadic cases and outbreaks of serious disease in humans (7, 12, 20, 46). Despite the isolation of these STEC serotypes, the vast majority of STEC strains recovered from the feces of healthy slaughter-age sheep and cattle do not possess *eae* although many possess *ehxA* (12, 13, 20, 41). EHEC serotypes O157:H7 and O111:H−, which are often found in association with patients with HUS and bloody diarrhea, are rarely isolated from healthy slaughter-age cattle and sheep in Australia (12, 13, 20). Contaminated mettwurst sausage containing O111:H− STEC (and several other STEC serogroups, including O157, O160, O91, O113, O123, and O128) was responsible for Australia's largest outbreak of HUS and bloody diarrhea (37), and this serotype and others, including O26:H−, O113:H21, O130:H11, OR:H9, O157:H−, Ont:H7, and Ont:H−, represent the most common EHEC isolates recovered from patients with HUS in Australia (15). Sursprisingly, despite a number of studies (11, 20), there is only a single report of the isolation of O111:H− STEC from ruminant sources in Australia (21).

The culture of STEC and EPEC involved in infections of humans or cattle can be problematic as there are no effective differential media available to discriminate between STEC and non-STEC and between EPEC and non-EPEC. The development of techniques such as PCR and Shiga toxin assays have contributed to the detection of more than 400 EHEC serotypes (http:/www.lugo.usc/ecoli, http://www.microbionet.com.au/vtectable.htm). The degree of success for the detection of STEC is also a function of the microbiological culture procedures employed. In one study of 65 patients with EHEC infections, direct culture of stool samples onto sorbitol MacConkey (SMAC) agar failed to detect four patients with O157 infections but these were detected by subculturing the enrichment broths (35). In a study of diagnostic bovine fecal samples, STEC cells were isolated from 23 (18.7%) of 123 samples; STEC cells were isolated from 14 samples by direct culturing, and 13 samples yielded STEC after enrichment in *E. coli* (EC) (modified) broths. In both instances, enterohemolytic colonies were identified by plating on vancomycin-cefixime-cefsulodin blood (BVCC) agar as described previously (30). Only four fecal samples produced STEC cells by using both culture methods.

In this study, we examined the presence of the virulence genes stx_1, stx_2, *eae*, and *ehxA* among *E. coli* samples cultured from 191 diagnostic bovine fecal samples with the aim of determining if cattle with the clinical signs indicative of gastrointestinal diseases excrete *E. coli* with virulence attributes akin to those commonly isolated from patients in Australia with HUS, hemorrhagic colitis, and diarrhea. Furthermore, we examined the effect of direct and enrichment culture protocols on the diversity of serotypes of *E. coli* isolates possessing combinations of the four virulence genes isolated from these diagnostic fecal samples. Using a multiplex PCR (36), we tested fecal samples for the presence of *E. coli* possessing O111 and O157 serogroups and compared the efficiency of both direct and enrichment culture procedures employing SMAC agar with PCR on the isolation of O157 *E. coli*.

MATERIALS AND METHODS

Fecal samples. Bovine diagnostic fecal samples (191 total) from 85 (44.5%) calves, 57 (29.8%) adult cattle, and 49 (25.7%) cattle of unspecified ages were used in this study. These beef or dairy cattle were suspected of having salmonellosis, yersiniosis, rotavirus, coccidiosis, parasites, colibacillosis, pestivirus, or ill thrift. Fecal samples were submitted to the Regional Veterinary Laboratory, Elizabeth Macarthur Agricultural Institute, New South Wales, over a 13-month period. Samples were usually submitted by courier and arrived at the laboratory within 24 h after their collection.

Detection of stx_1, stx_2, *eae*, *ehxA*, and O111 and O157 *rfb* regions in fecal samples by multiplex PCR. Two preparation methods were employed. Feces (50 mg) were inoculated into 10 ml of EC (modified) broth (CM853; Oxoid, Basingstoke, United Kingdom) and incubated at 37°C for 18 to 20 h. Feces (50 mg) were also inoculated into saline and vortexed. PCR for the specific detection of stx_1, stx_2, *eae*, and *ehxA* was carried out on the saline sample and on the EC (modified) broth after incubation as described previously (36). The detection of specific portions of the *rfb* (O-antigen-encoding) regions of *E. coli* serotypes O111 and O157 was carried out on the EC (modified) broths as described by Paton and Paton (36). Procedures used for the preparation of DNA for PCR have been described previously (16). Amplified DNA fragments were resolved by gel electrophoresis using 2% agarose and stained with ethidium bromide. Glycerol stocks of the overnight EC (modified) broth were stored at −80°C.

Isolation of STEC and EPEC. In this study, EPEC cells were defined as *eae*-positive *stx*-deficient *E. coli* cells. Two methods were used for the isolation of STEC and EPEC. One method examined the success of isolation of STEC and EPEC by direct culturing on BVCC agar and SMAC agar. Several studies have shown that there is a close association between the presence of *stx* and enterohemolysin (encoded by the *ehxA* gene) in STEC isolated from human and ruminant sources (6, 12, 20, 30), and BVCC agar was developed to identify

TABLE 1. Multiplex PCR data derived from 191 diagnostic bovine fecal samples

Virulence factor profile	No. detected in:	
	Saline	EC broth
stx$_1$	0	6
stx$_1$, *stx*$_2$	1	11
stx$_1$, *stx*$_2$, *eae*	0	2
stx$_1$, *stx*$_2$, *eae*, *ehxA*	0	10
stx$_2$	3	19
stx$_2$, *eae*, *ehxA*	1	6
eae	3	6
eae, *ehxA*	1	21
stx$_1$, *eae*	0	2
stx$_1$, *eae*, *ehxA*	3	13
stx$_2$, *ehxA*	0	16
stx$_1$, *stx*$_2$, *ehxA*	0	13
ehxA	2	7
Negative	177	59

TABLE 2. Virulence factors detected in SMAC and BVCC agar-positive colonies (one isolate per animal) from direct culture and enrichment in EC (modified) broth

Virulence profile	No. of isolates			
	Direct	SMAC agar enrichment	Direct	BVCC agar enrichment
eae	2	2	2	0
ehxA	5	4	2	6
ehxA, *eae*	2	7	25	16
stx$_1$	3	2	1	1
stx$_2$	7	6	0	0
stx$_1$, *stx*$_2$	0	2	0	0
stx$_1$, *eae*	0	0	0	1
stx$_2$, *eae*	0	0	1	0
stx$_2$, *ehxA*	5	4	15	25
stx$_1$, *stx*$_2$, *ehxA*	1	0	5	8
stx$_1$, *ehxA*, *eae*	0	1	8	8
stx$_2$, *ehxA*, *eae*	3	2	3	2
stx$_1$, *stx*$_2$, *ehxA*, *eae*	1	2	2	1
Total	29	32	64	68

enterohemolytic colonies. The second method compared the success of isolation of these *E. coli* subpopulations by plating EC (modified) fecal enrichment broths on both BVCC agar and SMAC agar.

Method 1: direct culturing on BVCC and SMAC agars. Feces (50 mg) were inoculated into 10 ml of saline and vortexed. Serial 10-fold dilutions were carried out to 10^{-3}, and the dilution which produced about 100 single colonies on MacConkey agar with a 100-μl inoculum was cultured onto BVCC agar to facilitate the detection of hemolysin variants of STEC. BVCC-agar-positive colonies produce a characteristic small turbid zone of hemolysis after incubation at 37°C for 18 to 24 h (30). SMAC agar was also inoculated for the detection of non-sorbitol-fermenting colonies. Up to five BVCC- and SMAC-positive colonies, if available, per fecal sample were examined by multiplex PCR as described above.

Method 2: culturing on BVCC and SMAC agars from EC (modified) fecal broth. Feces (50 mg) were inoculated into 10 ml of EC (modified) broth and incubated at 37°C for 18 to 20 h, and an aliquot was examined for the presence of four virulence genes (*stx*$_1$, *stx*$_2$, *eae*, and *ehxA*) by multiplex PCR as described previously (36). Serial 10-fold dilutions of enrichment broths positive for these virulence genes were plated onto MacConkey agar. The dilution that produced single colonies (ideally about 100 colonies per plate) was cultured onto BVCC and SMAC agar plates. Up to five BVCC-agar-positive and five SMAC-agar-positive colonies per aliquot of EC (modified) enrichment broth were subjected to multiplex PCR as described previously (36).

Biochemical and serological analyses of isolates. Isolates containing one or more virulence factors were confirmed as being *E. coli* as described by Bettelheim et al. (1) and serotyped as described by Bettelheim and Thompson (2) and Chandler and Bettelheim (10) using O antisera O1 through O181 and H antisera H1 through H56. Twenty-three isolates were not serotyped.

RESULTS

Detection of *E. coli* virulence factors and of O111 and O157 *rfb* regions in fecal samples by multiplex PCR. Virulence genes were detected in 14 (7.3%) fecal saline preparations and in 132 (69.1%) EC (modified) broths (Table 1), and all samples positive in fecal saline preparations were also positive in EC (modified) broths. The most common virulence gene combinations identified in the saline preparations were *stx*$_2$ alone, *eae* alone, and *stx*$_1$ with *eae* and *ehxA*; each were detected in 3 of 14 (21.4%) samples. The most common virulence gene combinations identified in the EC (modified) broths were *eae* with *ehxA* (21 of 132 [16.0%]), *stx*$_2$ alone (19 of 132 [14.4%]), and *stx*$_2$ and *ehxA* (16 of 132 [12.1%]). STEC isolates with the profiles *stx*$_1$ with *eae* and *ehxA* (13 of 132 [9.8%]) and *stx*$_1$ with *stx*$_2$ and *ehxA* (13 of 132 [9.8%]), *stx*$_1$ with *stx*$_2$ (11 of 132 [8.3%]), and *stx*$_1$ with *stx*$_2$, *eae*, and *ehxA* (10 of 132 [7.6%]) were all well represented. Multiplex PCR data showing the combinations of virulence genes detected are presented in Table 1.

Of 191 samples, 14 (7.3%) were positive for the O111 *rfb* region and 7 (3.7%) were positive for the O157 *rfb* region by PCR. One sample (0.5%) was positive for both O111 and O157 *rfb* regions (data not shown).

Isolation of STEC and EPEC. Of the 191 fecal samples, 90 (47.1%) yielded STEC or EPEC. Using both culture techniques STEC cells were cultured from 69 (36.1%) samples and EPEC cells were cultured from 30 (15.7%) samples, including 9 (4.7%) samples which yielded both STEC and EPEC.

Virulence genes and serotypes in STEC and EPEC cells isolated by direct culturing on SMAC agar. Of the 191 diagnostic fecal samples, 25 (13.1%) yielded non-sorbitol-fermenting colonies containing virulence factors, and 4 (2.1%) of these yielded two isolates with different virulence factors. Of these, 17 (75.0%) yielded STEC and 4 (16.3%) yielded EPEC (including one sample which also yielded STEC). Direct culture on SMAC detected seven STEC serotypes (O119:H−, O146:H21, O159:H9, O177:H−, Ont:H16, Ont:H38, and OR:H21) which were not detected elsewhere. The most common STEC profile was *stx*$_2$, which was detected on seven (24.1%) occasions and belonged to serotypes O119:H−, O159:H−, Ont:H−, Ont:H16, and Ont:H38. STEC strains possessing *ehxA* with *stx*$_2$ were detected on five (17.3%) occasions and belonged to serotypes O8:H19 and Ont:H11 (Table 2). Three isolates possessed *stx*$_2$ with *eae* and *ehxA* (serotypes Ont:H− and OR:H−), and one isolate (serotype O146:H21) possessed all four virulence genes. The 25 samples yielding non-sorbitol-fermenting colonies belonged to 13 STEC serotypes, none of which included the O157 serogroup. EPEC isolated by direct culture on SMAC agar belonged to serotypes O98:H−, O118:H− and OR:H− (Table 3). Of the 29 isolates recovered from direct culture on SMAC agar, there were 20 STEC and 4 EPEC.

Virulence genes and serotypes in STEC and EPEC cells isolated by direct culture on BVCC agar. Of the 191 samples that were cultured directly onto BVCC agar, 59 (30.9%) yielded BVCC-agar-positive colonies containing virulence

TABLE 3. Virulence gene profiles of STEC serotypes isolated from diagnostic bovine fecal samples

Serotype	No. of isolates (no. of cattle colonized)	Previously reported	Virulence factor profile				Culture method (no. of isolates)				Calf/cow/unknown[a]
							Direct		Enrichment		
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	SMAC	BVCC agar	SMAC	BVCC agar	
O2(rel):Hnt	1 (1)	No	+	+	−	+		1			Unknown
O5:H−	7 (4)	Yes	+	−	+	+		2	3	2	Calf/cow
O7:H21	1 (1)	No	−	+	−	−			1		Calf
O8:H19	11 (4)	Yes	−	+	−	+	4	2		5	Calf/unknown
O15rel:H−	2 (1)	No	−	+	+	+		2			Unknown
O22rel:H29	1 (1)	No	−	+	+	+				1	Unknown
O26:H−	6 (3)	Yes	+	−	+	+		4		2	Calf
O26:H11	1 (1)	Yes	+	−	+	+		1		1	Calf
O53:H41	1 (1)	No	−	+	−	+		1			Calf
O71:H9	2 (1)	No	+	−	−	−		1		1	Calf
O74:H28	2 (1)	Yes	+	−	+	+				2	Unknown
O75:H32	2 (1)	No	+	−	−	−			2		Unknown
O81:H−	1 (1)	No	−	+	−	+				1	Cow
O84:H−	2 (2)	Yes	+	−	+	+				2	Calf
O88:H−	1 (1)	Yes	−	+	+	+		1			Calf
O112a,b:H19	3 (2)	Yes	+	−	+	−	1		2		Unknown
O113:H21	9 (7)	Yes	−	+	−	+		1		8	Cow/unknown
O119:H−	1 (1)	Yes	−	+	−	−	1				Calf
O123:H11	6 (2)	Yes	+	−	+	+		4	2		Calf
O130:H11	3 (2)	Yes	−	+	−	+		2		1	Cow/unknown
	4 (3)		+	+	−	+		3		1	Calf/unknown
O130:H38	2 (1)	No	+	+	−	+			1	1	Cow
O146:H21	1 (1)	Yes	+	+	+	+	1				Cow
O157:H−	5 (2)	Yes	+	+	+	+		3	2		Calf
O157:H7	2 (1)	Yes	−	+	+	+			1	1	Calf
O159:H9	1 (1)	No	−	+	−	−	1				Calf
O159:H21	2 (1)	No	−	+	−	−		2			Cow
O163:H−	1 (1)	Yes	+	+	−	+		1			Cow
O174:H8	7 (3)	No	−	+	−	+		2	1	4	Calf/unknown
O175:H−	2 (1)	No	+	+	−	+		1		1	Unknown
O177:H−	3 (1)	Yes	−	+	+	+	3				Cow
O177:H32	1 (1)	No	−	+	+	+			1		Calf
O178:H19	1 (1)	Yes	+	+	−	+				1	Cow
O179:H8	1 (1)	No	−	+	−	+				1	Cow
O181:H49	1 (1)	No	−	+	−	+				1	Unknown
ONT:H−	1 (1)	Yes	+	−	+	+		1			Calf
	5 (5)		−	+	−	+		2		1	Cow
	5 (3)		−	+	−	−	2	1	2		Calf/cow
	1 (1)		−	+	+	+	1				Cow
ONT:H2	1 (1)	Yes	−	+	−	+				1	Unknown
	2 (1)		−	+	−	−			2		Calf
ONT:H11	7 (3)	No	−	+	−	+	1	2	1	3	Unknown
	1 (1)		+	+	−	+	1				Unknown
ONT:H16	2 (2)	Yes	−	+	−	−	2				Calf/cow
ONT:H19	3 (1)	Yes	−	+	−	+		2		1	Cow
ONT:H21	1 (1)	No	−	+	−	+				1	Unknown
ONT:H38	3 (2)	No	−	+	−	−	3				Calf
ONT:H41	1 (1)	No	+	−	+	−				1	Calf
ONT:H49	1 (1)	No	−	+	+	+				1	Unknown
ONT:HR	1 (1)	No	−	+	−	−			1		Calf
OR:H−	3 (2)	Yes	−	+	+	+	2		1		Calf
	5 (2)		+	−	+	+	3			2	Calf
OR:HNT	1 (1)	No	−	+	−	+		1			Unknown
OR:H21	1 (1)	Yes	+	−	−	−	1				Unknown

[a] Cattle of unknown age.

genes. One fecal sample yielded three isolates with different virulence gene profiles, and three samples possessed two isolates with two virulence gene profiles. Thirty-three (59.3%) of the BVCC-agar-positive samples yielded STEC (including two samples with two different STEC virulence factor profiles), and 26 (46.7%) yielded EPEC, including two samples that also yielded STEC and one sample that yielded two EPEC with different virulence gene profiles. Two samples yielded *E. coli* containing *ehxA*. Three O157:H− isolates were recovered using this approach. STEC with serotypes O8:H19, O53:H41, O113:H21, O130:H11, O174:H8, Ont:H−, Ont:H11, Ont:H19, and Ont:Hnt possessed the most common virulence gene pro-

file of *ehxA* with stx_2, which was isolated on 15 (25.4%) occasions. STEC cells that possessed *ehxA* with stx_1 and *eae* was recovered on eight occasions (13.6%) and belonged to serotypes O5:H−, O26:H−, O26:H11, O74:H28, O84:H−, O123:H11, Ont:H−, and OR:H− (Table 2). Seven STEC serotypes were detected only by direct culturing on BVCC agar [O2 (rel):Hnt, O15 (rel):H−, O53:H41, O88:H−, O159:H21, O163:H−, and OR:Hnt].

Virulence genes and serotypes in STEC and EPEC from EC (modified) broths and culturing on SMAC agar after enrichment in EC (modified) broth. Virulence factors were detected in 32 *E. coli* isolates cultured from 28 samples. Four samples contained two *E. coli* isolates with different virulence factor profiles. stx_2 alone was the most common STEC virulence gene profile (from six [18.8%] samples) and belonged to serotypes O7:H21, Ont:H−, Ont:H2, and Ont:HR. STEC cells with stx_2 and *ehxA* were detected on four (12.5%) occasions and belonged to serotypes O8:H19, O174:H8, and Ont:H11. Three isolates belonging to the O157 serogroup (two O157:H− isolates and one O157:H7 isolate) were recovered. Interestingly, three O5:H− isolates with *ehxA*, *eae*, and stx_1 were recovered; this important serotype has not previously been reported to be nonsorbitol fermenting. Four serotypes (O7:H21, O75:H32, O177:H−, and Ont:HR) were detected only on SMAC agar after enrichment in EC (modified) broth (Table 3). A total of 15 serotypes were shown to possess non-sorbitol-fermenting colonies (Table 3). EPEC cells were cultured from nine samples, of which seven contained *ehxA* and *eae*; two contained *eae* only (Table 2). Non-sorbitol-fermenting EPEC cells with *ehxA* and *eae* belonged to serotypes O41:H2, Ont:H7, Ont:H25, and OR:H−. EC (modified) enrichment on BVCC agar. Sixty-eight *E. coli* isolates (from 63 samples) contained virulence genes. Five samples yielded *E. coli* with two different virulence gene profiles. The most common STEC virulence gene profile was stx_2 with *ehxA*, which was detected in 25 samples (39.7%). Serotypes that possessed stx_2 with *ehxA* included O8:H19, O81:H−, O113:H21, O130:H11, O174:H8, O179:H8, O181:H49, Ont:H−, Ont:H2, Ont:H11, Ont:H19, and Ont:H21. Thirteen serotypes were detected only after enrichment followed by culturing on BVCC agar (O22rel:H29, O74:H28, O81:H−, O84:H−, O178:H19, O179:H8, O181:H49, Ont:H41, and Ont:HR). The most common EPEC profile was *ehxA* with *eae*, which was detected on 16 (25.4%) occasions (Table 2). Serotypes that possessed *eae* with *ehxA* included O98:H−, O111:H−, O111:H11, and O177:H11.

Isolation of *E. coli* O111 and O157 from O111 and/or O157 PCR-positive fecal samples. Eleven of the 14 fecal samples that were positive in an O111 *rfb* PCR yielded *E. coli* isolates with the O111 serogroup. These consisted of nine fecal samples that contained O111:H− isolates and three isolates that yielded O111:H11. One sample yielded both serotypes. All isolates were cultured on BVCC agar (from EC [modified] enrichment broths and via direct culture). One fecal sample produced an O111:H11 isolate that was negative with the O111 *rfb* PCR. Three of seven fecal samples positive with the O157 *rfb* PCR yielded *E. coli* isolates belonging to the O157 serogroup. One of these possessed an O157:H7 serotype, and the other two possessed an O157:H− serotype.

Identification of STEC and EPEC serotypes. All STEC and EPEC isolates were identified as *E. coli* by using standard biochemical assays (1). Of the 47 STEC serotypes identified, the most common belonged to serotypes Ont:H− (11 isolates from nine animals), O113:H21 (9 isolates from seven animals), O130:H11 (7 isolates from five animals), O8:H19 (11 isolates from four animals), O5:H− (7 isolates from four animals), O26:H−/H11 (7 isolates from four animals), Ont:H11 (8 isolates from four animals), and OR:H− (8 isolates from four animals). Two fecal samples yielded O157:H− (five isolates), and one sample yielded O157:H7 (two isolates) A description of all STEC serotypes, their virulence gene profiles, and their method of isolation is shown in Table 3. STEC cells isolated during this study with serotypes O5:H−, O26:H−, O26:H11, O113:H21, O146:H21, O157:H−, and O157:H7 have been recovered from patients with HUS, hemorrhagic colitis, and bloody diarrhea. Of the recently described *E. coli* serogroups O174-O181, we identified STEC with serotypes O174:H8, O175:H−, O177:H−/H32, O178:H19, O179:H8 and O181:H49. One of these (O179:H8) has previously been recovered from patients with bloody diarrhea. Twenty-three STEC serotypes are reported here for the first time (Table 3). Fifteen EPEC serotypes were identified, and the majority of these were isolated from calves. The vast majority of *eae*-positive isolates also possessed enterohemolysin, suggesting that many of these may represent atypical EPEC. The most common EPEC serotypes were O111:H− (18 isolates from nine animals) and O177:H11 (13 isolates from six animals). The isolation of eight O111:H11 isolates from three animals was also significant.

There were six fecal samples which yielded *E. coli* containing *ehxA* only, and these possessed serotypes O69:H32, O76.H−, O84:H38, O149:H−, O149:H7, and O168:H8.

DISCUSSION

Ruminants, particularly cattle and sheep, are regarded as the major reservoirs of STEC that contaminate food for human consumption. However, studies of STEC isolated from the feces of healthy, slaughter-age cattle and sheep show that most STEC strains do not possess the *eae* gene (3, 7, 12, 20, 29, 40, 46). The high prevalence of serologically diverse STEC cells containing *eae* (35 [29.2%] of 120 isolates) (Tables 2 and 3) was a feature of the current study. This observation reinforces findings from a previous preliminary Australian study of 123 bovine diagnostic fecal samples of which 23 yielded STEC cells containing *ehxA* and/or *eae*. Of these 23, 11 (47.8%) also possessed *eae* with stx_1, *eae*, and *ehxA*, representing the most common virulence gene profile (22). The high prevalence of *eae*-positive STEC cells in diagnostic bovine fecal samples is in marked contrast to the virulence factor profiles of STEC cells from healthy Australian cattle in a snapshot study where 37 of 1,692 (2.2%) isolates were colonized with STEC isolates containing *ehxA* and/or *eae* but only nine (0.5%) of these carried *eae* (20).

Many of the STEC isolates identified in our study (O5:H−, O26:H−/H11, O113:H21, O157:H−/H7) represent serotypes that are commonly recovered from humans with serious disease (http:/www.lugo.usc/ecoli, http://www.microbionet.com.au/vtectable.htm). Collectively, our data indicate that diagnostic bovine feces are a source of STEC with serotypes and virulence gene attributes that show many similarities to those identified

TABLE 4. Virulence gene profiles of EPEC serotypes isolated from diagnostic fecal samples

Serotype	No. of isolates (No. of cattle colonized)	Virulence factor profile		Culture method (no. of isolates)				Calf/cow/unknown[a]
				Direct		Enrichment		
		eae	*ehxA*	SMAC agar	BVCC agar	SMAC agar	BVCC agar	
O2:H−	1 (1)	+	+		1			Cow
O28:H−	1 (1)	+	+		1			Cow
O41:H2	1 (1)	+	−			1		Cow
O98:H−	3 (2)	+	+	1	1		1	Calf/unknown
O103:H2	1 (1)	+	+		1			Calf
O111:H−	18 (9)	+	+		11		7	Calf
O111:H11	8 (3)	+	+		5		3	Calf
O118:H−	1 (1)	+	−	1				Calf
O177:H11	13 (6)	+	+		8		5	Calf/cow
ONT:H2	1 (1)	+	+		1			Calf
ONT:H7	1 (1)	+	−			1		Calf
ONT:H11	2 (2)	+	+		2			Calf
ONT:H25	3 (3)	+	+		2	1		Calf
OR:H−	4 (2)	+	+	3		1		Calf
OR:H28	1 (1)	+	+		1			Calf

[a] Cattle of unknown age.

in EHEC from human patients with a range of gastrointestinal and other serious diseases.

Studies of STEC cells recovered from healthy calves and diarrheagenic calves and adult cattle, both within Australia and overseas, also show that a significant number possess *eae* (11, 19, 23, 28, 33, 48). STEC isolated during longitudinal studies of Australian dairy cattle (11) showed that (i) significantly more STEC isolates possessed stx_2, (ii) approximately 30% of STEC isolates possessed *eae*, particularly isolates from younger animals, (iii) *eae* and *ehxA* occurred more frequently together than individually, especially in younger animals, and (iv) weaning calves and preweaned calves possessed STEC with stx_1 predominantly. However, most *eae*-positive STEC strains isolated from healthy and diarrheagenic cattle in different regions of the world possess stx_1 (28, 31, 33, 34, 48) and our finding that 55.9% of *eae*-positive STEC isolates possess stx_1 is in keeping with these observations.

A serologically diverse collection (15 serotypes) of *eae*-positive, *stx*-deficient *E. coli* strains was isolated in this study. The majority of these were isolated from the feces of calves, were enterohemolytic on BVCC agar, and possessed *ehxA* (Table 4). The most prevalent serotypes were O111:H− (18 isolates from nine animals) followed by serotype O177:H11 (13 isolates from six animals). Although we have not determined adherence phenotype(s) or looked for the presence of the EAF plasmid for each of these *eae*-positive *stx*-deficient *E. coli*, these isolates are likely to represent atypical EPEC cells because they (i) do not possess serotypes representative of typical EPEC, (ii) were recovered from an animal reservoir (typical EPEC cells do not have an animal reservoir), and (iii) possess *ehxA*, a marker for the EHEC plasmid (9, 45). *eae*-positive, *stx*-deficient *E. coli* strains recently isolated from healthy cattle in Japan do not possess the *bfp* locus, possess the *ehxA* gene, and belong to a wide range of serotypes (28). Although a role for *eae*-positive *E. coli* as the causes of diarrhea and other gastrointestinal afflictions in humans is gaining momentum (see reference 45 and references therein), it is clear that healthy young cattle and diarrheagenic calves and cattle represent an important source of these potential pathogens.

Despite O111:H− STEC being the most common cause of HUS in Australia, reports of its isolation from Australian cattle are extremely rare (15). Extensive epidemiological investigations of STEC in the feces of healthy cattle and sheep in Australia failed to identify O111 STEC (11, 12, 13, 20). The only report of the isolation of the O111 serogroup to date has been from bovine diagnostic fecal samples from a 6-year-old cow with profuse watery diarrhea and a calf with a history of ill thrift and diarrhea (21). All 18 O111:H− isolates (from nine calves) and all eight O111:H11 isolates (from three calves) from our current study possessed *eae* with *ehxA* but did not contain *stx* genes. It is possible that the source of the O111 STEC serogroup that infects humans in Australia is sick cattle and that phage-borne genes transfer into these EPEC cells, converting them to STEC. O111 STEC cells that are shed by sick cattle may colonize healthy cattle that are sent to slaughter for human consumption. Alternatively, healthy cattle may carry O111 EPEC with the same outcome.

Although we suspect that healthy calves and diarrheagenic cattle and calves represent true reservoirs of O111 STEC in Australia, evidence for this remains limited (21). However, studies of diarrheagenic calves in Germany (48), Scotland (44), the United States (19, 23), and Canada (42) have all reported the isolation of O111 STEC (mostly with serotype O111:H−). Attempts to detect O111 STEC in the longitudinal studies of a cohort of beef calves and their dams in Scotland using a combination of immunomagnetic capture coupled with PCR and DNA hybridization assays failed (38), suggesting that O111 STEC cells are uncommon or shed sporadically. However, it is clear that diagnostic bovine feces represent a reservoir for O111 *E. coli* that possess *eae* and *ehxA*. Further work is required to examine if healthy slaughter-age cattle commonly carry these O111 EPEC cells.

O111 and O157 STEC cells are uncommonly isolated from the feces of Australian cattle compared to other STEC sero-

types. To increase the opportunity of isolating these serogroups, we screened all EC (modified) broths with a multiplex PCR specific for the *rfb* (O-antigen encoding) regions of both these serotypes. Furthermore, SMAC agar was also used to increase the chances of isolating non-sorbitol-fermenting STEC such as the O157 serogroup. Although O111 *E. coli* was isolated from 10 of 14 samples that were O111 PCR positive, none of these possessed *stx* genes. Three of the seven samples that were positive for the O157 *rfb* gene yielded O157. O157:H7 STEC was isolated from the feces of only 1 of 1,692 healthy cattle, and O157:H− was isolated from one feedlot animal in longitudinal studies of feedlot and pasture beef cattle (20). Furthermore, O157 STEC cells were rarely detected (1 in 505 fecal samples) in dairy cattle presented for slaughter in Victoria, Australia (18). These studies reinforce the findings that O157 and O111 STEC cells are not common inhabitants of the intestinal flora of most healthy, slaughter-age Australian cattle. However, O157:H7 STEC cells have been recovered with higher frequency from calves and weanlings during longitudinal studies of Australian dairy cattle (11).

In this study, 47 STEC serotypes were detected, including 23 that have not previously been reported (http://www.lugo.usc.es/ecoli). Of the 24 serotypes previously reported, most had been isolated from only cattle. These data further support observations that there is a predilection of STEC serotypes for specific animal species (12, 13, 20). Of the serotypes most commonly associated with sheep in Australia (1, 12, 13), such as O5:H−, O75:H8, O91:H−, O123:H−, and O128:H2, only O5:H− was isolated in this study.

In our study, 96 of 163 (58.9%) *E. coli* isolates containing *ehxA* also possessed at least one *stx* gene, suggesting that *ehxA* is often associated with STEC (Table 2). Although Beutin et al. (6) first reported the close association between enterohemolysin and Shiga toxin production and used washed sheep blood to identify such isolates, Lehmacher et al. (30) developed this medium further by adding vancomycin, cefixime, and cefsulodin, which has the added advantages of inhibiting the growth of non-*E. coli* bacteria and enhancing the hemolytic effect. Of the 120 STEC isolates recovered in our study, 81 (67.5%) were derived from BVCC agar and 17 of 23 (73.9%) serotypes that have not previously been reported were isolated on BVCC agar. We have previously shown that BVCC agar can be successfully used to isolate serologically diverse STEC from the feces of cattle and sheep (12, 13, 20, 22). However, our study shows that BVCC agar is also very effective for the detection of EPEC. A total of 41 of 43 (95.3%) EPEC isolates cultured on BVCC agar also contained *ehxA*, indicating a stronger association between *ehxA* and *eae* without *stx* than between *ehxA* with *stx* (Table 2).

The development of molecular biological techniques as well as immunologically based methods for rapidly diagnosing STEC in humans, food, and the environment has shown that non-O157:H7 serotypes are responsible for severe infections in humans (4, 7, 14, 25, 35). To date, 435 EHEC serotypes have been reported to cause disease in humans (4; http:/www.lugo.usc/ecoli, www.microbionet.com.au/vtectable.htm) and it is increasingly recognized that non-O157 STEC cells also play an important role in the less severe gastrointestinal complications such as diarrhea and bloody diarrhea (4). Most importantly, 31 previously unreported STEC serotypes were identified in that study. Non-O157 STEC strains are considered to be of greater clinical significance as causes of human disease than O157 STEC strains in Spain, Germany, France, Switzerland, Denmark, Belgium, Italy, Argentina, Australia, South Africa, and Chile but not in Canada, the United States, Japan, England, or Scotland, although the reported prevalence rates for non-O157 STEC cells continue to increase with the application of molecular and immunological screening methodologies (25, 35; see also reference 7 and references therein). Despite introducing nine new antisera against serogroups O174 through O182, we were unable to determine the serogroup of numerous STEC isolates with various flagella types, including H2, H11, H16, H19, H21, H38, H41, and H49 (including several OR types), and *eae*-positive, *stx*-deficient *E. coli* (flagella types H2, H4, H7, H11, and H25), suggesting that more STEC and EPEC serogroups remain to be identified.

The range of serotypes and number of samples containing STEC and EPEC cells were increased when both direct culture and enrichment in EC (modified) broth were used on diagnostic bovine fecal samples. Fifty-nine samples (30.9%) were positive for one or more of the four *E. coli* virulence factors by using direct culturing on BVCC agar, and 63 samples (33.0%) were positive after enrichment in EC (modified) broth and culturing on BVCC agar. Seven STEC serotypes were detected only by direct culturing on BVCC agar, and five of these had not previously been reported. Thirteen serotypes were detected only after enrichment followed by culturing on BVCC agar, of which six had not previously been reported. Similarly, direct culturing on SMAC detected seven serotypes which were not detected elsewhere and four serotypes were detected only on SMAC agar after enrichment in EC (modified) broth (Table 3). The use of a dual-culture approach and the use of SMAC agar as well as BVCC agar were also useful in detecting multiple serotypes in single samples. Thirteen samples yielded two STEC serotypes, one sample yielded three STEC serotypes, and two samples yielded three STEC and one EPEC serotypes. This type of approach may be useful in determining the diversity of STEC and EPEC in humans and healthy animals. However, the identification of multiple STEC serotypes in clinical specimens may raise questions as to the identity of the real etiological agent in EHEC infections.

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