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Molecular Detection of Sorbitol-Fermenting *Escherichia coli* O157 in Patients with Hemolytic-Uremic Syndrome

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Shiga-like toxin-producing *Escherichia coli* strains of serogroup O157 were identified in 26 of 104 patients with hemolytic-uremic syndrome and in 18 of 668 patients with diarrhea. All strains were identified by colony hybridization with DNA probes complementary to Shiga-like toxin I and Shiga-like toxin II gene sequences and characterized by biochemical tests and serotyping. Seventeen of these 44 patients had *E. coli* O157 strains which were unusual because they fermented sorbitol within 24 h of incubation and were positive for β-glucuronidase activity. Culture filtrates of these sorbitol-fermenting strains were highly toxic to Vero cells in culture. Serological tests and DNA analysis performed by restriction endonuclease digestion of B-subunit toxin genes revealed that all 17 isolates produced Shiga-like toxin II. Although by using molecular probes we established a high frequency of sorbitol-fermenting *E. coli* O157 strains in the patients we examined, further studies on the prevalence of such isolates in other areas of endemic disease are clearly warranted.

Shiga-like toxin (SLT)-producing *Escherichia coli* O157 is now well recognized to be associated with sporadic cases and outbreaks of diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS). Aside from *E. coli* O157, SLT production has also been observed in other serogroups of *E. coli* (12, 13).

Strains of serotype O157:H7 share some phenotypic characteristics in that they do not ferment sorbitol within 24 h of incubation (7, 14, 15, 17, 20) and show a negative reaction for β-glucuronidase (5, 20). Sorbitol MacConkey agar is used in many laboratories to screen for the presence of *E. coli* O157:H7 (12). In this procedure, non-sorbitol fermenters (colorless colonies) are screened with antisera to O157 and to H7 (14, 17). We have recently reported an outbreak of HUS in children (11) and some sporadic cases of HUS (2) caused by *E. coli* O157:H$^-$ strains that are sorbitol fermenters.

In the present study, we systematically investigated a total of 956 stool samples by DNA colony blot hybridization and present epidemiological evidence for the prevalence of sorbitol-fermenting *E. coli* O157 strains in stool samples of patients with diarrhea and HUS.

(Part of this work will appear in the M.D. thesis of F. Gunzer and H. Rüssmann, University Würzburg, Würzburg, Germany.)

MATERIALS AND METHODS

Stool samples. A total of 956 individual stool samples were available for investigation, of which 104 were collected from children (aged 8 months to 9 years) with classical HUS as defined previously (3, 13), 688 stool samples were collected from hospitalized patients with diarrhea (aged 1 month to 83 years), and 184 were collected from children without diarrhea or HUS (normal controls) who were in the hospital for other medical reasons (aged 6 months to 8 years). All stool samples were collected over a 3-year period from October 1988 to October 1991. Stool samples from patients with diarrhea or HUS were obtained throughout Germany, while stool samples from normal controls were obtained locally.

Bacterial strains. At least three different SLTs associated with HUS and hemorrhagic colitis have been described elsewhere (8, 12, 18, 21, 23, 24), with each of the following strains producing one of these toxins: *E. coli* C600 (H19J) as the SLT-I-producing reference strain (22), *E. coli* C600 (933W) as the SLT-II-producing reference strain (23), and *E. coli* O157:H7 strain 7279 producing a subtype of SLT-II (SLT-IIvhc) (18). We have shown (18) that the nucleotide sequence of the B subunit of strain 7279 is identical to that reported for the B2F1 toxin VT2ha (8) and for SLT-IIc of the *E. coli* O157:H$^-$ strain E32511 (21).

Biochemical tests. The biochemical properties of the isolated strains were determined by standard methods for identifying *E. coli* (4, 16). D-Sorbitol fermentation was examined in the tube test (4, 16), by the API 20E system (Bio Mérieux SA, Marcy-l'Etoile, France), and on D-sorbitol MacConkey agar (Oxoid, Wesel, Germany). The β-glucuronidase activity was measured in 4-methylumbelliferyl-β-D-glucuronide containing Fluorocult BRILA broth (Merck, Darmstadt, Germany) as described previously (1). Briefly, the strains were subcultivated on blood agar for 24 h and used to inoculate the medium. After incubation for 24 h at 36°C, the medium was tested for gas production and indole. Fluorescence was visualized at 366 nm in a dark chamber.

Cytotoxicity assay and dot blot ELISA. All *E. coli* strains that were positive by colony hybridization were assayed for SLT production. Single colonies were inoculated into Trypticase soy medium which was aerated and incubated overnight at 37°C. The culture filtrate was then tested for cytotoxicity on Vero cells (13). Dot blot enzyme-linked immunosorbent assay (ELISA) and neutralization of SLTs were performed as described previously (9).

Identification of SLT-producing *E. coli*. All SLT-producing *E. coli* strains isolated from the stool samples were identified

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TABLE 1. Identification of SLT-producing *E. coli* O157 by DNA colony hybridization, serotyping, and determination of sorbitol fermentation and β-glucuronidase activity

Source of stool samples	No. of stool samples tested	No. of isolates of *E. coli* O157:H⁻ sorbitol positive and β-glucuronidase positive	No. of isolates of *E. coli* O157:H7 or O157:H⁻ sorbitol negative and β-glucuronidase negative	No. of isolates of *E. coli* of serotype other than O157[a]
Patients with classical HUS	104	14	12	7
Patients with diarrhea	668	3	15	44
Normal controls	184			1

[a] These isolates belonged to serogroups O1, O2, O22, O23, O26, O45, O55, O73, O75, O91, O100, O104, O111, O113, O119, O121, O126, O128, O132, and O166; all were sorbitol positive.

by hybridization of single colonies with oligonucleotide probes 772 (SLT-I specific) and 849 (SLT-II specific) as described previously (9). If possible, from each stool sample which grew *E. coli*, a maximum of 64 lactose-fermenting colonies (average, 48) from MacConkey agar was tested for SLT genes by colony hybridization as described previously (9). Briefly, for colony hybridization, bacterial material from lactose-fermenting colonies was transferred by a toothpick from MacConkey agar plates onto nitrocellulose filters laid on top of Trypticase soy agar plates in duplicate. Moreover, bacterial material from the same colonies were transferred directly onto Trypticase soy agar plates to obtain probe-positive live bacteria for further characterization. The O and H antigens were determined by means of 170 O factor and 56 H factor sera which were prepared at the Deutsches Zentrum für Enteritiserreger (Hamburg, Germany) by the methods described by Orskov and Orskov (19). For confirmation purposes, a positive slide agglutination was followed by agglutination titration of the culture boiled to 100°C for 1 h (19).

PCR. Oligonucleotides used as primers in polymerase chain reaction (PCR) were synthesized as described previously (10). The sequences of the oligonucleotides were deduced from the published gene sequences of SLT-II (21). To amplify the A-subunit genes of both SLT-II and SLT-IIvhc, we used the following cloning primers: GK1, 5′-CCC GGA TCC ATG AAG TGT ATA TTA TTT AAA TGG-3′; GK2, 5′-CCC GAA TTC TTA TTT ACC CGT TGT ATA TAA AAA-3′.

The sequences of the cloning primers used to amplify the B subunit of SLT-II and SLT-IIvhc genes were as follows: GK3, 5′-CCC GGA TCC ATG AAG AAG ATG TTT ATG GCG-3′; GK4, 5′-CCC GAA TTC TCA GTC ATT ATT AAA CTG CAC-3′.

Primers GK1 and GK3 contain a *Bam*HI restriction site (GGA TCC); primers GK2 and GK4 contain an *Eco*RI restriction site (GAA TTC). The 5′ CCC triplet is added to increase the distance between the blunt end of the amplification product and the beginning of the restriction-enzyme-specific sequence.

Bacterial DNA was prepared by incubating 10 μl of bacterial suspension (10^4 bacteria) for 10 min at 95°C. Amplifications were performed in a total volume of 50 μl containing 200 μM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 100 pmol of each primer, 5 μl of dilution buffer, and 2.5 U of Taq polymerase (Amersham Laboratories, Buckinghamshire, United Kingdom). The samples were overlaid with 2 drops of mineral oil to prevent condensation and subjected to 30 cycles of amplification. The samples were incubated at 94°C for 1 min to denature the DNA, for 2 min at 52°C (for the primer pair GK1/GK2) and at 53°C (for the primer pair GK3/GK4) to anneal the primers, and at 72°C for 1 min to extend the annealed primers. Thermal cycling was carried out in a thermostatically controlled water bath. After the last cycle, the amplification products were subjected to submarine gel electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

Restriction enzyme digestion and restriction fragment length polymorphism. Restriction fragment length polymorphism was performed as described by Tyler et al. (24) with minor modifications. Briefly, 15-μl aliquots of the amplified products recovered after PCR with the SLT-II and SLT-IIvhc primers were subjected to restriction endonuclease digestion with *Hae*III (Boehringer Mannheim GmbH, Mannheim, Germany), *Rsa*I (Pharmacia LKB, Uppsala, Sweden), and *Nci*I (Bethesda Research Laboratories, Gaithersburg, Md.) as recommended by the suppliers. The DNA fragments recovered from the restriction endonuclease digestions were separated and visualized as described above.

RESULTS AND DISCUSSION

SLT-producing *E. coli* strains were identified by DNA hybridization in 33 of 104 stool samples from HUS patients, 62 of 668 stool samples from patients with diarrhea, and 1 of 184 stool samples from normal controls. Twenty-six of the 33 stool samples from HUS patients and 18 of the 62 stool samples from patients with diarrhea grew *E. coli* O157. The remaining 7 of 33 stool samples from HUS patients and 44 of 62 stool samples from patients with diarrhea yielded SLT-producing *E. coli* belonging to serogroups other than O157 (Table 1). These isolates belonged to 30 serotypes within 20 serogroups (Table 1).

With regard to D-sorbitol fermentation, 14 of 26 *E. coli* O157 isolates from HUS patients and 3 of 18 isolates from patients with diarrhea gave a positive sorbitol reaction within 24 h of incubation at 37°C, in the tube test, in the API 20E system, and on D-sorbitol MacConkey agar plates. Twelve of 26 *E. coli* O157 isolates from HUS patients and 15 of 18 isolates from patients with diarrhea were non-sorbitol fermenters. All *E. coli* O157 isolates which were sorbitol positive within 24 h of incubation were nonmotile and gave a positive reaction with the glucuronidase test and vice-versa.

All sorbitol-fermenting and non-sorbitol-fermenting *E. coli* O157 strains decarboxylated both ornithine and lysine. Among non-sorbitol-fermenting isolates, 10 of 29 had the H7 antigen and the remaining were nonmotile.

In liquid cultures, all sorbitol-fermenting and non-sorbitol-fermenting O157 isolates produced cytotoxin in comparable quantities, i.e., 10^4 to 10^7 50% cytotoxic doses per ml of culture suspension. The cytotoxin produced by the sorbitol-fermenting *E. coli* O157:H⁻ strains reacted with and was neutralized by antibodies against SLT-II. The SLT-II gene

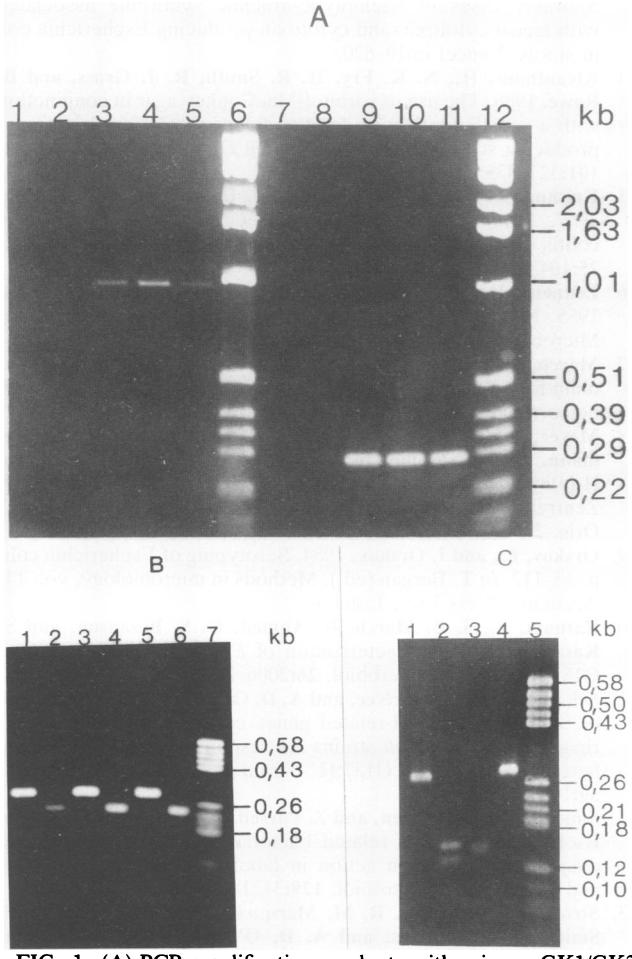

FIG. 1. (A) PCR amplification products with primers GK1/GK2 and GK3/GK4 to detect SLT-II and SLT-IIvhc A- and B-subunit genes. Lanes: 1 to 5, PCR amplification with primers GK1/GK2 (A subunit); 7 to 11, PCR amplification with primers GK3/GK4 (B subunit); 1 and 7, *E. coli* C 600 (negative control, i.e., nontoxicogenic); 2 and 8, *E. coli* C600 (H19J) (SLT-I-producing reference strain); 3 and 9, *E. coli* C600 (933W) (SLT-II-producing reference strain); 4 and 10, *E. coli* 5412 (sorbitol-fermenting strain); 5 and 11, *E. coli* 7279 (SLT-IIvhc-producing reference strain); 6 and 12, 1-kb DNA ladder (Gibco BRL). (B) Restriction fragment length polymorphism analysis of B-subunit gene product with SLT-II reference strain and two representative sorbitol-fermenting *E. coli* O157:H$^-$ strains. Lanes: 1 and 2, B-subunit gene of SLT-II reference strain digested with *Hae*III (lane 1) or *Rsa*I (lane 2); 3 and 4, sorbitol-fermenting *E. coli* 4999 digested with *Hae*III (lane 3) or *Rsa*I (lane 4); 5 and 6, sorbitol-fermenting *E. coli* 5412 digested with *Hae*III (lane 5) or *Rsa*I (lane 6); 7, DNA molecular weight marker V (Boehringer Mannheim GmbH). (C) Restriction endonuclease digestion of B-subunit genes of *E. coli* 7279. Lanes: 1, *Nci*I-digested B-subunit gene; 2, *Hae*III-digested fragments; 3, *Rsa*I-digested fragments; 4, *Nci*I-digested fragments of *E. coli* SLT-II reference strain C600 (933W); 5, DNA molecular weight marker V.

was identified in all sorbitol-fermenting *E. coli* O157:H$^-$ isolates with the oligonucleotide complementary to the SLT-II gene sequences. None of these isolates hybridized with the SLT-I probe.

Because variants of SLT-II have been recently reported within serogroup O157 (18, 21, 24), amplification of the A- and B-subunit genes were performed. Figure 1A shows the specificity of the primers for SLT-II and SLT-IIvhc sequences. There was no amplification product with nontoxicogenic or SLT-I-producing *E. coli*. The A- and B-subunit genes appeared at 978 and 288 bp, respectively.

The B-subunit genes were characterized by restriction fragment length polymorphism and visualized by submarine agarose gel electrophoresis and ethidium bromide staining as shown in Fig. 1B and C. Digestion of the B-subunit genes by *Hae*III and *Rsa*I can differentiate SLT-II from SLT-IIvhc. With the sorbitol-fermenting *E. coli* O157:H$^-$ strains, the SLT-II B-subunit genes remained undigested with *Hae*III (Fig. 1B, lanes 1, 3, and 5). Since *Rsa*I is known to digest 80-bp fragments (24), the B-subunit genes were digested into smaller fragments (Fig. 1B, lanes 2, 4, and 6), thus showing the presence of SLT-II genes in the sorbitol-fermenting *E. coli* isolates. Figure 1C shows the endonuclease digestion of SLT-IIvhc genes. As expected from the DNA sequences (15), *Hae*III yielded 137- and 151-bp fragments (Fig. 1C, lane 2), *Rsa*I produced a 148-bp fragment (Fig. 1C, lane 3) and two smaller fragments of 80 and 60 bp which could not be visualized in this gel system, and *Nci*I did not digest SLT-IIvhc and SLT-II amplification fragments (Fig. 1C, lanes 1 and 4). *Nci*I would have digested VT2hb into two fragments, while those amplicons from SLT-II and SLT-IIvhc should remain undigested (24).

By using molecular probes, SLT was demonstrated in *E. coli* recovered from the stools of 32% of the HUS patients, 9% of the patients with diarrhea, and 0.5% of the normal controls. Fourteen of 26 of the O157 strains from the HUS patients and 3 of 18 strains from the diarrheal patients were sorbitol positive. This was also a characteristic of all the SLT-producing strains from the non-O157 serogroups. Awareness of the existence of sorbitol-fermenting SLT-producing isolates among nonmotile *E. coli* O157 strains should be increased because such strains have not been described by other investigators. The 17 O157 sorbitol-fermenting strains described in this article include 11 isolates from patients who were separated in time and place; any epidemiological relationship among the patients, however, was unknown. Nevertheless, if a common source(s) existed for the strains, then the high incidence of sorbitol-positive SLT-producing O157 strains may well be misleading. Therefore, our results on the prevalence of such strains cannot be extrapolated to other populations, and future studies on their prevalence in other endemic areas are clearly warranted. If such attempts are undertaken by using sorbitol MacConkey agar, differentiation between the SLT-producing, sorbitol-fermenting *E. coli* strains and the physiological stool flora will not be possible since 95% of the latter also ferments sorbitol (6). Although sorbitol MacConkey agar is in wide use, laboratories should be aware of its limitations. Because there are no reliable biochemical screening tests for successful diagnosis of infections by SLT-producing *E. coli*, we recommended toxin assays or DNA hybridization as reliable and requisite detection methods.

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