JOURNAL OF CLINICAL MICROBIOLOGY, July 1993, p. 1700–1703
0095-1137/93/071700-04$02.00/0

Vol. 31, No. 7

Use of Digoxigenin-Labelled Oligonucleotide DNA Probes for VT2 and VT2 Human Variant Genes To Differentiate Vero Cytotoxin-Producing *Escherichia coli* Strains of Serogroup O157

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Digoxigenin-labelled oligonucleotide DNA probes specific for B-subunit genes of Vero cytotoxin 2 (VT2) and a variant of VT2 (VT2vha) were used to differentiate 116 strains of *Escherichia coli* serogroup O157 belonging to phage types 1, 2, 4, 8, 14, and 49. Of these strains, 38% had sequences for both VT2 and VT2vha, 38% had sequences for VT2 only, and 24% had sequences for VT2vha only. Oligonucleotide probe hybridization subdivided strains of all of the phage types except phage type 1. The greatest variation in toxin gene pattern was observed with strains of phage type 14, for which there were six distinct patterns when the presence or absence of VT1 genes was also considered. Two strains from each phage type group were examined for bacteriophages encoding VT production. Two of the six VT2vha-producing strains carried phage from which DNA hybridized with the VT2vha-specific probe. Phages were not detected in the remaining four VT2vha strains, suggesting that genes may be chromosomally located or associated with a defective prophage. In contrast, seven of the eight VT2 strains carried phages from which DNA hybridized with the VT2-specific probe. Two strains of E32511 (O157:H$^-$) were also investigated. One strain (E32511A) possessed gene sequences for both VT2 and VTvha and was shown to carry phage possessing gene sequences for VT2. With strain E32511B, however, phages were not detected and DNA hybridized only with the VT2vha probe. Analysis of total genomic DNA digested with restriction endonuclease *Eco*RI showed that polymorphisms were seen with VT2 strains and not with VT2vha strains.

Infection with Vero cytotoxin-producing *Escherichia coli* (VTEC) is an important cause of hemorrhagic colitis and hemolytic uremic syndrome. Strains of VTEC associated with bloody diarrhea are also referred to as enterohemorrhagic *E. coli* (11, 17).

Strains of *E. coli* serogroup O157 are most frequently associated with VTEC infection, although other serogroups have been implicated (11, 19). Studies in the United Kingdom have shown that isolates of *E. coli* O157 referred to the Laboratory of Enteric Pathogens have increased from 1 in 1982 to 532 in 1991 (23). Two immunologically distinct Vero cytotoxins (VT1 and VT2) are produced either singly or in combination. Since VT1 is almost identical to Shiga toxin, VT1 is often referred to as Shiga-like toxin I (SLT-I), and VT2 is often referred to as SLT-II. Gene sequences for VT1 and VT2 demonstrate 58% gene sequence homology (8), and VTEC strains can therefore be differentiated by using DNA probes.

In recent years, variants to VT2 have been described. VT2 is cytotoxic to both Vero and HeLa cells, whereas variants of VT2 usually show much-reduced or no cytotoxicity to HeLa cells. Five distinct toxin variants associated with human disease have been identified on the basis of gene sequence analysis, namely, VT2vha and VT2vhb from strain B2F1 of serotype O91:H21 (7), VTev (originally referred to as SLT-IIva) from strain H.1.8 of serotype O128:H$^-$ (4), SLT-IIc from strain E32511 of serotype O157:H$^-$ (18) (also referred to as VT2v [6]), and, most recently, SLT-IIvhc from strain 7279 of serotype O157:H7 (14). Sequence analysis by Schmitt et al. (18) of strain E32511 revealed that two VT genes were present, one that was identical to VT2 (except for 1 bp) and a second gene of which the A subunit was identical to VT2 and the B subunit was identical to VT2vha. The VT2 genes from two strains of *E. coli* O139 causing swine edema disease in pigs have also been sequenced and were identical; the toxins were termed VTe (5, 26). Sequence analysis of the B-subunit genes has shown that VT2vha demonstrates 95.5% sequence homology with VT2 and 82.8% homology with VTe. However, VTev exhibits 98% homology with VTe and 78.1% with VT2, suggesting that VT2vha is a variant of VT2 and VTev is a variant of VTe.

In recent years, molecular methods involving the use of polymerase chain reaction gene amplification, oligonucleotide DNA probes, and restriction enzyme analysis have been described to differentiate toxin gene type (1, 6, 9, 10, 15, 18, 25). All currently reported VT2 sequences, oligonucleotide probes, and polymerase chain reaction primers are summarized in a review by Smith et al. (20).

For epidemiological investigations, subdivision of VTEC serogroup O157 strains is achieved by phage typing (3, 12). A phage type (PT) is determined by the pattern of lysis caused by a set of 16 typing phages, and at least 66 PTs are recognized, of which 23 have been isolated in the United Kingdom (2). The most common United Kingdom PTs are 1, 2, 4, 8, 14, and 49, which, between 1982 and 1991, accounted for 91% of 1,425 VTEC serogroup O157 strains. Other epidemiological markers such as plasmid profiles and colicin types can be used during the investigation of outbreaks.

This study describes how digoxigenin-labelled oligonucle-

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otide DNA probes specific for gene sequences for VT2 and VT2vha were used to subdivide a collection of *E. coli* O157 strains belonging to six common PTs. The carriage of bacteriophages encoding VT production as well as the hybridization of the specific probes to restriction enzyme digest fragments of genomic DNA was also investigated.

MATERIALS AND METHODS

Bacterial strains. All randomly chosen isolates of certain PTs were from subjects with diarrhea, bloody diarrhea, or hemolytic uremic syndrome in the United Kingdom from 1989 to 1991. All strains belonged to serogroup O157, possessed flagellum antigen H7, or were nonmotile. The strains also hybridized with a digoxigenin (or ^{35}S)-labelled 0.85-kb *Sma*I-*Pst*I polynucleotide DNA probe specific for VT2 gene sequences. The presence of gene sequences for VT1 was determined by hybridization with a 0.75-kb *Hin*cII polynucleotide DNA probe specific for VT1 sequences (24, 27).

Phage typing was performed by the methods described previously (3). The panel of *E. coli* O157 strains examined included 30 of PT1, 35 of PT2, 29 of PT4, 26 of PT8, 30 of PT14, and 26 of PT49 (Table 1).

Sample preparation. (i) Cultures. Bacterial cultures were grown in broth overnight and then spotted in a grid formation on nylon membranes (Hybond N). After growth for 4 to 5 h, the cells were lysed, the DNA was denatured, and then the DNA was fixed to the membrane by baking (13).

(ii) Genomic DNA. Genomic DNA was prepared by the method of Stanley et al. (22). Purified DNA was digested with the restriction enzyme *Eco*RI, and fragments were separated on an 0.8% agarose gel in acetate buffer. Fragments were transferred to a nylon membrane by Southern blotting (21).

Detection of bacteriophages carrying VT genes. Phages were isolated by collecting the supernatant of centrifuged bacterial cultures. Phages were transfected into the phage-sensitive strain PAP1192μ (16). If no plaques were detected or numbers were low, induction with mitomycin (1 μg/ml) was performed. Phages were also purified by picking a single plaque into a log-phase growth of PAP1192μ and proceeding as described before. Phages were transferred onto nylon membranes by placing the membrane on the plaques for 1 min. DNA was released and denatured as described for the colony blots.

Oligonucleotide DNA probes. Probe sequences from regions of heterology internal to B-subunit gene sequences for VT2 and VT2vha were chosen from published data. VT2 was taken from positions 1320 to 1344 (5′-TAC AGT GAA GGT TGA CGG GAA AGA A-3′) (8), and VT2vha was taken from positions 1323 to 1347 (5′-CAC AGT AAA AGT GGC CGG AAA AGA G-3′) (7). The sequence which hybridizes with VT2vha would also hybridize with VT2vhb since they are identical in this region except for 1 nucleotide. Throughout this article, the probe will be referred to as a VT2vha probe, and it does not hybridize with any other VT2 variants such as VTe or VTev. Oligonucleotides were made with a DNA synthesizer (Applied Biosystems), and concentrations were calculated by their extinction coefficients and optical densities (13). The melting temperature was calculated as an indication of optimal hybridization temperatures.

Digoxigenin molecules were attached to the 3′ end of each oligonucleotide with terminal transferase. Two different procedures were used, the second being a modification of the first. For a 25-μl reaction volume, 35 pmol of DNA, $CoCl_2$ (5 mM), tailing buffer (Boehringer Corporation Limited), digoxigenin-dUTP (50 μM), and terminal transferase (50 U) were combined. Labelling took place at 37°C for 1.5 h. The probe was then ready for use. Alternatively, a reaction mixture was prepared as described above, except that 100 pmol of DNA was used, the labelling reaction was stopped by the addition of EDTA (0.2 M), and the probe was precipitated with LiCl (4 M) and ethanol as described by Boehringer Corporation Limited. Concentrations of labelled probe were estimated by electrophoresis with a 2% agarose gel and then by Southern blotting and immunological detection. Probes were stored at −20°C.

TABLE 1. Distribution of oligonucleotide gene sequences for VT2 and VT2vha within six PT groups

PT (total no. of strains)	Distribution of gene sequences			Strains with this distribution of gene sequences	
	VT1 (polynucleotide probe)	VT2 (oligonucleotide probe)	VT2vha (oligonucleotide probe)	No.	%
1 (30)	+	+	−	30	100
2 (35)	−	+	+	27	77
	−	+	−	6	17
	+	+	−	2	6
4 (29)	+	+	−	17	59
	−	+	+	7	24
	−	−	+	4	14
	−	+	−	1	3
8 (26)	+	+	+	1	4
	+	+	−	1	4
	+	−	+	23	88
	−	−	+	1	4
14 (30)	+	+	+	3	10
	+	+	−	8	27
	+	−	+	8	27
	−	+	+	4	13
	−	+	−	2	6
	−	−	+	5	17
49 (26)	−	+	+	24	92
	−	−	+	2	8

Oligonucleotide hybridization. Membranes were hybridized overnight with oligonucleotide probes (24) by using a probe concentration of approximately 30 to 50 pmol/ml and a hybridization temperature of 65°C. Hybrids were detected by using an antidigoxigenin antibody conjugated to alkaline phosphatase, which catalyzed the oxidation and reduction of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride, respectively.

RESULTS AND DISCUSSION

Oligonucleotide hybridization. One hundred seventy-six strains of *E. coli* O157, determined by polynucleotide DNA probe as having gene sequences for VT2, were examined by using oligonucleotide probes specific for sequences within the B subunit of VT2 and VT2vha toxin genes. Differences in hybridization patterns were observed in all PTs, except PT1, and are detailed in Table 1. All strains of PT1 possessed sequences for VT1 and VT2. PT2 and PT49, the most common PTs in the United Kingdom, were similar in that more than 77% of the strains examined carried sequences for both VT2 and VT2vha, the remainder for PT2 being VT2 and the remainder for PT49 being VT2vha. Two of the PT2 strains carried sequences for VT1. This contrasts strongly with PT8, for which 24 of 26 strains (92%) carried sequences

TABLE 2. Summary of VT gene type

VT type	Strains with VT type	
	No.	%
VT1, VT2, VT2vha	4	2
VT1, VT2	58	33
VT1, VT2vha	31	18
VT2, VT2vha	62	35
VT2	9	5
VT2vha	12	7

for VT2vha alone, only 1 strain carried sequences for VT2 alone, and 1 strain carried sequences for VT2 and VT2vha. In addition, all of these PT8 strains, except one, carried sequences for VT1. Within PT4, strains producing VT2 only were more common (62%), with strains producing VT2 and VT2vha (24%) and those producing VT2vha (14%) occurring less frequently. All except one of the PT4 strains positive with the VT2 oligonucleotide also carried genes for VT1. PT14 was the most diverse group, with six combinations, none of which contributed more than 27% of the total when the presence or absence of VT1 genes was taken into consideration.

There appears to be a relationship between the presence of the VT1 gene and the most likely VT2 gene combination (Table 2). There were 93 strains with VT1 genes (PT1, -2, -4, -8, and -14), and of these, only 4 (4%) had sequences for both VT2 and VT2vha. This compared with the 83 VT1-negative strains, of which 62 (75%) had both VT2 and VT2vha sequences. There were almost twice as many strains with both VT1 and VT2 sequences than strains with VT1 and VT2vha sequences. Of the VT1-negative strains, there were more strains with VT2vha sequences than strains with VT2 sequences. Irrespective of VT1 production, VT2 only or VT2 and VT2vha together were the most frequently encountered combinations (each at 38%), followed by VT2vha (24%).

Frost et al. (3) suggested that there is a relationship between the carriage of toxin-determining phage and the PT of the organism, since some VT2-encoding phages are known to restrict the typing phages. Some strains have been known to convert to other PTs. The most variable PT is PT1, which can give rise to PT4, -8, and -14 (3). In the current study, certain strains of PT4 and -14 (those which are VT1 and VT2) could have arisen from PT1. However, those with VT2vha sequences were not derived directly from PT1 since all PT1 strains had only VT2 sequences. PT1 can convert to PT8 with the loss of VT2. The strains of PT8 examined in this study did not therefore arise from PT1 (Table 1).

Bacteriophages carrying VT genes. Two strains from each PT were investigated further (Table 3). Phages encoding toxin genes were identified by using the specific oligonucleotide probes. All VT2 strains, except one PT2 strain, carried phages which hybridized with the VT2 oligonucleotide probe. Plaques hybridizing with the VT2vha probe were detected in only two of the six VT2 variant strains. This may be because the genes were chromosomally located, the number of phages was too low to be detected, or the genes were associated with a defective prophage. VT2-encoding bacteriophages were detected in strain E32511A but not in E32511B, suggesting that E32511B was derived from E32511A by loss of a bacteriophage encoding VT2. Transduction and cytotoxicity tests would need to be performed to show that the phages with toxin genes were actually toxin converting.

Restriction enzyme fragment analysis. Southern hybridization of genomic DNA digests showed that of the strains described above, those which carried VT2 genes, including E32511A, had an *Eco*RI fragment of either 4.6 (PT2), 4.9 (PT1, -4, -8, -14, and -49), or 6.5 (PT49) kb which hybridized with the VT2-specific oligonucleotide probe. Reference has already been made to the relatedness of PT1, -4, -8, and -14, which may explain why all of the strains of these types possessed a VT2-hybridizing *Eco*RI fragment of the same size. Strain E32511A, however, also has a hybridizing fragment of 4.9 kb and is a PT49 strain, but the other VT2-producing strain of PT49 had a VT2-probe-hybridizing fragment of 6.5 kb. All VT2vha strains (including E32511A and E32511B) possessed one fragment of 5.8 kb which hybridized with the VT2vha oligonucleotide probe. With the exception of two strains, all of the VT2 variant genes would

TABLE 3. Further investigations with two strains from each PT group and E32511

Strain	PT	VT1 genes	VT2 type (colony hybridization)	Plaque hybridization[a]	*Eco*RI restriction		
					No. of fragments	Fragment size (kb)	VT2 type[b]
E62388	1	+	VT2	+ (VT2)	1	4.9	VT2
E72490	1	+	VT2	+ (VT2)	1	4.9	VT2
E65235	2	−	VT2	+ (VT2)	1	4.6	VT2
E65520	2	−	VT2, VT2vha	−	2	4.6	VT2
						5.8	VT2vha
E58866	4	+	VT2	+ (VT2)	1	4.9	VT2
E60268	4	−	VT2vha	+ (VT2vha)	1	5.8	VT2vha
E61091	8	+	VT2	+ (VT2)	1	4.9	VT2
E62438	8	+	VT2vha	+ (VT2vha)	1	5.8	VT2vha
E66729	14	+	VT2	+ (VT2)	1	4.9	VT2
E66143	14	−	VT2vha	−	1	5.8	VT2vha
E65236	49	−	VT2vha	−	1	5.8	VT2vha
E65341	49	−	VT2, VT2vha	+ (VT2)	2	6.5	VT2
						5.8	VT2vha
E32511A	49	−	VT2, VT2vha	+ (VT2)	2	4.9	VT2
						5.8	VT2vha
E32511B	49	−	VT2vha	−	1	5.8	VT2vha

[a] Oligonucleotide probe hybridizing to plaques.
[b] Oligonucleotide probe hybridizing to *Eco*RI fragments.

appear to be chromosomally located or associated with a defective prophage because no phages were detected.

Within common PTs, there are a number of toxin gene combinations. Since VT2vha and VT2vhb are not identical (99% homology), one can subdivide the strains further by restriction fragment length polymorphism analysis of polymerase chain reaction products as described by Tyler et al. (25). A number of *E. coli* O157 strains have been examined by other workers. All 24 of the *E. coli* O157 strains (except E32511) studied by Hii et al. (6) carried only VT2 genes. Tyler et al. (25) examined eight O157 strains, all of which carried VT2vha genes. Examination of E32511 showed that both VT2 and VT2vha sequences were present. Schmitt et al. (18) examined 20 VT2-producing *E. coli* O157 strains for the number of fragments that were VT2 A subunit probe positive. Four had two fragments, suggesting that both gene types (VT2 and VT2vha) were carried or that genes were present in duplicate. The E32511 strain described by Schmitt et al. is thought to be the same as strain E32511A in our study, for which we also found the 4.9-kb *Eco*RI fragment to hybridize with the VT2 probe and the 5.8-kb fragment to hybridize with the VT2vha probe. E32511B is thought to be the same as that strain described by Hii et al. (6) which carried genes which hybridized only with the VT2v probe.

Polynucleotide DNA probes are particularly useful for screening for VTEC because they are not affected by small gene variations. The oligonucleotide probes, however, can provide important information about the diversity of the VT2 and VT2 variant genes and, as such, should prove useful for epidemiological investigations of infections with common phage types.

ACKNOWLEDGMENT

We thank the Virus Reference Division, Central Public Health Laboratory, for making the oligonucleotide DNA sequences.

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