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# Evaluation of Oligonucleotide Probes for Identification of Shiga-Like-Toxin-Producing *Escherichia coli*

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**Four synthetic oligonucleotide probes representing different regions of the Shiga-like toxin I (SLT-I) structural genes and one oligonucleotide derived from the SLT-II gene of *Escherichia coli* serotype O157:H7 strain 933 were examined for the identification of *E. coli* strains that produce cytotoxins for Vero or HeLa cells. *E. coli* strains that synthesize SLT-I alone or O157:H7 isolates that coexpress SLT-I and SLT-II hybridized with all four probes that were complementary to the SLT-I genes, suggesting that they have toxin genes with great homology in all the regions examined. In colony hybridization tests, these oligonucleotide probes did not react with *E. coli* strains that were nontoxigenic for Vero cells or that produced cytotoxins belonging to the SLT-II family. The probe derived from the *slt-IIA* gene distinguished *E. coli* strains that produced SLT-II alone from SLT-I-producing strains and hybridized to all *E. coli* O157:H7 strains that produced both SLT-I and SLT-II. Using two of these oligonucleotide probes that were complementary to *slt-IA* or *slt-IIA* sequences, we identified 50 of 52 cytotoxin-producing strains, whereas none of 416 nontoxigenic *E. coli* strains was reactive. The colony blot hybridization with the oligonucleotide probes described here can serve as a specific and sensitive test with potential diagnostic value.**

*Escherichia coli* strains that produce cytotoxins for HeLa or Vero cells are now recognized as important causes of diarrhea and hemorrhagic colitis (9, 32). Enteral infections with cytotoxigenic *E. coli* have also been correlated with the onset of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (4, 10, 20). The cytotoxins are termed in reference to the Vero cell cytotoxin originally described by Konowalchuk et al. (21) as verotoxins or because of their biological similarity to Shiga toxin as Shiga-like toxins (SLTs) (33). In *E. coli* isolates associated with human disease, two of these toxins have been purified (6, 17, 32, 34, 44) and their structural genes have been cloned and sequenced (3, 5, 12, 29, 30). These studies revealed that one of the *E. coli* cytotoxins, called SLT-I, is almost identical at the DNA sequence level to the Shiga toxin from *Shigella dysenteriae* type 1 (3, 5, 39), whereas a second toxin, termed SLT-II, is only 55% homologous with SLT-I (12). There is evidence that variants of SLT-II exist in *E. coli* isolates of human (35; S. C. Head, M. A. Karmali, M. E. Roscoe, M. Petric, N. A. Strockbine, and I. K. Wachsmuth, Letter, Lancet **ii**:751, 1988) and porcine (25a, 42) origin.

In intestinal and extraintestinal human diseases, in addition to *E. coli* serotype O157:H7, *E. coli* isolates of numerous serogroups have been recognized as producers of SLTs (2, 9, 15, 19, 20, 25). The identification of SLT producers that do not belong to serotype O157:H7 is extremely difficult because there seem to be no differential biochemical or serological markers for this heterogeneous group of toxin producers. Therefore, identification must be based solely on tissue culture assays or on immunoassay. Enzyme-linked immunosorbent assay (ELISA) and colony blot ELISA with monoclonal antibody (MAb) against SLTs represent significant advances in the diagnosis of SLT-associated infections (6, 18, 35, 40). Another line of research for identification of SLT-producing *E. coli* has recently been successfully directed toward the development of DNA probes. Levine et al. (23) have constructed a DNA probe from a 60-megadalton plasmid of *E. coli* serotype O157:H7 which is also present in most toxigenic *E. coli* isolates of serotypes O26:H11, O111:H8, and O111:H−. Although this plasmid does not carry the structural genes for SLT production, DNA probes from the plasmid accurately identify enterohemorrhagic *E. coli* serotype O157:H7 strains and about 70% of the SLT-producing *E. coli* strains that are non-O157 (23). Willshaw et al. (43) and Newland and Neill (28) constructed polynucleotide probes from the cloned SLT-I and SLT-II genes that were highly specific for the identification of SLT producers when compared with biological or serological assays.

In this study we constructed five synthetic oligonucleotides and tested them for the detection and characterization of SLT-I- and SLT-II-producing *E. coli*. We show that they specifically detect *E. coli* strains producing high levels of SLTs and examine the applicability of these probes for the colony hybridization technique.

(Part of this study appears in the thesis of T. Meyer.)

## MATERIALS AND METHODS

**Bacterial strains.** The *E. coli* serotype O157:H7 strains were obtained from the reference collections of the Centers for Disease Control, Atlanta, Ga.; from the Universitätskrankenhaus Hamburg; and from M. A. Karmali, Toronto, Ontario, Canada. *E. coli* HB101(pJN26) and *E. coli* HB101(pJN28) have been described previously (29) and were kindly provided by J. Newland (Bethesda, Md.); *E. coli* C600 was obtained from A. Pühler (Bielefeld, Federal Republic of Germany). Some *E. coli* strains that were used have been recently characterized for SLT production by cytotoxicity tests (15–18). In order to evaluate the sensitivities and specificities of the oligonucleotide probes, besides enterohemorrhagic *E. coli* serotype O157:H7 and the *E. coli* isolates referenced in Table 2, other clinical isolates of the major categories of diarrheagenic *E. coli* (enterotoxigenic [ETEC], enteroinvasive, and enteropathogenic) and *E. coli* isolates from patients with urinary tract infections were

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examined. We also included 125 separate *E. coli* isolates from the stools of healthy humans. A total of 90 isolates from this collection were from the stools of 90 infants and young children (ages, 1 day to 2 years) who visited the hospital for reasons other than gastrointestinal disorders, and 35 *E. coli* isolates were from the feces of 35 healthy laboratory workers and physicians. The strains in Table 3 also include 120 separate isolates of *E. coli* from the urine of 120 different patients with *E. coli*-associated urinary tract infections. These *E. coli* strains were of serotypes O1:K1, O4:K12, O6:K2, O7:K1, O18:K5, and O7:K1. Furthermore, 46 ETEC test strains representing serotypes O6:H16, O8:H9, O25:H−, O28:H?, O78:H11, and O148:H28 were investigated. These strains were derived from 46 patients in studies of infant diarrhea done in the Federal Republic of Germany (15). A total of 12 enteroinvasive *E. coli* strains positive by the Sereny test were isolated either from specimens from seven children with diarrhea (7 isolates) (15) or from five adult German travelers to southern Europe, Mexico, or Asia (5 isolates). The enteroinvasive *E. coli* belonged to serotypes O28:H−, O143:H−, O124:H−, and O167:H−. The 130 enteropathogenic *E. coli* isolates were cultured from the stools of 127 infants and young children (ages, between 1 day and 24 months) with diarrhea between January 1985 and July 1988. The enteropathogenic *E. coli* isolates belonged to serotypes O26:H11, O26:(H types 8, 25, and 32), O55:(H types 6 and 7), O111:H−, O111:(H types 2, 8, 12, 25, and 34), O114:H2, O119:H6, O125:H21, O126:H2, O126:H27, O125:H−, O127:H6, O128:H2, O128:H12, and O128:H?. The 15 *E. coli* isolates of porcine origin were collected from the stools of or enteral biopsy material from 15 different piglets (weight, 10 to 15 kg) with edema disease during a collaborative study with the Tierärztliche Ambulanz Schwarzenbek der Freien Universität Berlin. Of the 15 isolates, 9 belonged to serogroups O138 (4 strains), O139 (4 strains), and O141 (1 strain); the remaining isolates were not serotyped.

**Cytotoxicity assays.** The assay used to quantitate the amount of toxin was performed as described recently (14), with minor modifications. Briefly, Vero cells (ATCC CCL 81; Flow Laboratories, Meckenheim, Federal Republic of Germany) or HeLa-S3 cells (ATCC CCL2.2; Flow Laboratories) were grown as monolayers in minimal essential medium supplemented with L-glutamine, antibiotics (penicillin and streptomycin), and 3% fetal bovine serum. Purified SLTs or crude cytotoxin that was present in cell lysates prepared as described previously (18) was adjusted to 1 mg of protein per ml with 0.9% NaCl and diluted in tissue culture medium; portions of 0.1 ml were inoculated into the wells of a microtiter plate (A/S; Nunc, Roskilde, Denmark), and 0.1 ml of a freshly prepared HeLa or Vero cell suspension containing $1.2 \times 10^4$ viable cells was added and incubated at 37°C. For the detection of SLTs in bacterial culture filtrates, culture supernatants were filter sterilized, serially diluted in cell culture medium (10-fold dilutions), and transferred to tissue cell-containing wells as described above. The toxin titer was calculated as described previously (15). The reciprocal of the highest dilution causing cytopathic effects after 48 h in 50% of the wells (eight wells per each dilution) was multiplied by 10 and recorded as the 50% cytotoxic dose ($CD_{50}$) per milliliter of supernatant or per milligram of cell lysate protein.

**SLTs and antitoxins.** SLT-I from *E. coli* C600 lysogenized with coliphage 933J (41) of *E. coli* serotype O157:H7 strain 933 and SLT-II from *E. coli* C600 lysogenized with phage 933W (41) of *E. coli* 933 have been purified in our laboratory (17). Other Shiga-like cytotoxins used were the SLT-I from *E. coli* HUS-2, Hamburg (*E. coli* serotype O111:H−) (14). Shiga toxin was purified from *S. dysenteriae* type 1 strain S31 (14). The respective antitoxins have been raised in rabbits (14, 17). MAb 13C4 against the B subunit of SLT-I (40) was a kind gift from N. A. Strockbine (Bethesda, Md.).

**Neutralization of cytotoxicity and dot blot ELISA.** For neutralization studies, samples were diluted in tissue culture medium containing 100 to 200 $CD_{50}$/ml and incubated for 1 h at 37 and 4°C overnight with equal volumes of diluted rabbit antiserum against purified SLTs or preimmune rabbit serum. Samples were considered to be neutralized if dilutions of the antiserum up to 1:1,000 completely prevented cytopathic effects on Vero or HeLa cells but if preimmune rabbit serum diluted 1:10 did not.

For the dot blot ELISA, *E. coli* strains were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) to the late logarithmic phase (optical density at 572 nm, 0.8). Then, 0.1 mg of polymyxin B per ml was added (19), and the bacteria were grown for another 45 min. After the culture filtrates were tested by the cytotoxicity assay, they were adjusted to $10^4$ and $10^3$ $CD_{50}$/ml with phosphate-buffered saline. For the dot blot ELISA, 100 μl of each dilution was applied to a nitrocellulose membrane (Schleicher & Schüll, Dassel, Federal Republic of Germany) by a dot microfiltration apparatus (Schleicher & Schüll) and was incubated for 10 min before the membrane was attached to a vacuum source. The membranes were washed three times in phosphate-buffered saline–0.5% Tween and then incubated for 10 min with 100 μl of the rabbit antiserum against SLT-II (17). After the filters were washed with phosphate-buffered saline–0.5% Tween by vacuum pressure, they were reacted overnight with goat anti-rabbit immunoglobulin G coupled with peroxidase (Sigma Chemical Co., Munich, Federal Republic of Germany). The filters were washed three times as described above and developed by adding substrate (18).

**Synthetic probes.** Sequences representing four different regions of the SLT-I genes are given in Table 1. To compare probes of various lengths, oligonucleotides of 20 and 41 bases in length were constructed from the nucleotide sequences published by De Grandis et al. (5). Three probes were homologous to sequences of the *slt-IA* gene (probes 247, 772, and 818), and one probe represented nucleotides complementary to *slt-IB* (probe 1228). Another oligonucleotide (probe 849) was constructed from the sequence described for the *slt-IIA* gene of *E. coli* 933 (12). An automated phosphoramidite coupling method was used to synthesize the oligodeoxyribonucleotides on a synthesizer (Applied Biosystems). The oligonucleotides were radiolabeled at their 5′ ends by using T4 polynucleotide kinase (Boehringer GmbH, Mannheim, Federal Republic of Germany) and [$\gamma$-$^{32}$P]ATP (Amersham Buchler, Braunschweig, Federal Republic of Germany) and were purified by chromatography on Sephadex G-50.

**Colony blots and hybridization.** Colony blots were prepared by the method of Moseley et al. (27). The test nitrocellulose filters were prehybridized in 5 ml of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.4])–5× DET (1× DET is 0.02% Ficoll, polyvinylpyrrolidone, and bovine serum albumin each)–100 μg of sonicated salmon sperm DNA per ml–0.2% sodium dodecyl sulfate for 3 h at 46°C. Hybridization with $10^5$ cpm of radiolabeled oligonucleotides per ml of hybridization solution was carried out in the same buffer for 18 h at 50°C. The filters were washed twice in 5× SSPE–0.2% sodium dodecyl

TABLE 1. Nucleotide base sequence of the synthetic oligodeoxyribonucleotide probes

| Probe | Nucleotide base sequence (5′ to 3′) | Location[a] |
|---|---|---|
| 247[b] | CGACTGCAAAGACGTATGTAGATTCGCTGAATGTCATTCGC | 247–287 |
| 772[b] | GATGATCTCAGTGGGCGTTC | 772–791 |
| 818[b] | ATCTTACATTGAACTGGGGAAGGTTGAGTAGCGTCCTGCCT | 818–858 |
| 1228[c] | GATACCTTTACAGTTAAAGTTGGTGATAAAGAATTATTTAC | 1228–1268 |
| 849[d] | TCTGAAACTGCTCCTGTGTA | 849–868 |

[a] Location corresponds to the position of the bases in the previously published nucleotide sequences of the *slt-I* (5) and *slt-II* (12) genes.
[b] Sequences derived from the *slt-IA* gene (5).
[c] Sequence derived from the *slt-IB* gene (5).
[d] Sequence derived from the *slt-IIA* gene (12).

sulfate at 30°C for 10 min and then once for 5 min at 50°C, air-dried, and autoradiographed at −80°C.

**Southern hybridization.** Phage DNA, plasmid DNA, and chromosomal DNA were prepared as described by Maniatis et al. (24). After treatment with restriction enzymes, the resulting fragments were separated by electrophoresis through 0.7% agarose gels and transferred to zetaprobe membranes by an alkaline Southern blotting procedure (37). Prehybridization, hybridization, and washing of the membranes with covalently fixed DNA were carried out as described above for colony blots, except that $10^6$ cpm of radiolabeled oligonucleotides per ml of hybridization solution was used.

## RESULTS

**Specificity of the oligonucleotide probes.** The five oligonucleotides listed in Table 1 were tested for specificity by alkaline Southern hybridization. Representative results are shown for probes 772 (Fig. 1) and 849 (Fig. 2). Hybridization with probe 772, which was derived from the *slt-IA* gene, revealed the specific recognition of DNA from toxin-converting coliphage 933J (Fig. 1B, lane 1) and of SLT-I sequences present on pJN26 (29), which is a recombinant pBR328 plasmid containing sequences homologous to this oligonucleotide probe. In Fig. 1B (lanes 6 and 7), hybridization appeared with the excised SLT-I fragments of pJN26 but not with the vector pBR328. Furthermore, no hybridization signals occurred with pJN28 (29), a pBR328 derivative that lacks SLT sequences complementary to the probe used for hybridization (Fig. 1B, lane 5). Similarly, no hybridization signals were detectable with pBR328 (Fig. 1B, lane 11), plasmids of SLT-producing *E. coli* (Fig. 1B, lanes 2 and 3), or λ DNA (Fig. 1B, lane 4). The same specificity was observed with the three other probes derived from the SLT-I genes (data not shown).

When we used the DNA samples shown in Fig. 1A, probe 849 complementary to sequences of the *slt-IIA* gene gave no hybridization signals (data not shown). To evaluate the capacity of this probe to detect SLT-II DNA sequences, we used genomic DNA from *E. coli* serotype O157:H7 strain 933 that produces SLT-I and SLT-II (39) and genomic DNAs from *E. coli* C600 lysogenized either with the SLT-I-converting phage 933J or the SLT-II-converting phage 933W; *E. coli* C600 DNA was used as a control. The probe hybridized to single *Eco*RI fragments of about 5 kilobases from *E. coli* C600 lysogenized with phage 933W (Fig. 2B, lane 1) and *E. coli* serotype O157:H7 strain 933 (Fig. 2, lane 2) but not to DNA from the SLT-I-producing strain or the nontoxigenic strain (Fig. 2B, lanes 3 and 4, respectively). When this blot was reused for hybridization with the SLT-I-derived probe 772, a single *Eco*RI fragment of about 8.5 kilobases was only seen with *E. coli* 933 and *E. coli* C600 lysogenized with phage 933J (data not shown). The same specificities were obtained with the three other *slt-I*-derived probes.

**Serological classification of SLTs.** The *E. coli* strains used for colony hybridization with the oligonucleotides were first

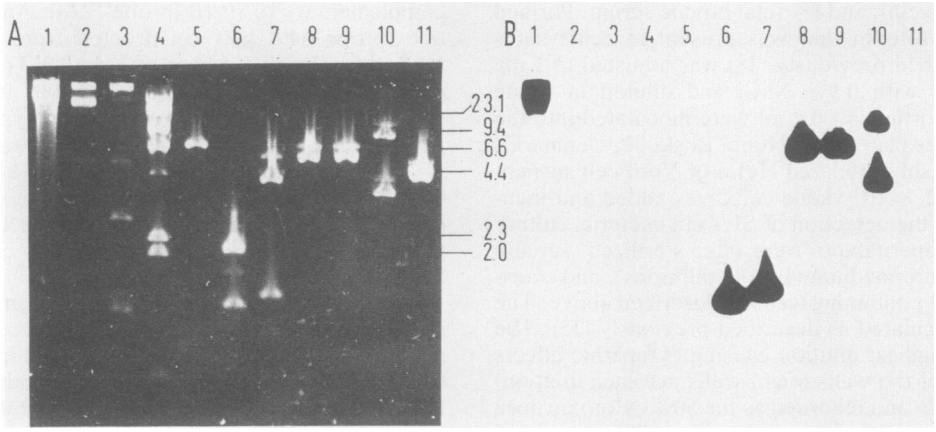


FIG. 1. Agarose gel electrophoresis of DNAs (A) and hybridization of these DNAs after Southern transfer with oligonucleotide probe 772 derived from the *slt-IA* gene (B). Lanes were labeled as follows: 1, purified undigested DNA of phage 933J; 2, plasmid DNA of SLT-I-producing *E. coli* 6545 (serotype O26:H11); 3, plasmid DNA of SLT-I-producing *E. coli* 245 (serotype O111:H8); 4, λ DNA digested with *Hin*dIII; 5, pJN28 digested with *Hin*dIII; 6, pJN26 digested with *Hin*cII; 7, pJN26 digested with *Hin*dIII-*Nco*I; 8, pJN26 digested with *Nco*I; 9, pJN26 digested with *Hin*dIII; 10, undigested pJN26; 11, pBR328 digested with *Eco*RI. Note that the probe specifically recognizes SLT-I sequences present on the recombinant plasmid pJN26 and coliphage 933J DNA. Numbers between the gels are in kilobases.

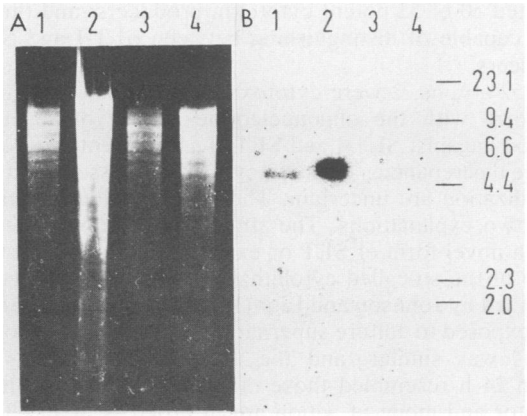


FIG. 2. Agarose gel electrophoresis of cellular DNAs (A) and hybridization with oligonucleotide 849 derived from the *slt-IIA* gene after Southern transfer (B). Lanes: 1, DNA from SLT-II-producing *E. coli* C600 lysogenized with phage 933W; 2, DNA from SLT-I- and SLT-II-producing *E. coli* 933 (serotype O157:H7); 3, DNA from SLT-I-producing *E. coli* C600 lysogenized with phage 933J; 4, DNA from *E. coli* C600. Numbers to the right of panel B are in kilobases.

characterized for cytotoxicity in sonic lysates and culture filtrates by using both HeLa-S3 and Vero cells. For serological characterization, SLT-I producers were identified after growth on trimethoprim-sulfamethoxazole-containing agar plates by colony blot ELISA with MAb 13C4 (40). For serological identification of SLT-II, filtrates of cultures grown overnight in Trypticase soy broth were examined by a dot blot ELISA with anti-SLT-II. In this assay cell-free cytotoxin that was present in culture supernatants was detected by anti-SLT-II, whereas the cytotoxic activity in culture supernatants from SLT-I producers was not reactive with anti-SLT-II.

**Colony blot hybridization.** Next, we used the synthetic oligonucleotides as hybridization probes in the DNA colony hybridization technique with strains classified as producers of SLT-I or SLT-II, producers of SLT-I and SLT-II, or producers of no toxin, as described above. Table 2 shows the ability of the five oligonucleotide probes to hybridize with some representative *E. coli* strains. Two *E. coli* serotype O157:H7 isolates (strains 933 and HC3159; Table 2) expressed both SLT-I and SLT-II. They were reactive with all five oligonucleotides. Three strains serologically characterized as SLT-I producers hybridized with all four probes derived from the SLT-I genes but not with probe 849 (Table 2). In contrast, the SLT-II-producing strains listed in Table 2 reacted with probe 849 but not with the SLT-I probes. No hybridization signals occurred with the nontoxigenic *E. coli* strains. The 12 isolates listed in Table 2 served as a standard set on each colony blot filter used for further screening of *E. coli* strains. After the initial testing of the probes, a variety of strains that were not of serotype O157:H7 were screened for the presence of SLT-I and SLT-II sequences. In Table 3, results of hybridization with the oligonucleotides are compared with the Vero cell cytotoxicity assay and ELISA techniques for SLT detection. Of 32 *E. coli* strains that were active on Vero cells, 27 hybridized with probe 772 and 3 hybridized with probe 849. None of the *E. coli* strains hybridized with both probes. Two Vero cytotoxin-producing strains belonging to serogroups O119:H6 and O128:H? were not identified by the use of the oligonucleotide probes. These strains were also negative in serological tests for SLT-I and SLT-II identification. When the 32 strains that produced cytotoxin in Vero cell tests were analyzed in immunoassays for SLT-I and SLT-II, only 28 strains were identified. The colony blot ELISA and MAb 13C4 identified 26 of the 27 SLT-I DNA probe-positive strains, and the dot blot ELISA detected 2 of the 3 SLT-II probe-positive strains. The specificity of the hybridization with the oligonucleotide probes in the colony blot assay was remarkable. Of the 416 *E. coli* strains that were negative in Vero cell tests for cytotoxin production, none hybridized with probes 772 and 849 (Table 3).

TABLE 2. Hybridization of the *E. coli* strains used as standards on filter blots with the different oligonucleotides

| Strain (serotype) | SLT[a] | Reactive probe(s)[b] | Reference |
|---|---|---|---|
| 933 (O157:H7) | I and II | 247, 772, 818, 1228, 849 | 38 |
| HC3159 (O157:H7) | I and II | 247, 772, 818, 1228, 849 | 16 |
| C600 lysogenized with phage 933J | I | 247, 772, 818, 1228 | 17 |
| C600 | None | None | 36 |
| 4859 (O55:H6) | None | None | This study |
| C600 lysogenized with phage 933W | II | 849 | 17 |
| HUS-2 (O111:H−) | I | 247, 772, 818, 1228 | 14 |
| HUS-1249 (O157:H7) | II | 849 | 16 |
| 240 (O111:H8) | I | 247, 772, 818, 1228 | 18 |
| 4157 (O119:H6) | None | None | This study |
| 2148 (O138:K81) | II | 849 | This study |
| $B_2C$ (O6:H16) | None | None | 22 |

[a] Kind of SLT defined by neutralization with anti-SLT-I or anti-SLT-II and reactivity in ELISA with the respective antibody.
[b] Reactivity of the oligonucleotide probes in colony hybridization.

**Hybridization of the oligonucleotides with *E. coli* serotype O157:H7 strains.** In Table 4 we compare the results of DNA hybridization, cytotoxicity assay, and immunodiagnosis of 20 clinical isolates of *E. coli* serotype O157:H7. From the DNA hybridization results, the serotype O157 strains fell into three classes; 15 isolates hybridized with probes 772 and 849, 1 isolate hybridized with probe 772 only, and 4 isolates hybridized with probe 849 only. The one *E. coli* serotype O157:H7 isolate that was positive with the SLT-I probe alone (strain A 9123-1; Centers for Disease Control) reacted with MAb 13C4 but not with anti-SLT-II. Of the 15 *E. coli*

TABLE 3. Identification of SLT-producing *E. coli* by hybridization with oligonucleotide probes, cytotoxicity assay, and serological tests

| Source of isolate[a] | Total no. | No. of strains reactive with: | | | | | |
|---|---|---|---|---|---|---|---|
| | | Oligonucleotide probes | | | Vero cells[b] | Antibody | |
| | | 772 | 849 | 772 and 849 | | MAb 13C4[c] | Anti-SLT-II[d] |
| ETEC, EIEC, UTI | 178 | 0 | 0 | 0 | 0 | 0 | 0 |
| EPEC | 130 | 27 | 1 | 0 | 30 | 26 | 0 |
| Normal flora | 125 | 0 | 0 | 0 | 0 | 0 | 0 |
| Diseased pigs | 15 | 0 | 2 | 0 | 2 | 0 | 2 |

[a] Abbreviations: ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*; UTI, uropathogenic *E. coli*; EPEC, enteropathogenic *E. coli*.
[b] Considered to be cytotoxic for Vero cells if sonic lysates contained $>10^3$ $CD_{50}$/mg of cell lysate protein or $>10^3$ $CD_{50}$/ml of culture supernatant.
[c] Described by Strockbine et al. (40) and tested by colony blot ELISA.
[d] Rabbit anti-SLT-II was prepared as described previously (17) and was tested by dot blot ELISA.

TABLE 4. Comparison of colony blot hybridization results for 20 *E. coli* serotype O157:H7 strains with cytotoxicity assay and serological detection of SLTs

| Hybridization with oligonucleotide probe: | No. of strains | No. of *E. coli* strains positive by: | | | |
|---|---|---|---|---|---|
| | | MAb 13C4 (SLT-I)[a] | Anti-SLT-II (SLT-II)[b] | Cytotoxicity[c] | |
| | | | | HeLa | Vero |
| 772 only | 1 | 1 | 0 | 1 | 1 |
| 772 and 849 | 15 | 15 | 14 | 15 | 15 |
| 849 only | 4 | 0 | 2 | 2 | 4 |

[a] Described by Strockbine et al. (40) and tested by colony blot ELISA (18, 40).

[b] Rabbit anti-SLT-II prepared as described previously (17) and tested by dot blot ELISA.

[c] Positive if there were $>10^3$ $CD_{50}$/ml of culture supernatant or $>10^3$ $CD_{50}$/mg of cell lysate.

serotype O157:H7 isolates that hybridized with both oligonucleotide probes, 14 reacted with anti-SLT-II and all of them hybridized with MAb 13C4. *E. coli* serotype O157:H7 strains EDL931, EDL932, and EDL933 (38); strain HUS-CL40 (M. A. Karmali, Toronto); A9167-1 (2); and A9047CS1 and A8959C7 (Centers for Disease Control) are examples of dual SLT producers. A third class comprising *E. coli* O157:H7 strains that were only reactive with the SLT-II probe showed considerable differences in the toxins with respect to their antigenicity and cytotoxicity to tissue cultures. Table 4 shows that the cytotoxin of only two of the four strains reacted with anti-SLT-II. Notably, the anti-SLT-II-negative strains were not cytotoxic for HeLa-S3 cells ($CD_{50}$, $<10^1$/ml of culture supernatant) but were cytotoxic for Vero cells ($CD_{50}$, $>10^4$/ml of culture supernatant). Both O157:H7 strains were associated with diarrheal disease. They were isolated from the stools of patients with nonbloody, watery diarrhea.

## DISCUSSION

*E. coli* serotype O157:H7 strains that produced cytotoxins active on HeLa and Vero cells were almost exclusively isolated from patients with hemorrhagic colitis, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome (9). Several investigators have identified other serotypes of *E. coli* that produce high levels of cytotoxins for cell cultures (1, 2, 15, 19–21, 25). In contrast to *E. coli* serotype O157:H7, to date these *E. coli* serotypes have not been implicated in recognized outbreaks of hemorrhagic colitis or hemolytic uremic syndrome. We have recently provided epidemiological observations of a significant association of these non-O157 toxin producers with enteric disease (15). To further support the association of these SLT producers with disease, there is a need to identify the sources and pathways of these enteric pathogens and to extend prospective case-control studies. For screening large numbers of individual strains for epidemiological surveys and for detecting small numbers of SLT-producing colonies among the physiological *E. coli* flora from stools of patients and healthy controls, we prepared synthetic oligonucleotides derived from the SLT-I and SLT-II genes. In this study we described the specificity of the oligonucleotides in detecting SLT-I and SLT-II sequences and compared the data from colony hybridization tests with those from cytotoxicity tests and serological assays. The usefulness of the probes was proved because (i) they displayed exquisite specificity; i.e., they did not hybridize to DNA sequences of 416 nontoxigenic strains; (ii) they detected 50 of 52 potent cytotoxin producers; and (iii) they were capable of distinguishing between SLT-I and SLT-II producers.

Of 52 strains, 2 were cytotoxic to Vero cells but did not hybridize with the oligonucleotide probes or react with antibody against SLT-I and SLT-II. At present, the reasons for the discrepancies between cytotoxicity assays and DNA hybridization are uncertain. These contrasting results may have two explanations. The strains in question either produce a novel form of SLT or express a toxin related to the group of the so-called cytolethal distending toxins recently described by Johnson and Lior (13). The appearance of Vero cells exposed to culture supernatants or sonic lysates of both strains was similar, and the morphological effects seen within 24 h resembled those of SLTs. Studies on the cell receptor and mode of action are in progress in an effort to further characterize these toxins.

Since the successful application of colony blot hybridization for detection of ETEC (26), both polynucleotide and oligonucleotide DNA probes have been used to identify ETEC in the clinical diagnostic laboratory. Oligonucleotides have been used to detect genes coding for classical heat-labile enterotoxins and heat-stable enterotoxins in ETEC. Hill et al. (11) have reported that there is a good correlation between radiolabeled synthetic probes and bioassays. Echeverria et al. (8) compared heat-labile enterotoxin oligonucleotide probes with cloned enterotoxin probes and biological assays and found significantly more heat-labile enterotoxin infections with the cloned probes than with the heat-labile enterotoxin oligonucleotide probes, whereas the sensitivity between the heat-stable enterotoxin oligonucleotide probe and the cloned probe was not significant when compared with the sensitivity of the suckling mouse model (7, 8).

Discrepancies between oligonucleotide probes and polynucleotide probes can be caused when the target DNA contains sequences that are not homologous with the sequence chosen for the oligonucleotide. If short oligonucleotides are used, the sensitivity may be affected by minor nucleotide differences in the target sequence. For the oligonucleotide probes prepared to detect *slt-I* sequences, we therefore investigated whether the length of the probes influenced the specificity in hybridization assays. The findings presented here with strains with defined phenotypic SLT profiles indicate that the 20-base probe appears to be as valid as the 41-base probes with regard to specificity and sensitivity of the hybridization reaction. This means that there was sufficient homology in all regions examined and indicates that the SLT-I toxins produced by different *E. coli* isolates were closely related genetically. Indeed, sequence data of SLT-I genes expressed by *E. coli* serotype O26:H11 strains (3, 5) and serotype O157:H7 strain 933 (12) have recently established that the SLT-I toxins of these strains are identical. Purification of the toxin of *E. coli* HUS-2 (serotype O111:H−) in our laboratory revealed that this SLT-I is structurally and immunologically identical to the SLT-I proteins of enterohemorrhagic *E. coli* serotype O25:H11 and O157:H7 strains.

For epidemiological screening of a large collection of *E. coli* isolates for SLT-I genes, we used probe 772 because it shares only 25% sequence homology with the *slt-II* sequences published by Jackson et al. (12). Similarly, oligonucleotide probe 849 derived from *slt-IIA* sequences shares only 25% sequence homology with the *slt-I* sequences. Thus, these oligonucleotides are thought to most properly distinguish between *slt-I* and *slt-II*. In clinical studies of entero-

hemorrhagic *E. coli*-induced diarrhea, a cocktail of the two oligonucleotides can identify SLT producers in a single hybridization experiment (unpublished data).

In contrast to SLT-I, there is evidence that *E. coli* SLT-II is not a homogeneous toxin (35, 42). On a molecular basis, at present, only a toxin termed SLT-IIv expressed by *E. coli* strains from porcine origin has been sequenced (42). This toxin associated with edema disease in pigs is neutralized by antibody to SLT-II and hybridizes with polynucleotide DNA probes derived from the SLT-II gene of *E. coli* serotype O157:H7 strain 933 (28). Oligonucleotide probe 849 derived from the *slt-IIA* gene of *E. coli* serotype O157:H7 strain 933 shared 95% sequence homology with the *slt-IIv* sequence published for *E. coli* serotype O139 strain S1191 (42). The oligonucleotide probe 849 hybridized with cytotoxic *E. coli* isolates of serotypes O138:K81 and O139:K82 (Table 2). Probe 849 was successfully used to locate the genes of an SLT-II variant in *E. coli* serotype O138:K81 strain E57 (25a).

The data presented in Table 4 provide preliminary evidence that variants among SLT-II are also expressed in *E. coli* serotype O157:H7 strains. The toxin present in culture supernatants and sonic lysates of two serotype O157:H7 strains isolated from patients with watery diarrhea in the Federal Republic of Germany were not toxic for HeLa-S3 cells but were highly toxic for Vero cells. The practical consequences for the clinical laboratory are twofold. First, if HeLa cells are used solely for establishing cytotoxicity in such individual strains, some serotype O157:H7 isolates could be misinterpreted as being nontoxigenic. Second, by serological screening with antibodies against SLT-I and SLT-II, not all serotype O157:H7 strains will be identified.

Since the genes of these toxins have not been sequenced and their mode of action has not been well characterized, we have not yet been able to define these toxins as a new category of SLTs (i.e., SLT-III). Moreover, hybridization of these strains with the oligonucleotide probe 849 suggests that DNA sequences in these strains are homologous to sequences of SLT-II. From the antigenic differences and the different spectrum of cell lines susceptible to the toxic effects, we expect nucleotide differences in other regions of the toxin genes.

Although there is no conclusive evidence that SLTs are the major virulence factors of enterohemorrhagic *E. coli*, at present the SLTs are the major important marker for identification of these pathogens. Therefore, the colony hybridization test with oligonucleotides derived from the toxin genes are an alternative approach to immunological assays for SLT detection and can serve as a useful tool in defining the epidemiology of infections by this new group of enteric pathogens.

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