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Serotypes, Virulence Genes, and Intimin Types of Shiga Toxin (Verotoxin)-Producing *Escherichia coli* Isolates from Healthy Sheep in Spain

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Fecal swabs obtained from 1,300 healthy lambs in 93 flocks in Spain in 1997 were examined for Shiga toxin-producing *Escherichia coli* (STEC). STEC O157:H7 strains were isolated from 5 (0.4%) animals in 4 flocks, and non-O157 STEC strains were isolated from 462 (36%) lambs in 63 flocks. A total of 384 ovine STEC strains were characterized in this study. PCR showed that 213 (55%) strains carried the *stx*$_1$ gene, 10 (3%) possessed the *stx*$_2$ gene, and 161 (42%) carried both the *stx*$_1$ and the *stx*$_2$ genes. Enterohemolysin (*ehxA*) and intimin (*eae*) virulence genes were detected in 106 (28%) and 23 (6%) of the STEC strains, respectively. The STEC strains belonged to 35 O serogroups and 64 O:H serotypes (including 18 new serotypes). However, 72% were of 1 of the following 12 serotypes: O5:H−, O6:H10, O91:H−, O117:H−, O128:H−, O128:H2, O136:H20, O146:H8, O146:H21, O156:H−, O166:H28, and ONT:H21 (where NT is nontypeable). Although the 384 STEC strains belonged to 95 different seropathotypes (associations between serotypes and virulence genes), 49% of strains belonged to only 11. O91:H− *stx*$_1$ *stx*$_2$ (54 strains) was the most common seropathotype, followed by O128:H− *stx*$_1$ *stx*$_2$ (33 strains) and O6:H10 *stx*$_1$ (25 strains). Three strains of serotypes O26:H11, O156:H11, and OX177:H11 had intimin type β1; 5 strains of serotype O157:H7 possessed intimin type γ1; and 15 strains of serotypes O49:H−, O52:H12, O156:H− (12 strains), and O156:H25 had the new intimin, intimin type ζ. The majority (82%) of ovine STEC strains belonged to serotypes previously found to be associated with human STEC strains, and 51% belonged to serotypes associated with STEC strains isolated from patients with hemolytic-uremic syndrome. Thus, this study confirms that healthy sheep are a major reservoir of STEC strains pathogenic for humans.

Shiga toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), has emerged as a pathogen that can cause food poisoning and severe and potentially fatal illnesses. It is a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic-uremic syndrome (HUS), which is the main cause of acute renal failure in children. Since its identification as a pathogen in 1982, STEC O157:H7 has been the cause of a series of outbreaks, especially in Canada, Japan, the United Kingdom, and the United States (19, 23, 27). Domestic ruminants, especially cattle, sheep, and goats, have been implicated as the principal reservoir of STEC strains that cause human infections, although other domestic animals, including pigs, poultry, cats, and dogs, can also harbor these bacteria. Transmission occurs through consumption of undercooked meat, unpasteurized dairy products, and vegetables or water contaminated by the feces of carriers, because STEC strains are found as part of the normal intestinal flora of the animals. Person-to-person transmission has also been documented (6, 9, 11, 12, 34).

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STEC strains elaborate two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or verotoxins (VT1 and VT2) (19, 27). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin, which is responsible for the intimate attachment of STEC to intestinal epithelial cells, causing attaching-and-effacing lesions in the intestinal mucosa (17). Intimin is encoded by the chromosomal gene *eae*, which is part of a pathogenicity island termed the locus for enterocyte effacement. Intimin type-specific PCR assays identified eight variants of the *eae* gene that encode eight different intimin types (types α, β1, β2, γ1, γ2, δ, ε, and ζ) (1, 23, 24, 30, 31, 33). Severe diarrhea (especially HC) and HUS are closely associated with STEC types carrying the *eae* gene for intimin. A factor that may also affect the virulence of STEC strains is the enterohemolysin (Ehly), also called enterohemorrhagic *E. coli* hemolysin (EHEC-HlyA), which is encoded by the *ehxA* gene (28).

STEC strains that cause human infections belong to a large number of O:H serotypes (a total of 435 serotypes are listed on the authors' website [http://www.lugo.usc/ecoli]; a review of the world literature by K. A. Bettelheim recording well over 1,000 reports of isolation of non-O157 STEC strains is also available [http://www.sciencenet.com.au]) (6, 23). Most outbreaks and sporadic cases of HC and HUS have been attributed to strains of enterohemorrhagic serotype O157:H7 (6, 10, 19, 23). How-

ever, as STEC non-O157 strains are more prevalent in animals and as contaminants in foods, humans are probably more exposed to these strains. Infections with some non-O157 STEC types, such as O26:H11 or O26:H−, O91:H21 or O91:H−, O103:H2, O111:H−, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2 or O128:H−, O145:H28 or O145:H−, and O146:H21, are frequently associated with severe illness in humans, but the roles of other non-O157 STEC types in human disease require further examination (4, 6, 10, 23).

Recently, STEC O157:H7 strains have been detected in sheep and goat feces or at slaughter, showing that small ruminants may also represent a source of contamination for humans. Transmission of STEC O157:H7 and other STEC serotypes to humans by raw goat milk or homemade cheese made from raw milk has been demonstrated (5, 6). However, small ruminants have been subjected to fewer surveys compared with the number of surveys done with cattle. The majority of existing studies have been performed with comparatively small numbers of sheep, have focused intensively on a single flock, or have examined small ruminants only for the presence of serotype O157:H7 (11, 12, 14, 16, 18, 32). Thus, the aim of this study was to establish the serotypes and the virulence genes of STEC strains isolated from sheep in Spain to determine whether sheep represent a source of STEC strains pathogenic for humans.

MATERIALS AND METHODS

Specimen collection and *E. coli* strains. A total of 1,300 healthy lambs from 93 flocks in Extremadura, Spain, were sampled between June and October 1997. A single fecal swab was obtained from each lamb up to 2 month of age. The swabs were placed in transport medium and taken to a laboratory for immediate processing. They were plated on MacConkey agar and on cefixime-tellurite-sorbitol MacConkey medium, and 10 suspect *E. coli* colonies (lactose positive, lactose negative, or sorbitol negative) were chosen from each sample, identified, and examined for Shiga toxin (verotoxin) production. Identification of *E. coli* was based on standard biochemical tests. Only one colony from animals for which all original isolates were identical with respect to the toxic genotype and serotype was selected as a test strain. When one lamb yielded colonies with different seropathotypes, one colony of each seropathotype was selected. Reference *E. coli* strains used as controls were 933 (O157:H7 stx_1 stx_2 *eae*-γ*1 ehxA*), EPEC-8 (O55:H6 *eae*-α), EPEC-4 (O26:H− *eae*-β*1*), EPEC-359 (O119:H6 *eae*-β2), VTB-308 (O111:H− stx_1 *eae*-γ2), EPEC-9 (O86:H34 *eae*-δ), VTB-286 (O103:H2 stx_1 *eae*-ε), VTO-50 (O156:H− stx_1 *eae*-ζ), and K-12 185 (negative for the stx_1, stx_2, *eae*, and *ehxA* genes). The strains were stored at room temperature in nutrient broth with 0.75% agar.

Production and detection of Shiga toxins (verotoxins) in Vero and HeLa cells. For production of Shiga toxins, one loopful of each isolated colony was inoculated in 50-ml Erlenmeyer flasks containing 5 ml of tryptone soy broth (pH 7.5) with mitomycin C and incubated for 20 h at 37°C (shaken at 200 rpm) and then centrifuged (6,000 × *g*) for 30 min at 4°C. The Vero and HeLa cell culture assays were performed with nearly confluent cell monolayers grown in plates with 24 wells. At the time of assay, the growth medium (RPMI with polymyxin B sulfate) was changed (0.5 ml per well), and 75 μl of undiluted culture supernatant was added. The cells were incubated at 37°C in a 5% CO_2 atmosphere, and after 24 and 48 h of incubation the morphological changes in the cells were observed with a phase-contrast inverted microscope (7, 8).

Detection of virulence genes by PCR. Bacteria were harvested from tryptone soy agar, suspended in 250 μl of sterile water, incubated at 100°C for 5 min to release the DNA, and centrifuged. The supernatant was used in the PCR as described below. The base sequences and the predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. We designed the majority of oligonucleotide primers according to the nucleotide sequences of the virulence genes. Multiplex PCR was used only for detection of the stx_1 and stx_2 genes. The DNA of isolates positive for the *eae* gene with primers EAE-1 and EAE-2 was later analyzed with all different variant primers. Amplification of bacterial DNA was performed by using 30-μl volumes containing 7 μl of the prepared sample supernatant; the oligonucleotide primers (see Table 1); 0.2 mM (each) dATP, dGTP, dCTP, and dTTP; 10 mM Tris-HCl (pH 8.8); 1.5 mM $MgCl_2$; 50 mM KCl, and 1 U of Biotaq DNA polymerase (Bioline, London, United Kingdom). The conditions for the PCR, which was performed with a thermal cycler (model PCR express; Hybaid, Teddington, United Kingdom), were 94°C for 2 min for initial denaturation of the DNA within the sample, followed by 35 cycles of 94°C for 1 min (denaturation), 55 to 63°C (Table 1) for 1 min (primer annealing), and 72°C for 1 min (DNA synthesis). The amplified products were visualized by standard submarine gel electrophoresis by using 10 μl of the final reaction mixture on a 2% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The samples were electrophoresed for 20 to 40 min at 130 V. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. Molecular size markers (*Hae*III digest of ϕX174 DNA; Promega, Madison, Wis.) were included in each gel.

Serotyping. The determination of the O and H antigens was carried out by the method described by Guinée et al. (15) with all available O (O1 to O181) and H (H1 to H56) antisera. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O antisera were produced in the Laboratorio de Referencia de *E. coli* (Lugo, Spain [http://www.lugo.usc.es/ecoli]), and the H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark).

RESULTS

Prevalence of VTEC in healthy sheep. Fecal swabs obtained from 1,300 healthy lambs in 93 flocks in Spain in 1997 were examined for STEC strains. We found STEC strains in 63 (68%) of the flocks. STEC O157:H7 strains were isolated from 5 (0.4%) animals in 4 flocks, and non-O157 STEC strains were isolated from 462 (36%) lambs in 63 flocks.

Virulence genes. A total of 384 ovine STEC strains were characterized in this study. PCR showed that 213 (55%) strains carried the stx_1 gene, 10 (3%) possessed the stx_2 gene, and 161 (42%) carried both the stx_1 and stx_2 genes. The *ehxA* and *eae* virulence genes were detected in 106 (28%) and 23 (6%) of the STEC strains, respectively.

Serotypes and seropathotypes. The STEC strains belonged to 35 O serogroups and 64 O:H serotypes. However, 76% of the strains were of 1 of 10 serogroups (O5, O6, O91, O117, O128, O136, O146, O156, O166, and O176), and 72% belonged to only 12 serotypes (O5:H−, O6:H10, O91:H−, O117:H−, O128:H−, O128:H2, O136:H20, O146:H8, O146:H21, O156:H−, O166:H28, and ONT:H21 [where NT is nontypeable]). A total of 18 new O:H serotypes not previously reported for STEC strains were found in this study. The majority (82%) of ovine STEC strains belonged to serotypes previously found to be associated with human STEC strains, and 51% belonged to serotypes previously associated with STEC strains isolated from patients with HUS (Table 2).

Although the 384 STEC strains belonged to 95 seropathotypes (associations between serotypes and virulence genes), 49% of strains belonged to only 11. O91:H− stx_1 stx_2 (54 strains) was the most common seropathotype, followed by O128:H− stx_1 stx_2 (33 strains) and O6:H10 stx_1 (25 strains) (Table 2).

Typing of *eae* (intimin) genes. Three strains of serotypes O26:H11, O156:H11, and OX177:H11, respectively, had intimin type β1; 5 strains of serotype O157:H7 possessed intimin type γ1; and 15 strains of serotypes O49:H− (1 strain), O52:H12 (1 strain), O156:H− (12 strains), and O156:H25 (1 strain) had the new intimin, intimin type ζ.

TABLE 1. Primer sequences and predicted lengths of PCR amplification products

Gene	Primer	Oligonucleotide sequence (5′-3′)	Fragment size (bp)	PCR conditions[a]	Primer coordinates	GenBank accession no.[b]	Reference or source
stx$_1$	VT1-A VT1-B	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	90 ng, 55°C	113–134 394–414	M17358	This study
stx$_2$	VT2-A VT2-B	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	60 ng, 55°C	50–69 543–565	M59432	This study
ehxA	HlyA1 HlyA4	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1.551	90 ng, 60°C	238–259 1767–1788	X79839	28
eae	EAE-1 EAE-2	GAGAATGAAATAGAAGTCGT GCGGTATCTTTCGCGTAATCGCC	775	150 ng, 55°C	1441–1460 2193–2215	M58154	This study
eae-α	EAE-F EAE-A	ATTACTGAGATTAAGGCTGAT CACTCTTCGCATCTTGAGCT	751	150 ng, 58°C	1978–1998 2709–2728	AF022236	This study
eae-β*1*	EA-B1-F EAE-B1	CGCCACTTAATGCCAGCG GCAGCACCCCATGTTGAAT	724	300 ng, 60°C	1928–1945 2633–2651	U38618	This study
eae-β*2*	EA-B2-F EAE-B2	CCCGCCACTTAATCGCACGT GCAGCACCCCATGTTGAGATA	720	300 ng, 60°C	1929–1948 2628–2648	AF043226	This study
eae-γ*1*	EAE-F EAE-C1	ATTACTGAGATTAAGGCTGAT CTCCAGAACGCTGCTCACT	739	150 ng, 58°C	1978–1998 2698–2716	S90827	This study
eae-γ*2*	EAE-F EAE-C2	ATTACTGAGATTAAGGCTGAT CTGATATTTTATCAGCTTCA	739	150 ng, 58°C	1978–1998 2697–2716	AF025311	This study
eae-δ	EAE-F EAE-D	ATTACTGAGATTAAGGCTGAT CTTGATACACCCGATGGTAAC	764	150 ng, 58°C	1978–1998 2721–2741	U66102	This study
eae-ε	EAE-F LP5	ATTACTGAGATTAAGGCTGAT AGCTCACTCGTAGATGACGGCAAGCG	653	150 ng, 60°C	1978–1998 2605–2630	AF116899	This study
eae-ζ	Z1 Z2	GAAGGCAAATGGATCTGA ATAGCAAGTGGGGTGAAG	258	150 ng, 63°C	2010–2027 2250–2267	AF449417	This study

[a] Amount of oligonucleotide primers, annealing temperature.
[b] The majority of oligonucleotide primers were designed by us according to the nucleotide sequences of the *eae* genes.

DISCUSSION

We isolated STEC O157:H7 strains from 0.4% of the swab samples from the animals tested in Spain in 1997, whereas we isolated non-O157 VTEC strains from 36% of the samples from lambs. The prevalence rate of STEC O157:H7 in that study was probably underestimated, as immunomagnetic separation was not used. In a subsequent study conducted in Spain in 2000 and 2001, we used immunomagnetic separation, and STEC O157:H7 strains were isolated from 1% of the lambs (unpublished data). The prevalence rates of non-O157 STEC strains were higher in sheep (67%) and goats (56%) than in cattle (21%) in Germany. Among the flocks, the percentage of positive animals varied greatly, from 7 to 84% of the animals in goat flocks and from 55 to 95% of the animals in sheep flocks (2). In Australia, STEC strains were identified in 40% of goats and 56 to 73% of sheep examined in three studies (13, 14, 29). In the United States, Kudva et al. (20, 21) detected STEC strains in 43% of sheep. Four groups have surveyed sheep populations using methods sensitive for STEC O157:H7 strains (11, 16, 18, 20, 21). In the United Kingdom, a survey of 1,000 sheep at a slaughterhouse found the agent in the feces of 22 (2%) animals, a rate lower than that obtained for cattle (16%) at the same site (11). Similarly, in The Netherlands, STEC O157:H7 strains were isolated from 2 (4%) of 52 ewes, 2 (4%) of 49 lambs, 57 (11%) of 540 adult cattle, and 2 (0.5%) of 397 veal calves (16). In Norway, although a sensitive method was used, no STEC O157:H7 strains were isolated from 364 sheep (18). In contrast, Kudva et al. (20, 21) detected STEC O157:H7 strains in a large percentage (31%) of samples from sheep in a flock in the United States. One flock was investigated for STEC O157:H7 strains over a 16-month period, and a variation in occurrence was observed (0 to 31%), with animals being culture positive only in the summer months and not in the spring, autumn, or winter months.

Sheep harbor many serotypes of STEC in their gastrointestinal tracts. Ovine STEC strains belonging to a total of 53 O serogroups and 105 O:H serotypes have been isolated in studies that we have reviewed (6, 23). The complete list of 105 O:H serotypes is available from the authors' website (http://www.lugo.usc/ecoli). The present study is the first, to our knowledge, that documents the verotoxigenicities of *E. coli* strains of 18 new O:H serotypes (Table 2). Only a small number of serotypes (O5:H−, O91:H−, O128:H2, O146:H8, and O146:H21) have been the most frequently and the most commonly found in the sheep populations of different countries (2, 13, 20, 21). Numerous ovine STEC serotypes were associated with

TABLE 2. Seropathotypes (serotypes and virulence genes) of ovine STEC strains[d]

Serotype	Total no. of strains tested	*stx*$_1$	*stx*$_2$	*eae*	*ehxA*	Serotype	Total no. of strains tested	*stx*$_1$	*stx*$_2$	*eae*	*ehxA*
O4:H−[b]	1	+	+	−	+	O126:H8[a]	1	+	+	−	+
O4:H−[b]	1	+	−	−	+	O128:H−[b]	1	+	+	−	+
O4:H10[a]	1	+	−	−	−	O128:H−[b]	33	+	+	−	−
O5:H−[b]	10	+	+	−	+	O128:H−[b]	1	+	−	−	+
O5:H−[b]	8	+	−	−	+	O128:H−[b]	11	+	−	−	−
O5:H−[b]	1	+	−	−	−	O128:H2[b]	3	+	+	−	+
O5:H28[c]	1	+	−	−	+	O128:H2[b]	7	+	+	−	−
O6:H−[b]	1	+	−	−	+	O128:H2[b]	4	+	−	−	−
O6:H−[b]	2	+	−	−	−	O128:H8[a]	1	+	+	−	−
O6:H2[b]	1	+	−	−	+	O128:H10[a]	1	+	+	−	−
O6:H10	25	+	−	−	−	O128:H21[c]	1	+	+	−	+
O6:H12[a]	1	+	−	−	+	O136:H−	1	+	−	−	−
O6:H28[a]	1	+	−	−	−	O136:H20	11	+	−	−	−
O8:H9[a]	1	−	+	−	−	O146:H−[a]	1	+	+	−	+
O9:H−[b]	1	+	+	−	−	O146:H−[a]	1	+	+	−	−
O15:H−[b]	1	+	−	−	+	O146:H8[b]	14	+	−	−	−
O26:H11[b]	1	+	−	+ (β1)	+	O146:H21[a]	14	+	+	−	+
O35:H7[c]	1	+	−	−	−	O146:H21[a]	3	+	+	−	−
O42:H?[c]	1	+	−	−	−	O146:H21[a]	2	+	−	−	+
O49:H−[b]	1	+	+	+ (ζ)	+	O146:H21[a]	6	+	−	−	−
O52:H12[c]	1	+	−	+ (ζ)	+	O146:H21[a]	2	−	+	−	+
O52:H45[c]	3	+	−	−	−	O156:H−[a]	11	+	−	+ (ζ)	+
O55:H−[b]	1	+	+	−	−	O156:H−[a]	1	+	−	+ (ζ)	−
O55:H−[b]	1	+	−	−	−	O156:H−[a]	1	+	−	−	+
O55:H2[c]	2	+	−	−	−	O156:H11[c]	1	+	−	+(β1)	+
O75:H−[b]	1	+	+	−	−	O156:H25[a]	1	+	−	+ (ζ)	+
O75:H55[c]	1	+	+	−	+	O156:H25[a]	1	+	−	−	−
O79:H14[a]	2	+	−	−	−	O157:H7[b]	5	−	+	+(γ1)	+
O87:H2[c]	1	+	+	−	−	O166:H−[c]	2	+	−	−	+
O91:H−[b]	6	+	+	−	+	O166:H28[a]	5	+	+	−	+
O91:H−[b]	54	+	+	−	−	O166:H28[a]	4	+	−	−	+
O91:H−[b]	3	+	−	−	−	O166:H28[a]	1	+	−	−	−
O91:H−[b]	1	−	+	−	−	O166:H28[a]	1	+	−	−	+
O91:H21[b]	1	+	−	−	−	O174:H21[b]	1	+	+	−	+
O91:H29[c]	1	+	−	−	−	OX176:H−[c]	1	+	−	−	+
O98:H7[c]	1	+	−	−	−	OX176:H4[c]	6	+	−	−	+
O104:H7[a]	9	+	−	−	−	OX176:H4[c]	3	+	−	−	−
O106:H2[c]	1	+	−	−	−	OX177:H11[c]	1	+	+	+(β1)	+
O106:H20[c]	1	+	−	−	−	ONT:H−[b]	3	+	+	−	+
O110:H−[a]	7	+	−	−	−	ONT:H−[b]	1	+	+	−	−
O112:H−[b]	7	+	−	−	−	ONT:H−[b]	1	+	−	−	+
O112:H21[a]	1	+	−	−	−	ONT:H−[b]	4	+	−	−	−
O117:H−[a]	16	+	−	−	−	ONT:H4[a]	2	+	−	−	−
O118:H12[b]	1	+	−	−	−	ONT:H16[b]	1	+	−	−	−
O118:H12[b]	1	−	+	−	−	ONT:H21[a]	1	+	+	−	−
O119:H−[a]	1	+	+	−	−	ONT:H21[a]	16	+	−	−	−
O123:H−[a]	1	+	+	−	+	ONT:H29	1	+	+	−	−
O123:H−[a]	2	+	+	−	−						

[a] Serotypes previously found for human VTEC strains.
[b] Serotypes previously associated with human VTEC strains that caused HUS.
[c] New serotypes not found for VTEC strains in previous studies.
[d] A total of 384 ovine STEC strains were tested.

disease in humans. Thus, 55 (52%) of the 105 ovine serotypes have been also recovered from humans, including 24 serotypes associated with HUS (6, 23). Furthermore, 10 of the 12 most prevalent STEC serotypes in sheep in Spain were also found among STEC strains that cause human infections, including 5 serotypes (O5:H−, O91:H−, O128:H−, O128:H2, and O146:H8) associated with HUS (Table 2). Although our results and those of other authors indicate that STEC strains of human and animal origin with the same serotype are similar regarding the presence of known virulence-associated factors, future studies are necessary to establish if animal and human strains represent the same clones or are only related subpopulations (2, 3, 6, 10, 13, 22). Thus, the results of Boerlin et al. (10) suggest that STEC isolates from humans form a population different from those found in the bovine reservoir or that they are only a subpopulation of the latter.

Examination of non-O157 STEC serotypes from cattle and sheep revealed remarkable differences, which could point out an animal-host serotype specificity for some serotypes. Thus, 8 of the 21 non-O157 STEC serotypes more frequently detected

in ovine strains in Spain (serotypes O52:H45, O91:H−, O104:H7, O110:H−, O112:H−, O123:H−, O128:H2, and O136:H20) are not included among the 323 non-O157 STEC serotypes detected in cattle to date (http://www.lugo.usc/ecoli) (6, 23). More than half (54%) of bovine non-O157 STEC strains have only the stx_2 gene, whereas only 1% of strains of ovine origin have only the stx_2 gene. Furthermore, the *eae* gene was detected in significantly fewer ovine non-O157 STEC (5%) than in bovine (17%) and human strains (45%) (6, 23).

The *eae* gene, which has been shown to be necessary for attaching-and-effacing activity, encodes a 94- to 97-kDa outer membrane protein termed intimin. Analysis of the nucleotide sequences of the intimin genes from different STEC and enteropathogenic *E. coli* strains has shown that a high degree of homology exists in the 5′ two-thirds of the genes and that a significant degree of heterogeneity exists in the 3′ one-third of the genes. Eight variants of the *eae* gene were identified by intimin type-specific PCR assays that used oligonucleotide primers complementary to the 3′ ends of the specific intimin genes that encode intimin types α, β1, β2, γ1, γ2, δ, ε, and ζ (1, 23, 24, 30, 33). In the present study, the new intimin, intimin type ζ, was detected in 15 (65%) of 23 ovine STEC strains which possessed the *eae* gene. In contrast, we found intimin type ζ in only 8 (13%) of 63 human STEC strains with the *eae* gene and 8 (5%) of 149 bovine STEC strains with the *eae* gene. We observed that intimin type ζ is especially associated with serotype O156:H−. Thus, of 31 STEC strains that possessed intimin type ζ, 17 (12 ovine strains, 4 bovine strains, and 1 human strain) belonged to serotype O156:H− (23). As in previous studies, all ovine STEC O157:H7 strains possessed intimin type γ1. Intimin types β1 (mainly associated with serotype O26:H11) and γ1 are the most frequently found in both human and bovine STEC strains.

Numerous investigators have underlined the strong association between carriage of the *eae* gene and the capacity of STEC strains to cause severe human disease, especially HUS. As this important virulence gene is present in only a minimal (5%) proportion of ovine non-O157 STEC strains, some of these ovine strains probably have low levels of virulence for humans. Nevertheless, production of intimin is not essential for pathogenesis, because a number of sporadic cases of HUS have been caused by *eae*-negative non-O157 STEC strains. Thus, STEC O104:H21 and O113:H21 strains lacking the *eae* gene were responsible for an outbreak and a cluster of three HUS cases in the United States and Australia, respectively (25). Furthermore, Paton and Paton (26) recently described a novel megaplasmid-encoded adhesin (Saa) which we have detected in most STEC strains lacking the *eae* gene (unpublished data). This adhesin may be an important virulence factor of *eae*-negative STEC strains capable of causing severe disease in humans.

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