Serotypes, Virulence Genes, and Intimin Types of Shiga Toxin (Verotoxin)-Producing *Escherichia coli* Isolates from Cattle in Spain and Identification of a New Intimin Variant Gene (*eae*-ξ)

M. Blanco,[1] J. E. Blanco,[1] A. Mora,[1] G. Dahbi,[1] M. P. Alonso,[1,2] E. A. González,[1]
M. I. Bernárdez,[1] and J. Blanco[1]*

*Laboratorio de Referencia de E. coli, Departamento de Microbioloxía e Parasitoloxía, Facultade de Veterinaria,
Universidade de Santiago de Compostela, 27002 Lugo,[1] and Unidade de Microbioloxía, Complexo Hospitalario
Xeral-Calde, 27004 Lugo,[2] Spain*

A total of 514 Shiga toxin-producing *Escherichia coli* (STEC) isolates from diarrheic and healthy cattle in Spain were characterized in this study. PCR showed that 101 (20%) isolates carried stx_1 genes, 278 (54%) possessed stx_2 genes, and 135 (26%) possessed both stx_1 and stx_2. Enterohemolysin (*ehxA*) and intimin (*eae*) virulence genes were detected in 326 (63%) and in 151 (29%) of the isolates, respectively. STEC isolates belonged to 66 O serogroups and 113 O:H serotypes (including 23 new serotypes). However, 67% were of one of these 15 serogroups (O2, O4, O8, O20, O22, O26, O77, O91, O105, O113, O116, O157, O171, O174, and OX177) and 52% of the isolates belonged to only 10 serotypes (O4:H4, O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, O171:H2, and ONT:H19). Although the 514 STEC isolates belonged to 164 different seropathotypes (associations between serotypes and virulence genes), only 12 accounted for 43% of isolates. Seropathotype O157:H7 stx_2 *eae*-γ1 *ehxA* (46 isolates) was the most common, followed by O157:H7 stx_1 stx_2 *eae*-γ1 *ehxA* (34 isolates), O113:H21 stx_2 (25 isolates), O22:H8 stx_1 stx_2 *ehxA* (15 isolates), O26:H11 stx_1 *eae*-β1 *ehxA* (14 isolates), and O77:H41 stx_2 *ehxA* (14 isolates). Forty-one (22 of serotype O26:H11) isolates had intimin β1, 82 O157:H7 isolates possessed intimin γ1, three O111:H- isolates had intimin type γ2, one O49:H- strain showed intimin type δ, 13 (six of serotype O103:H2) isolates had intimin type ε and eight (four of serotype O156:H-) isolates had intimin ζ. We have identified a new variant of the *eae* intimin gene designated ξ (xi) in two isolates of serotype O80:H-. The majority (85%) of bovine STEC isolates belonged to serotypes previously found for human STEC organisms and 54% to serotypes associated with STEC organisms isolated from patients with hemolytic uremic syndrome. Thus, this study confirms that cattle are a major reservoir of STEC strains pathogenic for humans.

Shiga toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), strains are the most important recently emerged group of food-borne pathogens. These bacteria can cause severe disease in humans, such as hemorrhagic colitis and hemolytic uremic syndrome (20, 29). Cattle, especially young animals, have been implicated as a principal reservoir of STEC, undercooked ground beef and raw milk being the major vehicles of food-borne outbreaks (2, 5).

Human and bovine STEC strains elaborate two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or verotoxins (VT1 and VT2) (20, 29). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin, which is responsible for intimate attachment of STEC to intestinal epithelial cells, causing attaching and effacing lesions in the intestinal mucosa (16). Intimin is encoded by the chromosomal gene *eae*, which is part of a pathogenicity island termed the locus for enterocyte effacement (19). Severe diarrhea (specially hemorrhagic coli-

tis) and hemolytic uremic syndrome were closely associated with STEC types carrying the *eae* gene for intimin (19, 29).

Differentiation of intimin alleles represents an important tool for STEC typing in routine diagnostics as well as epidemiological and clonal studies. The C-terminal end of intimin is responsible for receptor binding, and it has been suggested that different intimins may be responsible for different host tissue cell tropism (23, 32, 42). Intimin type-specific PCR assays identified 14 variants of the *eae* gene that encode 14 different intimin types and subtypes (α1, α2, β1, β2, γ1, γ2/θ, δ/κ, ε, ζ, η, ι, λ, μ,ν) (1, 6, 10, 18, 26, 36, 37, 42; Blanco et al., submitted for publication). A factor that may also affect the virulence of STEC is the enterohemolysin, also called enterohemorrhagic *E. coli* hemolysin, which is encoded by the *ehxA* gene (35).

STEC strains that cause human infections belong to a large number of O:H serotypes (a total of 472 serotypes are listed at our website, http://www.lugo.usc/ecoli). Most outbreaks of hemorrhagic colitis and hemolytic uremic syndrome have been attributed to strains of the enterohemorrhagic serotype O157:H7 (5, 20). However, 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 H-, O91:H21 or H-, O103:H2, O111:H-, O113:H21, O117:H7, O118:H16, O121:H19, O128:H2 or H-, O145:H28 or H- and

O146:H21 are frequently associated with severe illness in humans, but the role of other non-O157 STEC types in human disease needs further examination (4, 5, 6, 11, 20). Although more than 400 different O:H serotypes of STEC have been isolated from cattle (a total of 435 serotypes are listed at our website, http://www.lugo.usc/ecoli), there is a lack of information regarding associations between serotype, intimin types, and virulence factor profiles among bovine STEC isolates (12, 24, 34, 40).

Thus, the aim of this study was to establish the serotypes, virulence genes, and intimin types of STEC strains isolated from cattle to establish if bovine STEC strains possess the same serotypes and virulence factor profiles as STEC strains that cause human infections.

MATERIALS AND METHODS

E. coli **isolates and control strains.** A total of 514 STEC isolates from diarrheic and healthy cattle in Spain were characterized in this study. Only one isolate for each animal was included. *E. coli* strains used as controls were EPEC-2348 (human, O127:H6, *eae*-α1), AEEC-IH2498a (human, O125:H6, *eae*-α2), REPEC-RDEC-1 (rabbit, O15:H-, *eae*-β1), EPEC-359 (human, O119:H6, *eae*-β2), STEC-EDL933 (human, O157:H7, stx_1 stx_2 *eae*-γ1 *ehxA*), STEC-VTB308 (bovine, O111:H-, stx_1 *eae*-γ2), STEC-TW07926 (human, O111:H8, stx_1 stx_2 *eae*-θ), EPEC-BP12665 (human, O86:H34, *eae*-δ), AEEC-6044/95 (human, O118:H5, *eae*-κ), STEC-VTB-286 (bovine, O103:H2, stx_1 *eae*-ε), STEC-VTO-50 (ovine, O156:H-, stx_1 *eae*-ζ), AEEC-CF11201 (human, O125:H-, *eae*-η), AEEC-7476/96 (human, O145:H4, *eae*-ι), AEEC-68-4 (human, O34:H-, *eae*-λ), EPEC-373 (human, O55:H51, *eae*-μ), AEEC-IH1229a (human, O10:H-, *eae*-ν), and K12-185 (negative for stx_1, stx_2, *eae* and *ehxA*). Strains were stored at room temperature in nutrient broth with 0.75% of 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 sulfate) was changed (0.5 ml per well) and 75 µl of undiluted culture supernatant was added. Cells were incubated at 37°C in a 5% CO$_2$ atmosphere and the morphological changes in cells were observed after 24 and 48 h of incubation with a phase contrast inverted microscope (8, 9).

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. Base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. The majority of oligonucleotide primers were designed by us according to the nucleotide sequences of the virulence genes (Blanco et al., submitted). Multiplex PCR was used only for detection of stx_1 and stx_2 genes. Isolates positive for the *eae* gene with the EAE-1 and EAE-2 primers were afterwards analyzed with all different variant primers.

Amplification of bacterial DNA was performed with 30-µl volumes containing 7 µl of the prepared sample supernatant; 150 ng of the oligonucleotide primers; 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, United Kingdom). The conditions for the PCR were 94°C for 2 min for initial denaturation of DNA within the sample, followed by 35 cycles of 94°C for 1 min (denaturation), 55°C to 66°C (see Table 1) for 1 min (primer annealing), and 72°C for 1 min (DNA synthesis) performed with a thermal cycler (model PCR express; Hybaid, United Kingdom). The amplified products were visualized by standard submarine gel electrophoresis with 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 being stained with ethidium bromide. Molecular size markers (*Hae*III digest of φx174DNA) (Promega) were included in each gel (10).

Sequencing of the *eae* genes. The nucleotide sequence of the amplification products was determined by the dideoxynucleotide triphosphate chain termina-

tion method of Sanger, with the BigDye Terminator v3.1 cycle sequencing kit with an ABI 3100 genetic analyzer (Applied Bio-Systems).

Phylogenetic analyses. Genetic distances and phylogenetic trees of *eae* sequences were calculated and constructed with the CLUSTAL W program (38) included in the EMBL software (http://www.ebi.ac.uk/clustalw/).

Serotyping. The determination of O and H antigens was carried out by the method described by Guinée et al. (14) employing 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*, Departamento de Microbioloxía e Parasitoloxía, Facultade de Veterinaria, Universidade de Santiago de Compostela (Lugo, Spain, http://www.lugo.usc.es/ecoli), and the H antisera were obtained from the Statens Seruminstitut (Copenhagen, Denmark).

Nucleotide sequence accession number. The *eae*-ξ sequence of strain B49 O80:H- was deposited in the European Bioinformatics Institute (EMBL Nucleotide Sequence Database) and has been assigned accession number AJ582912.

RESULTS

Virulence genes. A total of 514 STEC isolates from diarrheic and healthy cattle in Spain were characterized in this study. PCR showed that 101 (20%) isolates carried stx_1 genes, 278 (54%) possessed stx_2 genes, and 135 (26%) possessed both stx_1 and stx_2. Enterohemolysin (*ehxA*) and intimin (*eae*) virulence genes were detected in 326 (63%) and in 151 (29%) of the isolates, respectively.

Serotypes and seropathotypes. STEC isolates belonged to 66 O serogroups and 113 O:H serotypes (including 23 new serotypes). However, 67% were of one of these 15 serogroups (O2, O4, O8, O20, O22, O26, O77, O91, O105, O113, O116, O157, O171, O174, and OX177) and 52% of isolates belonged to only 10 serotypes (O4:H4, O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, O171:H2, and ONT:H19). Although the 514 STEC isolates belonged to 164 different seropathotypes (associations between serotypes and virulence genes), only 12 accounted for 43% of isolates (Table 2). Seropathotype O157:H7 stx_2 *eae* *ehxA* (46 isolates) was the most common, followed by O157:H7 stx_1 stx_2 *eae* *ehxA* (34 isolates), O113:H21 stx_2 (25 isolates), O22:H8 stx_1 stx_2 *ehxA* (15 isolates), O26:H11 stx_1 *eae* *ehxA* (14 isolates), and O77:H41 stx_2 *ehxA* (14 isolates). The majority 85% of bovine STEC isolates belonged to serotypes previously found among human STEC strains and 54% to serotypes associated with STEC strains isolated from patients with hemolytic uremic syndrome.

Typing of *eae* (intimin) genes. Forty-one strains of serotypes O26:H11, O26:H-, O118:H16, O118:H-, O126:H-, OX177:H11, OX177:H-, and ONT:H- had intimin β1, 82 strains of serotype O157:H7 possessed intimin γ1, three strains of serotype O111:H- had intimin type γ2, one strain of serotype O49:H- showed intimin type δ, 13 strains of serotypes O103:H2, O103:H-, O103:H21, O136:H-, O163:H21, O165:H21 and O165:H25 had intimin type ε and eight strains of serotypes O84:H2, O138:H48, O150:H-, O156:H-, O156:H25 and ONT:H25 had intimin ζ. We have identified a new variant of the *eae* intimin gene designated ξ (xi) in two isolates of serotype O80:H-.

Sequence comparison and evolutionary analysis of *E. coli* intimin genes. An approximately 682-bp fragment of the 3′ variable region of the *eae* gene was amplified with universal *eae* primers EAE-F and EAE-RB from the STEC-B49 O80:H-strain and sequenced. As the *eae* sequence of STEC-B49 strain (accession number AJ582912) represents a new variant of the

TABLE 1. PCR primer and conditions for amplification of STEC virulence genes

Gene	Primers	Oligonucleotide sequence (5′-3′)[c]	Fragment size (bp)	Annealing temp (°C)	Primer coordinates	GenBank accession no.
stx_1	VT1-A VT1-B	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	55	113–134 394–414	M17358
stx_2	VT2-A VT2-B	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	55	50–69 543–565	M59432
ehxA	HlyA1 HlyA4	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1,551	60	238–259 1767–1788	X79839
eae[a]	EAE-1 EAE-2	GGAACGGCAGAGGTTAATCTGCAG GGCGCTCATCATAGTCTTTC	775	55	1441–1460 2193–2215	AF022236
eae[b]	EAE-F EAE-RB	ATTACTGAGATTAAGGCTGAT ATTTATTTGCAGCCCCCCAT	682	55	1978–1998 2640–2659	AF022236
eae-α1	EAE-FB EAE-A	AAAACCGCGGAGATGACTTC CACTCTTCGCATCTTGAGCT	820	60	1909–1928 2709–2728	AF022236
eae-α2	IH2498aF IH2498aR	AGACCTTAGGTACATTAAGTAAGC TCCTGAGAAGAGGGTAATC	517	60	2099–2122 2597–2615	AF530555
eae-β	EAE-FB EAE-B	AAAACCGCGGAGATGACTTC CTTGATACACTTGATGACTGT	830	64	1909–1928 2718–2738	AF453441
eae-β1	EA-B1-F EAE-B	CGCCACTTAATGCCAGCG CTTGATACACCTGATGACTGT	811	60	1928–1945 2718–2738	AF453441
eae-β2	EA-B2-F EAE-B	CCCGCCACTTAATCGCACGT CTTGATACACCTGATGACTGT	807	60	1929–1948 2715–2735	AF043226
eae-γ1	EAE-FB EAE-C1	AAAACCGCGGAGATGACTTC AGAACGCTGCTCACTAGATGTC	804	60	1909–1928 2691–2712	AF071034
eae-γ2/θ	EAE-FB EAE-C2	AAAACCGCGGAGATGACTTC CTGATATTTTATCAGCTTCA	808	58	1909–1928 2697–2716	AF025311
eae-δ/κ	EAE-FB EAE-D	AAAACCGCGGAGATGACTTC CTTGATACACCCGATGGTAAC	833	60	1909–1928 2721–2741	U66102
eae-ε	EAE-FB LP5	AAAACCGCGGAGATGACTTC AGCTCACTCGTAGATGACGGCAAGCG	722	66	1909–1928 2605–2630	AF116899
eae-ζ	Z1 Z2	GGTAAGCCGTTATCTGCC ATAGCAAGTGGGGTGAAG	206	62	2062–2079 2250–2267	AF449417
eae-η	EAE-FB LP8	AAAACCGCGGAGATGACTTC TAGATGACGGTAAGCGAC	712	62	1899–1918 2593–2610	AJ308550
eae-ι	EAE-FB LP7	AAAACCGCGGAGATGACTTC TTTATCCTGCTCCGTTTGCT	807	66	1909–1928 2695–2715	AJ308551
eae-λ	68.4F 68.4R	CGGTCAGCCTGTGAAGGGC ATAGATGCCTCTTCCGGTATT	466	64	2061–2079 2506–2526	AF530557
eae-μ	FV373F FV373R	CAACGGTAAGTCTCAGACAC CATAATAAGCTTTTTGGCCTACC	443	64	114–133 534–556	AJ579305
eae-ν	IH1229aF IH1229aR	CACAGCTTACAATTGATAACA CTCACTATAAGTCATACGACT	311	60	269–289 559–579	AJ579306
eae-ξ	EAE-FB B49R	AAAACCGCGGAGATGACTTC ACCACCTTTAGCAGTCAATTTG	468	66	1909–1928 363–384	AF116899 AJ582912

[a] Universal oligonucleotide primer pair EAE-1 and EAE-2 with homology to the 5′ conseved region of *eae* (detects all types of *eae* variants described at the moment).
[b] Universal oligonucleotide primer pair EAE-F and EAE-RB with homology to the 3′ variable region of *eae* (detects all types of *eae* variants described at the moment).
[c] HlyA1 and Hly4 primer pair was designed by Schmidt et al. (35). The remaining primer pairs were designed by us according to the nucleotide sequences of the genes (10; Blanco et al., submitted).

TABLE 2. Seropathotypes (serotypes and virulence genes) of 514 bovine STEC isolates

Serotype	Total no. of strains	stx_1	stx_2	eae	ehxA	Serotype	Total no. of strains	stx_1	stx_2	eae	ehxA
O2:H27[a]	7	−	+	−	−	O91:H21[b]	6	−	+	−	+
O2:H29[b]	5	−	+	−	−	O96:H-[a]	1	+	+	−	+
O4:H4	1	−	+	−	+	O103:H-[b]	2	+	−	+ (ε)	+
O4:H4	10	−	+	−	−	O103:H2[b]	5	+	−	+ (ε)	+
O6:H8[c]	1	+	+	−	+	O103:H2[b]	1	+	−	−	+
O6:H34[a]	1	−	+	−	+	O103:H21[a]	1	+	−	+ (ε)	−
O7:H6[a]	1	+	−	−	−	O104:H21[b]	1	+	+	−	+
O8:H2[b]	5	+	+	−	+	O104:H21[b]	1	+	+	−	−
O8:H2[b]	1	+	−	−	−	O105:H18[b]	12	+	+	−	+
O8:H2[b]	2	−	+	−	+	O105:H18[b]	3	+	−	−	+
O8:H2[b]	1	−	+	−	−	O110:H2	1	−	+	−	−
O8:H8	1	+	−	−	−	O111:H-[b]	3	+	−	+ (γ2)	+
O8:H19[b]	1	+	+	−	+	O113:H-[a]	3	−	+	−	−
O8:H19[b]	1	−	+	−	+	O113:H4[a]	1	+	+	−	−
O8:H19[b]	1	−	+	−	−	O113:H4[a]	7	−	+	−	−
O9:H-[b]	1	+	−	−	+	O113:H7[a]	1	+	−	−	−
O15:H16	1	−	+	−	−	O113:H21[b]	1	+	+	−	−
O15:H19[c]	1	+	+	−	−	O113:H21[b]	7	−	+	−	+
O15:H27[a]	1	−	+	−	−	O113:H21[b]	25	−	+	−	−
O17:H18[a]	1	+	+	−	+	O116:H-[a]	1	−	+	−	+
O17:H18[a]	1	+	−	−	+	O116:H21[a]	9	−	+	−	+
O20:H7[a]	1	+	−	−	−	O117:H28[a]	2	+	+	−	−
O20:H19[b]	10	+	+	−	+	O118:H-[b]	2	+	−	+ (β1)	+
O20:H19[b]	2	−	+	−	+	O118:H16[b]	2	+	−	+ (β1)	+
O20:H19[b]	6	−	+	−	−	O123:H16[c]	2	+	−	−	−
O22:H8[b]	15	+	+	−	+	O126:H-[a]	1	+	−	+ (β1)	+
O22:H8[b]	2	+	+	−	−	O126:H20[a]	4	+	+	−	+
O22:H8[b]	1	+	−	−	+	O127:H21[a]	1	−	+	−	−
O22:H8[b]	1	+	−	−	−	O128:H-[b]	4	+	−	−	−
O22:H8[b]	4	−	+	−	+	O132:H-[a]	1	+	−	−	−
O22:H8[b]	2	−	+	−	−	O136:H-	1	+	−	−	−
O22:H16[a]	1	−	+	−	+	O136:H-	1	−	+	+ (ε)	−
O22:H16[a]	1	+	−	−	−	O136:H12	3	+	−	−	−
O26:H-[b]	1	+	−	+ (β1)	+	O136:H16	2	+	−	−	−
O26:H-[b]	2	−	+	+ (β1)	+	O136:H16	1	−	+	−	−
O26:H11[b]	14	+	−	+ (β1)	+	O138:H48[c]	1	+	−	+ (ζ)	−
O26:H11[b]	8	+	−	+ (β1)	−	O138:H48[c]	1	+	−	−	−
O26:H11[b]	1	+	−	−	+	O140:H21[c]	1	−	+	−	+
O28:H9[c]	1	+	+	−	+	O141:H2[a]	1	+	+	−	+
O38:H21[a]	1	−	+	−	+	O141:H8[a]	1	+	+	−	−
O39:H21	1	+	+	−	−	O141:H8[a]	3	−	+	−	+
O39:H48[a]	1	−	+	−	−	O146:H21[a]	1	−	+	−	+
O41:H2[a]	3	−	+	−	+	O146:H32[c]	1	+	−	−	+
O41:H14	1	+	−	−	−	O148:H8[c]	1	+	−	−	−
O41:H51[c]	1	+	−	−	−	O149:H1[c]	1	+	−	−	−
O49:H-[b]	1	+	−	+ (δ)	+	O150:H-[a]	1	+	−	+ (ζ)	+
O60:H19[a]	1	−	+	−	+	O156:H-[a]	3	+	−	+ (ζ)	+
O64:H-[c]	4	+	−	−	−	O156:H-[a]	1	−	+	+ (ζ)	+
O64:H34[c]	1	−	+	−	−	O156:H-[a]	5	−	+	−	−
O65:H16[a]	1	−	+	−	−	O156:H19[c]	1	−	+	−	−
O74:H28[c]	1	+	−	−	−	O156:H25[a]	1	+	−	+ (ζ)	+
O77:H41[a]	5	+	+	−	+	O157:H7[b]	34	+	+	+ (γ1)	+
O77:H41[a]	14	−	+	−	+	O157:H7[b]	46	−	+	+ (γ1)	+
O77:H41[a]	2	−	+	−	−	O157:H7[b]	2	+	−	+ (γ1)	+
O79:H-	1	+	+	−	+	O162:H4[a]	1	−	+	−	+
O80:H-[a]	2	+	−	+ (ξ)	+	O162:H7[c]	1	+	+	−	+
O81:H28[c]	1	+	+	−	+	O162:H7[c]	1	−	+	−	−
O81:H28[c]	1	+	+	−	−	O162:H21[c]	1	−	+	−	+
O81:H31[a]	1	+	+	−	+	O162:H21[c]	3	−	+	−	−
O82:H8[a]	7	+	+	−	+	O162:H28[c]	1	+	−	−	−
O84:H2[a]	1	+	−	+ (ζ)	+	O163:H19[b]	3	−	+	−	+
O88:H8	1	+	+	−	+	O163:H21[c]	1	+	+	−	+
O88:H25[a]	1	−	+	−	+	O163:H21[c]	1	+	−	+ (ε)	−
O90:H-[a]	1	+	−	−	−	O163:H21[c]	1	−	+	−	+
O91:H19[c]	1	+	−	−	+	O165:H21[a]	1	−	+	+ (ε)	+
O91:H19[c]	1	−	+	−	−	O166:H28[a]	1	−	+	−	−
O91:H21[b]	2	+	+	−	+	O167:H21[c]	1	+	−	−	−

TABLE 2—*Continued*

Serotype	Total no. of strains	stx_1	stx_2	*eae*	*ehxA*	Serotype	Total no. of strains	stx_1	stx_2	*eae*	*ehxA*
O168:H8	1	+	−	−	−	OX178:H11[c]	1	−	+	−	+
O168:H8	2	−	+	−	−	OX178:H19[a]	2	+	+	−	+
O171:H2[a]	20	−	+	−	−	ONT:H-[b]	1	−	+	+ (β1)	+
O171:H25	1	+	−	−	+	ONT:H-[b]	5	−	+	−	+
O171:H25	3	−	+	−	−	ONT:H-[b]	1	−	+	−	−
O174:H-[b]	3	+	+	−	+	ONT:H2[a]	3	+	+	−	+
O174:H-[b]	2	−	+	−	−	ONT:H2[a]	2	−	+	−	+
O174:H2[b]	5	+	+	−	+	ONT:H8[a]	1	+	+	−	+
O174:H2[b]	2	−	+	−	+	ONT:H19[a]	13	−	+	−	+
O174:H2[b]	1	−	+	−	−	ONT:H19[a]	3	−	+	−	−
O174:H21[b]	6	−	+	−	−	ONT:H21[a]	1	+	+	−	−
OX177:H-[a]	5	−	+	+(β1)	+	ONT:H21[a]	1	−	+	−	−
OX177:H11	5	+	−	+(β1)	+	ONT:H25[b]	1	+	−	+ (ζ)	+
OX178:H11[c]	1	+	+	−	+	ONT:H32[c]	1	+	−	−	−

[a] Serotype previously found in human STEC.
[b] Serotype previously associated with human STEC strains that cause hemolytic uremic syndrome.
[c] New serotype not found in STEC in previous studies.

intimin gene, it has been designated *eae*-ξ (xi) in order to follow the Greek alphabet.

We determined the genetic relationship of the 3′ variable region of the *eae*-ξ gene (accession number AJ582912, fragment size 600 bp, primer coordinates 1 to 600) and the remaining 16 *eae* variants: α1 (AF022236, 635 bp, 2002 to 2636, aligned score 60%), α2 (AJ579368, 635 bp, 2002 to 2636, 54%), β1 (AF453441, 635 bp, 2002 to 2636, 64%), β2 (AF043226, 629 bp, 2005 to 2633, 63%), γ1 (AF071034, 620 bp, 2002 to 2621, 58%), γ2 (AF025311, 623 bp, 2002 to 2624, 59%), δ (U66102, 638 bp, 2002 to 2639, 61%), ε (AF116899, 644 bp, 2002 to 2645, 93%), ζ (AF449417, 629 bp, 2002 to 2630, 58%), η (AJ308550, 644 bp, 1992 to 2635, 91%), θ (AF449418, 623 bp, 2002 to 2624, 59%), ι (AJ308551, 632 bp, 2002 to 2633, 63%), κ (AJ308552, 635 bp, 1992 to 2636, 62%), λ (AF530557, 632 bp, 2002 to 2633, 56%), μ (AJ579305, 622 bp, 1 to 622, 59%), and ν (AJ579306, 635 bp, 16 to 650, 60%). Since the nucleotide sequences analyzed were of different lengths (600 to 644 bp), we used CLUSTAL W for optimal sequence alignment. Thus, the *eae*-ξ sequence is similar to *eae*-ε (identity of 93%) and *eae*-η (identity of 91%) sequences. The phylogenetic tree (Fig. 1) supports the data of the sequence identity values. Phylogenetic analyses revealed five groups of the closely related intimin genes: (i) α1, α2, ζ and ν; (ii) β1, β2, δ and κ; (iii) γ1, γ2, θ and μ; (iv) ι and λ; and (v) ε, η and ξ.

DISCUSSION

The results of our previous studies indicate that STEC colonization is widespread among cattle in Spain. Between 1993 and 1995, 1,069 healthy cattle were examined for STEC colonization. STEC-positive animals were found in 95% of the farms examined and the estimated proportion of positive cattle in each farm ranged from 0 to 100%. The overall prevalence rates of STEC colonization were estimated to be 37% in calves and 27% in cows. In Spain, stx_2 and stx_1 stx_2 isolates were present in similar proportions in calves and cows. In contrast, stx_1 and *eae* isolates were more commonly recovered from calves than from cows. STEC O157:H7 was detected in only 8 (0.7%) of the 1,069 animals investigated. Interestingly, the majority of *eae* non-O157 STEC and the eight STEC O157:H7

were isolated from calves, confirming that young animals are the most important reservoir of highly pathogenic *eae* STEC (5, 8, 9).

In a survey realized between 1998 and 1999, STEC O157:H7 was isolated from 55 (12%) of 471 feedlot calves (4 to 8 months of age). Although only a mean of three animals per herd were sampled, STEC O157:H7 isolates were detected in 32 (22%) of 145 feedlots examined. When we sampled a higher number of animals (9 to 56), STEC O157:H7 was found in 3 (60%) of five feedlots investigated. Individual prevalences in the three positive feedlots were 23% (13 of 56), 22% (two of nine) and 8% (1 of 13). Farms were visited only in one occasion to collect fecal samples (5). While the total numbers of healthy and diarrheic calves positive for STEC did not vary significantly, a significantly higher percentage of Stx1-producing *E. coli* was found in diarrheic calves, suggesting a pathogenic role in neonatal calf diarrhea (5, 7). Similar prevalences of STEC were found in other countries (3, 15, 21, 22, 25, 33, 39, 41, 43, 44).

In the present work, we have established the serotypes, virulence genes, and intimin types of STEC isolated from cattle in previous studies (5, 7, 8, 9) to know if bovine STEC possess the same serotypes and virulence factor profiles that STEC strains that cause human infection. STEC isolates belonged to 66 O serogroups and 113 O:H serotypes (including 23 new serotypes). However, 67% were of one of these 15 serogroups (O2, O4, O8, O20, O22, O26, O77, O91, O105, O113, O116, O157, O171, O174, and OX177) and 52% of isolates belonged to only 10 serotypes (O4:H4, O20:H19, O22:H8, O26:H11, O77:H41, O105:H18, O113:H21, O157:H7, O171:H2, and ONT:H19), including 10 serotypes also found among STEC strains that cause human infections and six serotypes associated with hemolytic uremic syndrome (O20:H19, O22:H8, O26:H11, O105:H18, O113:H21, and O157:H7). Similarly, among the 20 serotypes most frequently isolated in cattle or cattle products in Canada by Johnson et al. (17), 18 have been isolated from humans, and 11 of these are serotypes associated with bloody diarrhea/hemorrhagic colitis and/or hemolytic uremic syndrome. Pradel et al. (33) in France, Beutin et al. (3), and Montenegro et al. (25) in Germany, Wells et al. (39) in the United States, and Parma et al. (28) in Argentina have also
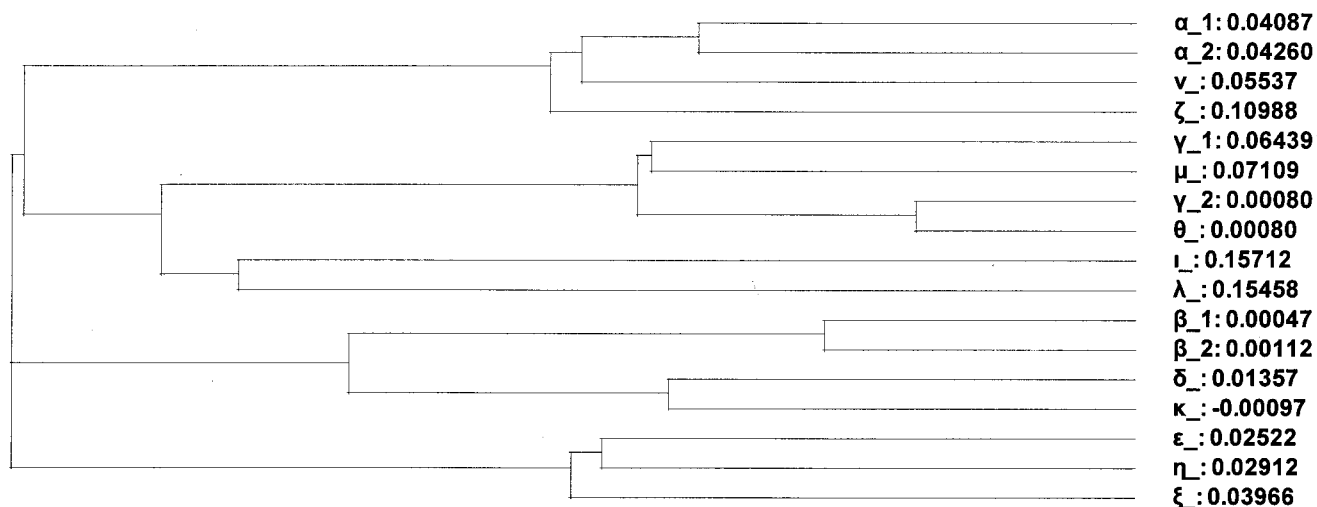
FIG. 1. Phylogenetic tree of the intimin variant genes constructed with the CLUSTAL W program. Numbers on the branches denote the genetic distance. Phylogenetic analyses revealed five groups of the closely related intimin genes: (i) α1, α2, ζ, and ν; (ii) β1, β2, δ, and κ; (iii) γ1, γ2, θ, and μ; (iv) ι and λ; and (v) ε, η, and ξ.

found that many STEC isolates recovered from cattle belong to serotypes previously associated with human disease. Like other authors (12, 17, 24, 40), we have observed that bovine and human STEC isolates of the same serotype have similar known virulence-associated properties.

The *eae* gene, which has been shown to be necessary for attaching and effacing activity, encodes a 94- to 97-kDa outer membrane protein which is termed intimin (16, 20). Numerous investigators have underlined the strong association between the carriage of the *eae* gene and the capacity of STEC strains to cause severe human disease, especially hemolytic uremic syndrome (1, 27, 29). This important virulence gene was detected in 100% of STEC O157:H7 and in 17% of non-O157 bovine isolates assayed in the present study. Nevertheless, production of intimin is not essential for pathogenesis, because a number of sporadic cases of hemolytic uremic syndrome 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 hemolytic uremic syndrome cases in the United States and Australia, respectively (30, 31). Furthermore, recently Paton and Paton (31) described a novel megaplasmid-encoded adhesin (Saa) which we have detected in some of bovine STEC strains lacking the *eae* gene (data not published). This adhesin may be an important virulence factor of *eae*-negative STEC strains capable of causing severe diseases in humans.

Analysis of the nucleotide sequences of the intimin genes from different STEC and enteropathogenic *E. coli* strains has shown a high degree of homology in the 5′ two-thirds of the genes and a significant degree of heterogeneity in the 3′ one-third of the genes (1, 13). Fourteen variants of the *eae* gene were identified by intimin type-specific PCR assays with oligonucleotide primers complementary to the 3′ end of the specific intimin genes that encode the intimin types α1, α2, β1, β2, γ1, γ2/θ, δ/κ, ε, ζ, η, ι, λ, μ, and ν (1, 6, 10, 18, 26, 27, 36, 37, 42; Blanco et al., submitted).

In the present study, we have identified a new intimin vari-

ant, designated intimin type ξ (xi) to follow the Greek alphabet. The *eae*-ξ (xi) sequence of our bovine STEC-B49 O80:H- strain is almost identical (>99%) to the *eae* genes sequenced by K. G. Zehmke (accession numbers AJ276416, AJ275092, AJ275103, and AJ275107, unpublished data) from four STEC strains isolated from calves in Germany and Belgium. As the *eae*-ξ sequence is similar to the *eae*-ε variant, K. G. Zehmke assumed that his bovine STEC strains were positive for intimin type ε. Our phylogenetic analysis revealed that the *eae*-ξ gene is closely related to intimin genes *eae*-ε and *eae*-η. As in human strains, the intimin types β1 and γ1 are the most frequently found among bovine STEC isolates. The intimin β1 was mainly found among strains belonging to serotype O26:H11, whereas the intimin type γ1 was detected in all 82 STEC O157:H7 assayed.

Seropathotypes O26:H11 stx_1 *eae*-β1, O157:H7 stx_1 stx_2 *eae*-γ1 and O157:H7 stx_1 stx_2 *eae*-γ1 are the most frequently observed in STEC strains that cause human infections in Spain (6). These highly virulent seropathotypes were detected in 102 bovine STEC strains characterized in the present study. Although our and other authors' results indicate that STEC strains of human and animal origin with the same serotype are similar in relation to the presence of known virulence-associated factors (4, 11, 12, 24, 34), the results of Boerlin et al. (11) suggest that STEC isolates from humans form a different population from those found in the bovine reservoir or that they are only a subpopulation of the latter. Thus, future studies are necessary to establish if animal and human strains represent the same clones or are only related subpopulations.

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