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Differentiation of Genes Coding for *Escherichia coli* Verotoxin 2 and the Verotoxin Associated with Porcine Edema Disease (VTe) by the Polymerase Chain Reaction

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Two sets of synthetic oligonucleotide primers were used in a polymerase chain reaction adaptation to distinguish the closely related genes for type 2 verotoxin (VT2 or Shiga-like toxin [SLT-II]) and the verotoxin associated with porcine edema disease (VTe or SLT-II variant [SLT-IIv]) in *Escherichia coli*.

Strains of *Escherichia coli* producing a family of related cytotoxins are termed verotoxigenic *E. coli* (VTEC). Clinically, VTEC has been closely associated with sporadic diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (6). In animals, VTEC has been associated with porcine edema disease, a frequently fatal disease of weanling pigs (2, 7, 9).

Three antigenically distinct verotoxins (VT) or Shiga-like toxins (SLT), i.e., VT1 (SLT-I), VT2 (SLT-II), and VTe (SLT-II variant, [SLT-IIv]), have been isolated, purified, and characterized (6). VT1 and VT2 are cytotoxic to HeLa and Vero cells, whereas VTe is cytotoxic only to Vero cells (9). Although toxin-converting bacteriophages have been described for both VT1 and VT2, none have been isolated from VTe-producing porcine edema disease strains of *E. coli*, and the genes are likely chromosomal (8, 9). Cloning and sequencing of the VT genes have been completed, and the A toxin subunit genes of VT2 and VTe were found to be highly homologous (94%), whereas the B toxin subunit genes were less homologous (79 to 80%) (3, 14).

In this article, we report the development of a polymerase chain reaction (PCR) adaptation which will rapidly and specifically distinguish VT2 and VTe genes in VTEC. The PCR protocol was tested with template nucleic acid (NA) extracted from reference *E. coli* and VTEC.

A list of *E. coli* strains tested and their sources appears in Table 1. Details of serotypes, growth conditions, VT and enterotoxin determinations, NA isolation, and general PCR conditions were previously described (13). The PCR for the two sets of primers varied only in terms of annealing temperature, with 2 min at 55°C being optimal for VT2 and 2 min at 45°C being optimal for VTe. VTe production was confirmed by lack of cytotoxicity to HeLa cells and neutralization by polyclonal anti-VT2 and anti-VTe.

A summary of cytotoxicity to Vero and HeLa cells, toxigenicity, and PCR probe results appears in Table 1. Primers used in a previous study for VT2 (13) were directed at a 346-base-pair (bp) fragment of the *slt*-IIA gene. VTe primers described in Table 2 delineated at 230-bp fragment encompassing regions for the proposed promoter and a portion of the A toxin subunit of the *slt*-IIvA gene (3, 14). Figure 1 shows the presence and distribution of the two amplified products when NA samples from representative VTEC strains of human and nonhuman origin were used as template. The sensitivity limit of the PCR application in detecting VT2 or VTe genes was 1 ng of total NA for VT2 and 100 pg for VTe (data not shown). There was no correlation between titers of VT and sensitivity of gene detection.

The identification of VTEC strains carrying VT2 or VTe genes correlated without exception with VT activity determined by toxin-specific-antibody neutralization and differential cytotoxicity of the VTe to Vero and HeLa cells. The PCR clearly identified 31 human and nonhuman VT2-producing and 16 nonhuman VTe-positive strains of *E. coli* and did not give amplification with NA from VT1-producing strains or from 3 strains of Shiga-toxin-positive *Shigella dysenteriae* 1 (data not shown). NA samples from reference nontoxigenic, toxigenic, invasive, adherent, cytotoxic (4), and hemolytic strains of *E. coli* were negative in the PCR. When NA from nine *E. coli* isolates from ground pork were used, the PCR clearly distinguished three groups of VTEC, with four isolates confirmed as VTe-positive, four confirmed as VT2-positive, and one confirmed as VT1-producing (13). None of the human VTEC strains tested in this study were positive in PCR tests with the VTe primers.

Although most DNA hybridization probes described to date have failed to differentiate VT2- and VTe-producing VTEC (1, 5, 8, 10, 11), variation in the degrees of stringency has permitted identification of *E. coli* isolates producing VT2 and VTe at 53°C but not at 45°C (1). Using these probes, Brown et al. reported VTe-producing isolates of *E. coli* associated with human disease, thereby suggesting a possible pathogenic role for VTe in humans (1). Our results with the PCR corroborate previous views of a host-specific pathogenic role for VTe-positive VTEC in pigs (2, 3, 6–8). None of the human VTEC produced VTe or contained *slt*-IIv gene sequences detected by the VTe primers, and all VTe strains were consistently associated with porcine edema disease or ground pork.

PCR results using NA template from a hemolytic uremic syndrome isolate of *E. coli* O91:H21 identical with that described by Oku et al. (12) appear in lanes f of Fig. 1A (VT2 primers) and Fig. 1B (VTe primers). Oku et al. tentatively designated this strain a VT2 human variant in view of remarkably reduced cytotoxicity to HeLa cells compared with cytotoxicity to Vero cells. The PCR clearly established the presence of the VT2 gene, which produced a VT expressing reduced toxicity for HeLa cells. A similar observation

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TABLE 1. Summary of organisms, cytotoxicity, toxin profiles, and PCR probe results

E. coli strain type and serotype[a]	No. of strains	Cytotoxicity to[b]:		Toxin(s)[c]	Result of PCR amplification	
		Vero cells	HeLa cells		VT2 (346 bp)	VTe (230 bp)
Human						
—	5	+	+	VT2	+	−
O91:H21	1	+	+	VT2, CLDT	+	−
—	17	+	+	VT1, VT2	+	−
O157:H43	1	−	−	LT	−	−
O111:H2[d]	1	−	−	None	−	−
—[e]	2	−	−	None	−	−
O22:H43	1	−	−	HLY	−	−
—	2	−	−	CLDT	−	−
—	5	−	−	None	−	−
Nonhuman						
O2:H−	3	+	−	VTe	−	+
O2:H32	1	+	−	VTe	−	+
O8:H9[f]	2	+	−	VTe	−	+
O8:H19[f]	1	+	−	VTe	−	+
O65:H9	1	+	−	VTe	−	+
O120:H−	1	+	−	VTe	−	+
O121:H−	1	+	−	VTe	−	+
O157:H19	2	+	−	VTe	−	+
O?:H−	1	+	−	VTe	−	+
O?:H−[f]	1	+	−	VTe	−	+
O1:H20[f]	1	+	+	VT2	+	−
O2:H29[f]	1	+	+	VT2	+	−
O6:H34[f]	1	+	+	VT2	+	−
O82:H8[f]	1	+	+	VT1	−	−
O91:H21	1	+	+	VT1, VT2, CLDT	+	−
O132:H−	1	+	+	VT2	+	−
O?:H2[f]	1	+	+	VT2	+	−
O157:H10	1	−	−	None	−	−
Reference (strain)						
O26:H11 (H19)	1	+	+	VT1	−	−
OI57:H− (E32511)	1	+	+	VT2	+	−
O139:H1 (412)	1	+	−	VTe	−	+
— [*E. coli*(pCG6)]	1	+	−	VTe	−	+
— [*E. coli*(pEB1)]	1	+	+	VT2	+	−
O25:H− (TD427c2)	1	−	−	LT	−	−
O128 (TD213c2)	1	−	−	ST	−	−
O78:H11 (H10407)	1	−	−	LT, ST	−	−
— (HB101)	1	−	−	None	−	−
— (C600)	1	−	−	None	−	−
O6:H1 (ATCC 25992)	1	−	−	None	−	−

[a] —, Serotypes not specified for individual isolates; see reference 13.
[b] Cytotoxicity to monolayers of Vero or HeLa cells.
[c] LT, Heat-labile enterotoxin; ST, heat-stable enterotoxin; CLDT, cytolethal distending toxin; HLY, hemolytic *E. coli* strain.
[d] Strain demonstrates localized adherence in HeLa and HEp-2 cells.
[e] Invasive *E. coli* strains.
[f] Isolated from ground pork.

involving reduced HeLa cell cytotoxicity included two human strains of *E. coli* O157:H7 isolated from patients with nonbloody, watery diarrhea (5). These strains produced VT which was not neutralized by rabbit anti-VT2 to *E. coli* 933W but which did hybridize with an oligonucleotide probe constructed from the *E. coli* 933 *slt*-IIA sequence, thereby leading the authors to speculate on a new category of VT. Application of the PCR protocol to these particular strains may differentiate these genes as VTe or VT2 analogous to those of the *E. coli* O91:H21.

TABLE 2. Base sequences, locations and predicted sizes of amplified products for the VT-specific oligonucleotide primers

Primer	Oligonucleotide sequence (5′–3′)[a]	Location within gene[b]	Size of amplified product (bp)
VT2-a	ttaaccacacccacggcagt	426–445	
VT2-b	gctctggatgcatctctggt	752–771	346
VTe-a	ccttaactaaaaggaatata	217–236	
VTe-b	ctggtggtgtatgattaata	427–446	230

[a] Sequences derived from *slt*-II and *slt*-IIv genes (3, 14).
[b] In nucleotides.

In animals, VTEC has commonly been encountered in bovine and porcine reservoirs and has been shown to have pathogenic potential in piglets. PCR technology has now been applied to the rapid and specific detection of three distinct verotoxin genes coding for VT1, VT2, and VTe across a wide spectrum of VTEC strains. Although VT2 and VTe genes share extensive sequence homology, they can now be differentiated and clearly distinguished by the PCR. The capacity to rapidly and inexpensively differentiate VT2-

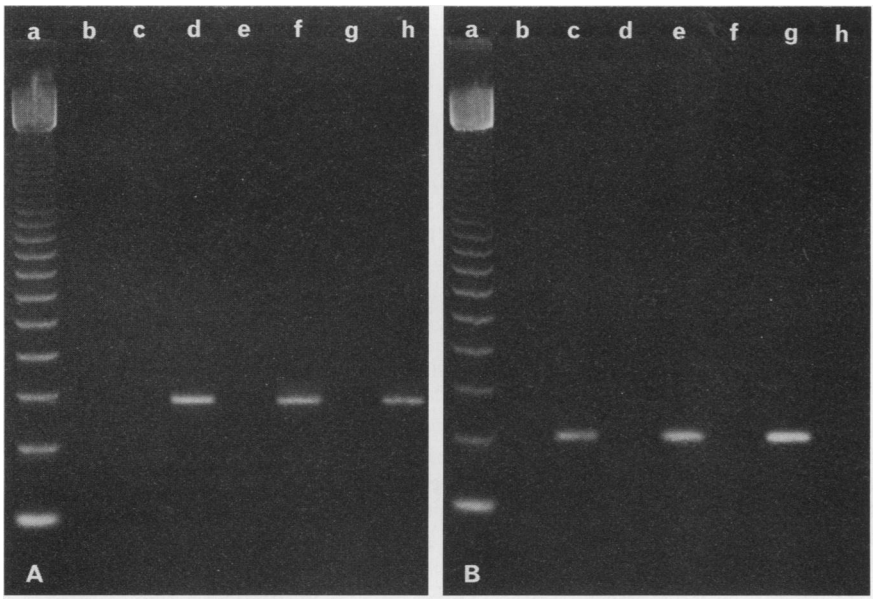

FIG. 1. Occurrence and distribution of PCR amplification products, 346-bp and 230-bp fragments, which specifically detect VT2 and VTe genes, respectively. PCR was performed with VT2 (A) and VTe (B) primers. Lanes: a, 123-bp ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); b, *E. coli* O6:H12 (VT negative); c, *E. coli* O139:H1 (VTe reference strain 412); d, *E. coli* O157:H− (VT2 reference strain E32511); e, *E. coli* O2:H− (VTe); f, *E. coli* O91:H21 (VT2); g, *E. coli* O65:H9 (VTe); h, *E. coli* O157:H7 (VT1 plus VT2).

and VTe-producing VTEC will allow microbiologists and epidemiologists to definitively classify VTEC isolates from certain meats and meat products and help identify any phenotypic variants.

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LITERATURE CITED

1. **Brown, J. E., O. Sethabutr, M. P. Jackson, S. Lolekha, and P. Echeverria.** 1989. Hybridization of *Escherichia coli* producing Shiga-like toxin I, Shiga-like toxin II, and a variant of Shiga-like toxin II with synthetic oligonucleotide probes. Infect. Immun. **57**:2811–2814.
2. **Dobrescu, L.** 1983. New biological effect of edema disease principle (*Escherichia coli* neurotoxin) and its use as an in-vitro assay for this toxin. Am. J. Vet. Res. **44**:31–34.
3. **Gyles, C. L., S. A. De Grandis, C. MacKenzie, and J. L. Brunton.** 1988. Cloning and nucleotide sequence analysis of the genes determining verocytotoxin production in a porcine edema disease isolate of *Escherichia coli*. Microb. Pathog. **5**:419–426.
4. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. Microb. Pathog. **4**:103–113.
5. **Karch, H., and T. Meyer.** 1989. Evaluation of oligonucleotide probes for identification of Shiga-like-toxin-producing *Escherichia coli*. J. Clin. Microbiol. **27**:1180–1186.
6. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2**:15–38.
7. **Linggood, M. A., and J. M. Thompson.** 1987. Verotoxin production among porcine strains of *Escherichia coli* and its association with oedema disease. J. Med. Microbiol. **25**:359–362.
8. **Mainil, J., G. Daube, P. Deprez, A. Kaeckenbeeck, and P. Pohl.** 1989. Detection and identification of pathotypes of verocytotoxigenic *Escherichia coli* isolated from weaned piglets using gene probes for seven *E. coli* toxins. FEMS Microbiol. Lett. **59**: 345–350.
9. **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.
10. **Meyer, T., M. Bitzan, O. Sandkamp, and H. Karch.** 1989. Synthetic oligodeoxyribonucleotide probes to detect verocytotoxin-producing *Escherichia coli* in diseased pigs. FEMS Microbiol. Lett. **57**:247–252.
11. **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.
12. **Oku, Y., T. Yutsudo, T. Hirayama, A. D. O'Brien, and Y. Takeda.** 1989. Purification and some properties of a Vero toxin from a human strain of *Escherichia coli* that is immunologically related to Shiga-like toxin II (VT2). Microb. Pathog. **6**:113–122.
13. **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.
14. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J. Bacteriol. **170**:4223–4230.