JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1997, p. 2958–2963 Vol. 35, No. 11
0095-1137/97/$04.00+0

NOTES

Genes Coding for Enterotoxins and Verotoxins in Porcine *Escherichia coli* Strains Belonging to Different O:K:H Serotypes: Relationship with Toxic Phenotypes

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Received 31 December 1996/Returned for modification 19 June 1997/Accepted 12 August 1997

Seventy-four *E. coli* strains isolated from piglets with diarrhea or edema disease in Spain were serotyped and examined for production of heat-labile (LT) and heat-stable (ST) enterotoxins (LT-I, LT-II, STaH, STaP, and STb) and verotoxins (VT1, VT2, and VT2v = VTe) by phenotypic (Vero cell assay and infant mouse test) and genotypic (colony hybridization and PCR) methods. In general, an excellent correlation was found between the results obtained with a PCR approach and those determined with biological assays. DNA probes used in the hybridization also showed a very good agreement with phenotypic results, with the exception of a VT1 probe that initially produced 10 false-positive reactions. The gene coding for STb (58 strains) was the most prevalent gene detected by PCR, followed by those coding for STa (46 strains), LT (19 strains), VT2v (11 strains), and VT1 (1 strain). Apparently, in Spain three seropathotypes are predominant: (i) O149:K91:H10 K88$^+$ LT-I$^+$ STb$^+$, (ii) O141:K85ab:H− P987$^+$ STaP$^+$, and (iii) O138:K81:H14 or H− STaP$^+$ VT2v$^+$. We conclude that PCR is a fast, specific, and practical method for the identification of enterotoxin and VT genes in clinical and epidemiological studies.

Enterotoxigenic *Escherichia coli* (ETEC) and verotoxigenic *E. coli* (VTEC) are the main categories of bacteria that cause enteric infections in pigs (2, 10). ETEC strains can cause severe diarrhea in newborn and weaned piglets by the production of heat-labile enterotoxin (LT) and/or a group of heat-stable enterotoxins (STs) (2). STs are classified as STa (also called STI, ST1, or STmouse) and STb (also called STII, ST2, or STpig) (11, 19, 32). Many porcine ETEC strains have fimbrial structures on their surface which, like LT, STa, and STb enterotoxins, are plasmid mediated. These fimbriae are termed colonization antigens (K88, K99, F41, and P987) and enable the bacteria to colonize the epithelial surface of the small intestine (29, 31). On the other hand, porcine VTEC strains produce the edema verotoxin (VTe), also named VT2 variant (VT2v) or Shiga-like toxin II variant (SLT-IIv) (18). ETEC and VTEC strains can be phenotypically detected by testing isolates for toxin production with bioassays or immunoassays (6, 11, 12, 16, 32). Alternatively, ETEC and VTEC strains can be genotypically identified by detection of genes encoding toxin production by DNA hybridization techniques or PCR (13, 15, 16, 22, 27, 33). PCR is rapid, sensitive, and highly specific and gives results within a working day (22). However, no studies have compared the sensitivities and specificities of PCR and biological assays for the detection of enterotoxins and VTs in a wide range of serotypes of porcine strains. Thus, the objectives of this study were to determine the presence of enterotoxin and VT genes in ETEC and VTEC strains isolated from piglets with diarrhea or edema disease in Spain, to compare the sensitivity and specificity of PCR with those of the DNA hybridization test and conventional biological assays for the detection of enterotoxins and VTs, and to establish the O:K:H serotypes and colonization factors of Spanish porcine ETEC and VTEC strains.

From 1986 to 1991, 1,334 *E. coli* colonies were isolated from 414 pigs sampled at 65 piggeries located in different areas of Spain (6). ETEC strains were isolated from 64 (20.2%) of 317 piglets with diarrhea, 5 (25%) of 20 piglets with edema disease, and 1 (1.3%) of 77 healthy piglets. ETEC strains were statistically associated ($P < 0.001$) with piglets with diarrhea or edema disease. VTEC strains were isolated from 13 (4.1%) piglets with diarrhea, 3 (15.0%) piglets with edema disease, and only 1 (1.3%) piglet who was healthy. VTEC strains were statistically associated with pigs with edema disease ($P < 0.05$) but not with diarrheic piglets. With only biological assays (Vero cell assay for detection of LT and VTs, and infant mouse test [IMT] for STa), a total of 273 ETEC and VTEC isolates were detected (6). ETEC (LT$^+$ and/or STa$^+$) and VTEC strains isolated in Spain from pigs with diarrhea or edema disease belonged almost exclusively (91.2%) to only seven se-

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TABLE 1. Primers used in PCR to amplify specific fragments from genes for enterotoxins (LT, STa, and STb) and VTs (VT1, VT2, and VT2v)

Target gene coding for toxin(s)	Primer code	Oligonucleotide sequence (5′→3′)	Predicted size of amplified product (bp)	Reference
LT[a]	LTA-1	GGCGACAGATTATACCGTGC	696	27
	LTA-2	CCGAATTCTGTTATATATGTC		
STa	STI-1	TTAATAGCACCCGGTACAAGCAGG	147	22
STaP and STaH[b]	STI-2	CTTGACTCTTCAAAAGAGAAAATTAC		
STb	STb-1	ATCGCATTTCTTCTTGCATC	172	This study[f]
	STb-2	GGGCGCCAAAGCATGCTCC		
VT1	VT1-A	GAAGAGTCCGTGGGATTACG	130	25
	VT1-B	AGCGATGCAGCTATTAATAA		
VT2all[c]	SLTII-1	CTTCGGTATCCTATTCCCGG	478	22
	SLTII-2	GGATGCATCTCTGGTCATTG		
VT2hb[d]	VT2-3	CCGTCAGGACTGTCTGAAAC	726	33
	VT2-5	GAGTCTGACAGGCAACTGTC		
VT2v=VTe[e]	VT2e-A	CCTTAACTAAAAGGAATATA	230	13
	VT2e-B	CTGGTGGTGTATGATTAATA		

[a] Detects LT-I enterotoxin produced by human and porcine ETEC strains. However, it is possible that sufficient homology exists with the LT-II variety produced by bovine strains.
[b] Detects the two varieties of STa active in the infant mouse test.
[c] Detects VT2 produced by human and bovine VTEC strains and the VT2 variant (VT2v=VTe) synthesized by porcine strains.
[d] Only detects VT2 produced by human and bovine strains associated with hemorrhagic colitis and the hemolytic uremic syndrome.
[e] Only detects the VT2 variant (VT2v=VTe) synthesized by porcine strains associated with the edema disease.
[f] The primer sequences were designed in this study and were based on the published sequences for the STb gene (14).

rogroups (O8, O9, O20, O101, O138, O141, and O149) which were detected at lower frequencies among nontoxigenic isolates (7). Seventy-four representative strains of these 273 ETEC and VTEC strains were included in the present study for additional characterization. Reference *E. coli* strains used as positive and negative controls were H510/76 (O149:K91:[K88ac] LT-I$^+$ STb$^+$), H255/82 (O9:K103:[P987] STaP$^+$), Bd206/75 (O101:K30:H1 [K99,F41] STaP$^+$), m452-C_1 (O63:K-:H− LT-I$^+$ STaH$^+$), 933 (O157:H7 VT1$^+$ VT2$^+$), E57 (O138:K81 STaP$^+$ VTe$^+$), and K-12-185 (nontoxigenic).

The phenotypic techniques (Vero cell assay and IMT) were comprehensively described in previous papers (1, 3, 8). The following DNA sequences were used as probes for *E. coli* toxin genes: the 850-bp *Hin*dIII fragment of pEWD299 and the 800-bp *Hin*dIII-*Pst*I fragment of pCP2725 for detection of LT-I (20) and LT-II (23), respectively; the 157-bp *Hin*fI fragment of pRIT10036 (STaP) and a 215-bp *Hpa*II fragment obtained from digestion of a *Hin*dIII-*Eco*RI fragment of pSLM004 (STaH) for detection of STa (20); the 460-bp *Eco*RI-*Bam*HI fragment of pCHL6 for detection of STb (14); and the 1,154-bp *Bam*HI fragment of pNN37-19 and the 842-bp *Pst*I fragment of pNN110-18 for detection of VT1 and VT2 (21), respectively. Probe fragments were labelled with [α-^{32}P]dATP by nick translation (26). Filters containing the test strains for colony hybridization were prepared and hybridized under stringent conditions as described by Maas (17). *E. coli* K-12 strains bearing the recombinant plasmids were used as positive controls, and *E. coli* HB101(pBR322) was used as the negative control. In the PCR for detection of toxin genes, the DNA to be amplified was released from the whole organism by boiling. Base sequences, locations, and predicted sizes of amplified products for the specific oligonucleotide primers used in this study for detection of LT, STa, STb, and VTs are shown in Table 1. Amplification of bacterial DNA was performed with 50-μl volumes containing 10 μl of the DNA sample; the oligonucleotide primers (300 ng for LT and STa primers; 90 ng for STb, VT2all, and VThb primers; 180 ng for VT1 primers; and 450 ng for VT2v 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 DynaZyme DNA polymerase (Finnzymes OY, Finland). The reaction mixtures were overlaid with an equal volume of mineral oil. The PCR was performed with a thermal cycler at 94°C for 3 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified product was visualized by standard submarine gel electrophoresis of 10 μl of the final reaction mixture on a 2% agarose gel. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. The serotyping was carried out by the method described by Guinée et al. (10), employing all available O (O1 to O171) and H (H1 to H56) antisera and the specific OK antisera against the K antigens usually associated with each O antigen determined. For phenotypic expression of the K88, K99, P987, and F41 antigens, bacteria were inoculated into Mueller-Hinton broth (Difco) and incubated at 37°C for 5 days until a definitive pellicle was formed. From this pellicle, bacteria were inoculated on colonization factor antigen agar and Minca-Is agar and incubated at 37°C for 18 h. The presence of colonization antigens in *E. coli* strains was revealed by using specific polyclonal antisera in a staphylococcal coagglutination test (8).

The results of the phenotypic and genotypic assays for detection of enterotoxins and VTs are shown in Table 2. The study of toxigenicity was performed in four stages. (i) The 74 porcine *E. coli* strains were originally identified with biological assays from 1986 to 1991. (ii) In 1993, the presence of enterotoxin and VT genes was established by colony hybridization (CH) in 34 of the strains. (iii) During 1996, the PCR results

TABLE 2. Relationship of enterotoxin and VT genes to serotypes, colonization factors, and phenotypic production of enterotoxins and VTs

Strain	O:K:H serotype [colonization factor(s)]	Phenotypic result		Genotypic result (PCR/hybridization)[a]				
		Vero and HeLa cell assay	IMT	LT[b]	STa[c]	STb	VT1	VT2all[d]
PD34a	O8:K85:H2 [K99]		0.166 (STa)	−	+	−	−	−
PD36b	O8:K85:H2 [K99]		0.137 (STa)	−	+	−	−	−
PD37a	O8:K85:H2 [K99]		0.144 (STa)	−	+	−	−	−
PD215a	O8:K201:H6 [K88]	LT	0.064	+	−	+	−	−
IP101b	O8:K201:H9	LT	0.070	+	−	+	−	−
PD205a	O8:K−:H11 [K88]	LT	0.159 (STa)	+/+	+/+	+/+	−/+[f]	−/−
PDV5a	O8:K201:H14 [K88]	LT	0.070	+	−	+	−	−
PD69d	O8:K48:H31	LT	0.058	+	−	+	−	−
PDV26e	O8:K?:H31 [K88]	LT	0.082	+	−	+	−	−
PD23a	O9:K103:H− [P987]		0.065 (−)[e]	−	−	−	−	−
PD26c	O9:K103:H− [P987]		0.143 (STa)	−	+	−	−	−
PD114b	O9:K103:H− [P987]		0.066 (−)	−/−	−/−	−/−	−/−	−/−
IP19a	O9:K?:H− [K99,F41]		0.113 (STa)	−/−	+/+	−/−	−/−	−/−
PD204a	O9:K?:H−		0.069 (−)	−/−	−/−	−/−	−/−	−/−
PD206c	O9:K?:H−		0.050 (−)	−/−	−/−	+/+	−/−	−/−
PD205d	O9:K?:H−		0.068 (−)	−/−	−/−	−/+	−/−	−/−
PE12b	O9:K(A)?:H−	VT	0.059 (−)	−/−	−/+	+/+	−/+	+/+
IP30a	O20:K101:H− [P987]		0.194 (STa)	−/−	+/+	−/−	−/−	−/−
PDV36a	O20:K101:H−		0.174 (STa)	−	+	+	−	−
PD30a	O20:K?:H−		0.167 (STa)	−/−	+/+	+/+	−/−	−/−
IP40a	O20:K?:H− [K99,F41]		0.163 (STa)	−/−	+/+	−/−	−/−	−/−
PD30b	O51:K?:H42	LT	0.070	+/+	−/−	+/+	−/+	−/−
PDV36b	O64:K−:H− [K99,F41]		0.125 (STa)	−	+	−	−	−
PD18e	O91:K−:H−	VT	0.054	−	−	−	+	−
PD88e	O92:K?:H−		0.130 (STa)	−/−	+/+	+/+	−/+	−/−
IP20c	O101:K−:H9 [F41]		0.195 (STa)	−/−	+/+	−/−	−/−	−/−
PD114a	O101:K−:H9 [K99,F41]		0.108 (STa)	−/−	+/+	−/−	−/−	−/−
IP18a	O101:K30:H9 [F41]		0.175 (STa)	−	+	−	−	−
PDV46a	O101:K30:H9		0.094 (STa)	−	+	−	−	−
IP75b	O101:K?:H9 [K99,F41]		0.115 (STa)	−/−	+/+	−	−/−	−/−
PE5d	O138:K?:H−	VT	0.166 (STa)	−/−	+/+	+/+	−/+	+/+
PE9b	O138:K?:H−	VT	0.122 (STa)	−	+	+	−	+
PD32b	O138:K81:H−	VT	0.154 (STa)	−/−	+/+	+/−	−/−	+/+
PD87a	O138:K81:H−		0.163 (STa)	−/−	+/+	+/+	−/−	−/−
PD87c	O138:K81:H−	VT	0.140 (STa)	−/−	+/+	+/+	−/−	+/+
PD88a	O138:K81:H−		0.152 (STa)	−	+	+	−	−
PD90d	O138:K81:H−		0.137 (STa)	−/−	+/+	+/+	−/−	−/−
PD131a	O138:K81:H−		0.171 (STa)	−	+	+	−	−
PD8d	O138:K81:H14	VT	0.166 (STa)	−	+	+	−	+
PD27a	O138:K81:H14	VT	0.148 (STa)	−/−	+/+	+/+	−/+	+/+
PD59a	O138:K81:H14	VT	0.185 (STa)	−/−	+/+	+/+	−/−	+/+
PD89d	O138:K81:H14	VT	0.146 (STa)	−	+	+	−	+
PD90b	O138:K81:H14	VT	0.093 (STa)	−/−	+/+	+/+	−/−	+/+
IP10a	O141:K?:H−		0.060 (−)	−/−	−/−	−/−	−/−	−/−
IP23a	O141:K85ab:H− [P987]		0.149 (STa)	−/−	+/+	+/+	−/+	−/−
IP42a	O141:K85ab:H− [P987]		0.125 (STa)	−	+	+	−	−
IP49a	O141:K85ab:H− [P987]		0.162 (STa)	−	+	−	−	−
IP51a	O141:K85ab:H− [P987]		0.057 (−)	−	−	−	−	−
IP57a	O141:K85ab:H− [P987]		0.063 (−)	−	−	−	−	−
IP71e	O141:K85ab:H− [P987]		0.051 (−)	−/−	−/+	−/−	−/−	−/−
IP84a	O141:K85ab:H− [P987]		0.133 (STa)	−	+	−	−	−
IP89a	O141:K85ab:H− [P987]		0.069 (−)	−	−	−	−	−
IP92b	O141:K85ab:H− [P987]		0.104 (STa)	−	+	−	−	−
PDV131a	O141:K85ab:H4		0.173 (STa)	−	+	+	−	−
PDV7a	O149:K91:H10 [K88]	LT	0.195 (STa)	+	+	+	−	−
PDV8a	O149:K91:H10 [K88]	LT	0.041	+	−	+	−	−
PDV8g	O149:K91:H10 [K88]	LT	0.078	+	−	+	−	−
PDV9a	O149:K91:H10 [K88]	LT	0.064	+	−	+	−	−
PDV132a	O149:K91:H10 [K88]	LT	0.078	+	−	+	−	−
IP19b	O149:K91:H10 [K88]	LT	0.069	+/+	−/−	+/+	−/−	−/−
IP20a	O149:K91:H10 [K88]	LT	0.068	+/+	−/−	+/+	−/+	−/−
IP62e	O149:K91:H10 [K88]	LT	0.130 (STa)	+	+	+	−	−
IP102a	O149:K91:H10 [K88]	LT	0.064 (−)	+	−	+	−	−
PE2a	O149:K91:H19	LT	0.069	−	−	+	−	−
PD116a	O149:K91:H19	LT	0.070	+/+	−/−	+/+	−/+	−/−
PD117a	O149:K91:H19	LT	0.054	+	−	+	−	−

Continued on following page

TABLE 2—*Continued*

Strain	O:K:H serotype [colonization factor(s)]	Phenotypic result		Genotypic result (PCR/hybridization)[a]				
		Vero and HeLa cell assay	IMT	LT[b]	STa[c]	STb	VT1	VT2all[d]
PD216a	O149:K91:H19	LT	0.069	+/+	−/−	+/+	−/−	−/−
IP10c	O157:K−:H43		0.116 (STa)	−	+	+	−	−
PD88b	ONT[g]:H−		0.113 (STa)	−	+	+	−	−
PD129a	ONT:H−		0.101 (STa)	−/−	+/+	+/+	−/−	−/−
PD204b	ONT:H−		0.186 (STa)	−/−	+/+	+/+	−/+	−/−
PD206a	ONT:H−		0.190 (STa)	−/−	+/+	+/+	−/−	−/−
PDV42d	ONT:H−		0.152 (STa)	−	+	−	−	−
PE5e	ONT:H−	VT	0.140 (STa)	−/−	+/+	+/+	−/−	+/+

[a] All 74 strains were tested by PCR to determine the presence of the gene coding for the toxin listed. However, only 34 of them were examined by CH.
[b] The six LT^+ strains tested by hybridization were LT-I^+ and LT-II^-.
[c] The 24 STa^+ strains examined by hybridization were $STaP^+$ and $STaH^-$.
[d] Initially, the 74 strains were assayed by PCR and hybridization with a set of primers (VTall) and a DNA probe (842-bp *Pst*I fragment of pNN110-18) that detect both VT2 and the VT2 variant (VT2v=VT2). Afterwards, differentiation between VT2 and VT2v was achieved by PCR with two specific primer sets (VT2hb and VTe). All 11 positive strains were $VT2hb^-$ and VTe^+ in the PCR assays.
[e] Twelve strains originally recorded as STa^+ in the IMT gave negative results (−), apparently as a result of loss of plasmids carrying the enterotoxin genes during storage.
[f] −/+, false-positive reaction for the VT1 gene obtained by CH during 1993. When the CH tests were repeated in 1997, all gave true-negative reactions.
[g] ONT, O not typeable.

were obtained. (iv) During 1997, the presence of VT1 genes was again assayed by CH in 10 *E. coli* strains. Because toxin genes may be lost during storage, the 74 strains included in this study were tested again with the Vero cell assay and the IMT at the same time as the PCR test was carried out. Twelve strains originally recorded as STa^+ in the IMT gave negative results, whereas the phenotypic production of LT and VTs has not changed since this study was started. A nearly total correlation was observed between the results with the PCR approach and those determined by using biological assays with respect to LT and STa enterotoxins and VT1 and VT2. DNA probes used in the hybridization also showed a very good agreement with phenotypic results, with the exception of the VT1 probe that produced 10 false-positive cases in the test performed during 1993. However, when the CH tests were repeated in 1997, all false-positive reactions for the VT1 gene were now true-negative reactions.

In this study, a pair of oligonucleotide primers were specifically designed to amplify a 172-bp fragment of the STb gene. The primer sequences were based on the published sequences for STb (14). The use of an oligonucleotide primer pair homologous to the nucleotide sequence of the STb gene generated PCR products of the predicted size with all porcine STb^+ reference strains, but not with reference ETEC and VTEC strains producing other toxins. The toxin genotypes determined by PCR-STb test for the 34 strains tested corresponded to the results of DNA hybridization, with only two exceptions (PD205d and PD32b strains) (Table 2).

The STb gene (58 strains) was the most prevalent gene detected, followed by the STa (46 strains), LT (19 strains), VT2v (11 strains), and VT1 (1 strain) genes. The 6 LT^+ strains and 22 STa^+ strains in the PCR that were assayed with DNA probes hybridized with LT-I and STaP, respectively. None of 34 strains assayed hybridized with LT-II and STaH. A total of eight different combinations of enterotoxin and VT genes were observed (Table 3). However, four main toxic genotypes predominated: STa^+ only (including 18 strains belonging to a wide variety of serotypes), LT^+ STb^+ (including 16 strains, many of which belong to serotype O149:K91:H10 or O149:K91:H19), STa^+ STb^+ (including 15 strains which belong to a wide number of different serotypes), and $STa^+STb^+VT2v^+$ (including 10 strains, 9 of which belong to serotype O138:K81:H− or O138:K81:H14). Interestingly, all 19 of the swine ETEC strains that carried the LT gene also carried the STb gene. Both LT and STb genes were detected in the 13 $K88^+$ strains. In contrast, the majority of $P987^+$, $K99^+$, and/or $F41^+$ isolates presented only the STa gene. Strains that do not possess any of the four colonization factors examined in this study ($4P^-$ strains) presented seven different toxic genotypes (Table 4).

Various studies have compared the conventional biological assays for detection of enterotoxin production with different types of DNA probes used in colony hybridization (11, 12, 16), but to the authors' knowledge, this is the first survey that compares PCR for detection of different types of enterotoxins and VTs with the conventional methods. In this study, the PCR assays used for detection of LT and STa enterotoxins showed a very good agreement with phenotypic methods and the DNA probes used in colony hybridization. As in previous studies, we observed that some ETEC strains lost the STa phenotype during storage. These phenotypically negative strains were also negative in the PCR, indicating that the strains probably lost the STa gene-bearing plasmids by segregation during storage. Our results further indicate that STaP is the STa enterotoxin active in IMT produced by porcine ETEC strains and that LT-I is the variety of LT synthesized by ETEC strains of porcine origin. In the majority of studies, the STaH gene was detected

TABLE 3. Relationship between toxic genotypes and O serogroups

Toxic genotype					No. of strains	Serogroup(s) (no. of strains)
LT	STa	STb	VT1	VT2v		
+	+	+	−	−	3	O8 (1), O149 (2)
+	−	+	−	−	16	O8 (5), O51 (1), O149 (10)
−	+	+	−	−	15	O20 (2), O92 (1), O138 (4), O141 (3), O157 (1), ONT[a] (4)
−	+	−	−	−	18	O8 (3), O9 (2), O20 (2), O64 (1), O101 (5), O141 (4), ONT (1)
−	−	+	−	−	3	O9 (2), O149 (1)
−	+	+	−	+	10	O138 (9), ONT (1)
−	−	+	−	+	1	O9 (1)
−	−	−	+	−	1	O91 (1)

[a] ONT, O not typeable.

TABLE 4. Relationship between colonization factors and toxic genotypes

Colonization factor(s)	No. of strains	Toxic genotype(s) (no. of strains)
K88	13	LT STb (10), LT STa STb (3)
P987	13[a]	STa (5), STa STb (2)
K99 and F41	5	STa (5)
K99	3	STa (3)
F41	2	STa (2)
$4P^{-}$[b]	38[a]	LT STb (6), STa STb (13), STa (2), STb (3), STa STb VT2v (10), STb VT2v (1), VT1 (1)

[a] Six $P987^{+}$ strains and two $4P^{-}$ strains that were originally STa^{+} in the IMT lost the STa genes during the storage in the laboratory.
[b] Negative for K88, K99, P987, and F41.

only in human ETEC strains, whereas the LT-II gene was observed only in bovine strains (2, 19, 33). However, Celemin et al. (5) found that 30 (25%) of 118 *E. coli* strains isolated from healthy pigs in Spain hybridized with both LT-II and STaH probes. However, when the phenotypic production of LT-II was tested with cell culture assays, all $LT\text{-}II^{+}$ strains gave unclear or negative results, indicating that the results obtained by the hybridization were probably false positives (5). False-positive reactions were also observed in our study when the VT1 DNA probe was used in the hybridization.

STb enterotoxin appears to be a significant contributor to swine diarrhea. In fact, the STb gene is the toxin gene most commonly found in association with ETEC strains from diseased swine in the present study, and this finding is in agreement with those of others (11, 12, 19). However, epidemiological studies to determine the scope and significance of ETEC strains producing STb enterotoxin in diseased piglets have been limited by the lack of simple and rapid assays, since the animal assays are very complicated and time-consuming (11, 16). The PCR protocol outlined in this study was sensitive, specific, and simple and required very little technical time compared with other methodologies for the detection of STb enterotoxin.

Three distinct types of VTs or Shiga-like toxins (SLTs) have been well characterized: VT1 (SLT-I), VT2 (SLT-II), and VT2v (SLT-II variant = VTe) (15). VT1 and VT2 are produced by human and bovine strains associated with hemorrhagic colitis and hemolytic-uremic syndrome in humans (4, 28), whereas VT2v is synthesized by porcine *E. coli* strains responsible for edema disease (13, 18). In the present study, a total correlation between the results obtained with the PCR approach and those determined with the Vero cell assay was observed. In total, 12 strains were verocytotoxic: 11 were $VT2v^{+}$, and 1 was $VT1^{+}$. Thus, our results confirm that porcine *E. coli* strains usually produce the edema VT. Interestingly, 10 of 11 $VT2v^{+}$ strains also presented the STa and STb genes, and the majority of them belonged to serotype O138:K81:H− or O138:K81:H14. Porcine VTEC strains do not appear to be associated with human disease. However, Piérard et al. (24) reported the first episode of human infection with a strain producing the porcine toxin VT2v. The infecting strain belonged to serotype O101:H9. Interestingly, the porcine $VT1^{+}$ strain identified in our study belongs to a serotype (O91:K−:H−) previously found in VTEC strains that cause hemorrhagic colitis in humans (28).

The results obtained in this study indicate that the majority of ETEC strains that cause diarrhea in pigs in Spain belong to classical enteropathogenic serotypes and present the same enterotoxin status as ETEC strains that cause pig diarrhea in other geographical zones. Apparently, in Spain three seropathotypes predominate: (i) O149:K91:H10$K88^{+}$ $LT\text{-}I^{+}$ STb^{+}, (ii) O141:K85ab:H− $P987^{+}$ $STaP^{+}$, and (iii) O138:K81:H14 or H− $STaP^{+}$ $VT2v^{+}$. These three pathotypes are also frequently isolated in other countries, especially the strains of serogroup O149 (9, 12, 29, 32). However, in some studies, LT^{+} STb^{+} $K88^{+}$ strains have decreased in frequency, whereas STa^{+} ($P987^{+}$, $K99^{+}$, or $F41^{+}$) strains have become more prominent (29, 31). In the present study, STa-producing strains with colonization factors other than K88 were nearly twice as common as LT^{+} STb^{+} $K88^{+}$ strains (23 versus 13 strains). Surprisingly, other authors found that porcine ETEC strains isolated in Spain possess atypical characteristics (many belong to serotypes not included among those considered classic enteropathogens, some express both K88 and P987 colonization factors at the same time, and others possess LT-II and STaH enterotoxin genes) and suggest the existence of indigenous serovars and pathotypes which are probably specific for each area (5, 30). These findings could not be confirmed in the present study, because the majority of ETEC strains examined in our study showed the same serotypes and produced the same enterotoxins (LT-I, STaP, and STb) as porcine ETEC strains isolated in other countries (12, 19, 29, 32).

We conclude that porcine ETEC and VTEC strains isolated in Spain belong to the same O:K:H serotypes and possess the same toxic genotypes as strains associated with pig diarrhea or edema disease in other geographical zones. Furthermore, it was concluded that PCR was a fast and reliable method for the identification of enterotoxin and VT genes in clinical and epidemiological studies. The results obtained in our study also have special relevance for the design and development of *E. coli* vaccines against enteric infections in pigs to be applied in Spanish piggeries.

This study was supported by grants from the Xunta de Galicia (XUGA 26105B97 and 26106B97), from the CICYT (AGF92-0570), from the DGICYT (PTR 91-0050), and from the FIS (94/1056). M.B., J.E.B., and A.M. acknowledge the receipt of research fellowships from the DGICYT, Xunta de Galicia, and Universidad de Santiago.

Our grateful thanks is given to Cristina Prado, Marta Rio, and Lucia Fernández for skillful technical assistance.

REFERENCES

1. **Blanco, J., M. Blanco, E. A. González, M. P. Alonso, and J. I. Garabal.** 1990. Comparative evaluation of three tests for the detection of *Escherichia coli* cytotoxic necrotizing factors (CNF1 and CNF2) using filtrates of cultures treated with mitomycin C. FEMS Microbiol. Lett. **69:**311–316.
2. **Blanco, J., M. Blanco, J. I. Garabal, and E. A. González.** 1991. Enterotoxins, colonization factors and serotypes of enterotoxigenic *Escherichia coli* from humans and animals. Microbiol. SEM **7:**57–72.
3. **Blanco, M., J. Blanco, J. E. Blanco, and J. Ramos.** 1993. Enterotoxigenic, verotoxigenic and necrotoxigenic *Escherichia coli* isolated from cattle in Spain. Am. J. Vet. Res. **54:**1446–1451.
4. **Blanco, M., J. Blanco, J. E. Blanco, E. A. González, T. A. T. Gomes, L. F. Zerbini, T. Yano, and A. F. Pestana de Castro.** 1994. Genes coding for Shiga-like toxins in bovine verotoxin-producing *Escherichia coli* (VTEC) strains belonging to different O:K:H serotypes. Vet. Microbiol. **42:**105–110.
5. **Celemin, C., J. Anguita, G. Naharro, and S. Suárez.** 1994. Evidence that *Escherichia coli* isolated from the intestine of healthy pigs hybridize with LT-II, ST-Ib and SLT-II DNA probes. Microb. Pathog. **16:**77–81.
6. **Garabal, J. I., E. A. González, F. Vazquez, J. Blanco, and M. Blanco.** 1995. Toxigenic *Escherichia coli* in Spanish piggeries from 1986 to 1991. Vet. Microbiol. **47:**17–25.
7. **Garabal, J. I., E. A. González, F. Vazquez, J. Blanco, M. Blanco, and J. E. Blanco.** 1996. Serogroups of *Escherichia coli* isolated from piglets in Spain. Vet. Microbiol. **48:**113–123.
8. **González, E. A., and J. Blanco.** 1986. Colonization antigens, antibiotic resistance and plasmid content of enterotoxigenic *Escherichia coli* isolated from piglets with diarrhoea in Galicia (north-western Spain). Vet. Microbiol. **11:** 271–283.
9. **Guinée, P. A. M., and W. H. Jansen.** 1979. Detection of enterotoxigenicity and attachment factors in *Escherichia coli* strains of human, porcine and

bovine origin: a comparative study. Zentralbl. Bakteriol. Hyg. A **243:**245–257.
10. **Guinée, P. A. M., W. H. Jansen, T. Wadström, and R. Sellwood.** 1981. *Escherichia coli* associated with neonatal diarrhoea in piglets and calves. Curr. Top. Vet. Anim. Sci. **13:**126–162.
11. **Handl, C. E., E. Olsson, and J. I. Flock.** 1992. Evaluation of three different STb assays and comparison of enterotoxin pattern over a five-year period in Swedish porcine *Escherichia coli*. Diagn. Microbiol. Infect. Dis. **15:**505–510.
12. **Harel, J., H. Lapointe, A. Fallara, L. A. Lortie, M. Bigras-Poulin, S. Larivière, and J. M. Fairbrother.** 1991. Detection of genes for fimbrial antigens and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with diarrhea. J. Clin. Microbiol. **29:**745–752.
13. **Johnson, W. M., D. R. Pollard, H. Lior, S. D. Tyler, and K. B. Rozee.** 1990. Differentiation of genes coding for *Escherichia coli* verotoxin 2 and the verotoxin associated with porcine edema disease (VTe) by the polymerase chain reaction. J. Clin. Microbiol. **28:**2351–2353.
14. **Lee, C. H., S. L. Moseley, H. W. Moon, S. C. Whipp, C. L. Gyles, and M. So.** 1983. Characterization of the gene encoding heat-stable toxin II and preliminary molecular epidemiological studies of enterotoxigenic *Escherichia coli* heat-stable toxin II producers. Infect. Immun. **42:**264–268.
15. **Lin, Z., H. Kurazomo, S. Yamasaki, and Y. Takeda.** 1993. Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. Microbiol. Immunol. **37:**543–548.
16. **Lortie, L. A., J. Harel, J. M. Fairbrother, and J. D. Dubreuil.** 1991. Evaluation of three new techniques for the detection of STb-positive *Escherichia coli* strains. Mol. Cell. Probes **5:**271–275.
17. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10:**296–298.
18. **Marques, L. R. M., J. S. M. Peiris, S. J. Cryz, and A. D. O'Brien.** 1987. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. FEMS Microbiol. Lett. **44:**33–38.
19. **Moon, H. W., R. A. Schneider, and S. L. Moseley.** 1986. Comparative prevalence of four enterotoxin genes among *Escherichia coli* isolated from swine. Am. J. Vet. Res. **47:**210–212.
20. **Moseley, S. L., P. Echeverria, J. Seriwatana, C. Typarat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow.** 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. **145:**863–869.
21. **Newland, J. W., and R. J. Neill.** 1988. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26:**1292–1297.
22. **Olsvik, Ø., and N. A. Strockbine.** 1993. PCR detection of heat-stable, heat-labile, and Shiga-like toxin genes in *Escherichia coli*, p. 271–276. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology. Principles and applications. American Society for Microbiology, Washington, D.C.
23. **Pickett, C. L., E. M. Twiddy, B. W. Belisle, and R. K. Holmes.** 1986. Cloning of genes that encode a new heat-labile enterotoxin of *Escherichia coli*. J. Bacteriol. **165:**348–352.
24. **Piérard, D., L. Huyghens, S. Lauwers, and H. Lior.** 1991. Diarrhoea associated with *Escherichia coli* producing porcine oedema disease verotoxin. Lancet **338:**762.
25. **Pollard, D. R., W. M. Johnson, H. Lior, S. D. Tyler, and K. R. Rozee.** 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. J. Clin. Microbiol. **28:**540–545.
26. **Rigby, P. W. J., M. Diekman, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase. J. Mol. Biol. **113:**237–251.
27. **Schultsz, C., G. J. Pool, R. van Ketel, B. de Wevek, P. Speelman, and J. Dankert.** 1994. Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. J. Clin. Microbiol. **32:**2393–2397.
28. **Smith, H. R., and S. M. Scotland.** 1988. Vero cytotoxin-producing strains of *Escherichia coli*. J. Med. Microbiol. **26:**77–85.
29. **Söderlind, O., B. Thafvelin, and R. Möllby.** 1988. Virulence factors in *Escherichia coli* strains isolated from Swedish piglets with diarrhea. J. Clin. Microbiol. **26:**879–884.
30. **Suárez, S., C. Paniagua, I. Ørskov, and F. Ørskov.** 1988. Unusual serotypes of *Escherichia coli* from piglet diarrhea in North Spain. Vet. Microbiol. **17:**375–377.
31. **Supar, R. G. Hirst, and B. E. Patten.** 1991. The importance of enterotoxigenic *Escherichia coli* containing the 987P antigen in causing neonatal colibacillosis in piglets in Indonesia. Vet. Microbiol. **26:**393–400.
32. **Wilson, R. A., and D. H. Francis.** 1986. Fimbriae and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with colibacillosis. Am. J. Vet. Res. **47:**213–217.
33. **Woodward, M. J., P. J. Carroll, and C. Wray.** 1992. Detection of entero- and verocyto-toxin genes in *Escherichia coli* from diarrhoeal disease in animals using the polymerase chain reaction. Vet. Microbiol. **31:**251–261.