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Frequent Loss of Shiga-Like Toxin Genes in Clinical Isolates of *Escherichia coli* upon Subcultivation

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Forty-five consecutive patients with various gastrointestinal disorders were identified as having Shiga-like toxin (SLT)-producing *Escherichia coli* infections. This was shown by the cytotoxic effect of stool extracts in Vero cell cultures which was neutralizable by antibodies to SLTs and by isolation of *E. coli* that hybridized with DNA probes complementary to SLT-I and SLT-II sequences. When we tested the same strains for SLT genes after subcultivation, the isolates from 15 patients became negative by colony hybridization and polymerase chain reaction and failed to produce SLTs. The instability of SLT genes warrants direct screening methods for clinical material and the development of new culture methods to prevent the loss of SLT genes.

It is now well recognized that *Escherichia coli* strains producing Shiga-like toxins (SLTs; verocytotoxins) induce diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and neurological symptoms. The ability to produce SLTs has been transferred to *E. coli* laboratory strains from clinical isolates belonging to different serogroups, and the genes encoding SLT-I and SLT-II have been shown to be carried on bacteriophages (3, 5, 15, 17–19, 21). Laboratory diagnosis of SLT-producing *E. coli* relies on detection of either SLTs—be it in the stools or culture filtrates—or toxin-producing bacteria by colony blot enzyme-linked immunosorbent assay or DNA hybridization (6, 7, 9, 10, 14, 20, 22). Monoclonal antibodies (20) and polynucleotide and oligonucleotide probes (6, 14) have been constructed and used successfully for detecting SLT-producing colonies. Both phenotypical and genotypical detection methods necessitate that the genes encoding the toxins remain stable subsequent to subcultivation. Loss of SLT genes may account for the discrepancy in the neutralization data and the type of SLT produced by individual strains. Schmitt et al. (16), for example, have shown that the *E. coli* O157:H$^-$ strain E32511 harbors SLT-II and SLT-IIc genes. On the other hand, Hii et al. (2) have shown that the same strain grown in their laboratory harbors only the SLT-IIc genes, and they have postulated that the strain has been spontaneously cured of a phage carrying SLT-II genes. These investigators have presented data for a possible partial loss of SLT genes because a specific type of SLT has disappeared but the isolate is still capable of producing measurable cytotoxic activity because of the presence of SLT-IIc genes.

In this paper we report on unequivocal evidence of clinical SLT-producing *E. coli* isolates that lost their toxin genes during subcultivation and became nontoxigenic.

The reference strains for SLT-I and SLT-II were *E. coli* C600/H19J and *E. coli* C600/933W, respectively (provided by A. D. O'Brien, Bethesda, Md.). *E. coli* O157:H7 strains 4821 and 7279 produce variants of SLT-II and have been described previously (6, 7, 13). The amino acid sequence of the B subunit of strain 7279 is identical to that reported for SLT-IIc (16). The pig isolate E57 originally described by Konowalchuck et al. (11) produces a variant of SLT-II (12). *E. coli* K-12 strain W3110 (provided by D. Hantke, Tübingen, Germany) was used in the transduction experiments. Those experiments and the purification of phage were performed as described previously (4).

Stool samples of 784 patients with watery or bloody diarrhea, of whom 28 had the clinical diagnosis of ulcerative colitis, were examined for the presence of fecal SLT as described by Karmali et al. (10) with minor modifications. Briefly, fecal samples were suspended in an equal volume of phosphate-buffered saline, vortexed, and centrifuged. Supernatants were filter sterilized and tested for cytotoxicity on Vero cell monolayers. Double dilutions of stool filtrates were made with culture medium, starting with a dilution of 1:20. The titer of the cytotoxin activity was expressed as the reciprocal of the maximum dilutions inducing a cytopathic effect on Vero cells after 48 h of incubation at 37°C.

Fecal SLT was identified by neutralization tests performed by adding an equal volume of rabbit antiserum to SLT-I and SLT-II (1:100 dilutions) (8) separately or together to the serially diluted fecal extracts. The mixture was incubated at 37°C for 1 h and added to the cells. A sample was defined as being positive for SLT if the cytotoxic titer was decreased by four 50% cytotoxic doses in the presence of one or both antiserum types. Uninoculated monolayers and filtrates of SLT-producing reference strains C600/H19J and C600/933W and preimmune rabbit serum were used as controls.

If possible, from each stool sample which grew *E. coli*, a maximum of 64 lactose-fermenting colonies (average, 45) from MacConkey agar was tested for SLT genes by colony hybridization. Material from single colonies grown on MacConkey agar was transferred with a toothpick to Trypticase soy agar (TSA) (these primary cultures are referred to as TSA-I) and transferred in parallel to two nitrocellulose filters laid on top of the TSA. One of the filters was used for hybridization with oligonucleotide 772, which is complementary to SLT-I sequences, and the other was used for hybridization with oligonucleotide 849, which is complementary to SLT-II sequences (6). The positions of the colonies on the nitrocellulose filters were identical to those on the TSA-I plates. Probe-positive colonies were transferred from TSA-I plates to a second subcultivation and hybridization on TSA plates (these cultures are referred to as TSA-II). In some experiments, in addition to MacConkey agar, the stool was

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TABLE 1. Serotyping and type of SLT in stool filtrates and primary cultures (TSA-I) in clinical *E. coli* isolates

Patient no. (age [yr])	Clinical diagnosis	Type of free fecal SLT	No. of colonies tested by DNA hybridization on TSA-I	No. of SLT probe-positive colonies with:		*E. coli* serotype of probe-positive colonies	No. of TSA-I probe-positive colonies tested after subcultivation[a]
				SLT-I probe	SLT-II probe		
1 (16)	Ulcerative colitis	SLT-I	64	64	0	O2:H5	64
2 (49)	Ulcerative colitis	SLT-II	64	0	64	O2:H5	64
3 (34)	Ulcerative colitis	SLT-II	45	0	12	O2:H5	3
4 (39)	Ulcerative colitis	SLT-II	58	0	24	O2:H5	24
5 (27)	Ulcerative colitis	SLT-II	64	0	4	O2:H5	3
6 (64)	Ulcerative colitis	SLT-II	38	0	3	O2:H5	3
7 (48)	Ulcerative colitis	SLT-II	60	0	42	O2:H5	42
8 (22)	Ulcerative colitis	SLT-I	26	26	0	O2:H5	26
9 (2)	Bloody diarrhea	SLT-I	64	48	0	O26:H11	48
10 (4)	Bloody diarrhea	SLT-I	29	13	0	O26:H11	13
11 (1)	Bloody diarrhea	SLT-I	64	3	0	O26:H11	3
12 (27)	Bloody diarrhea	SLT-I	64	4	0	O100:H32	3
13 (34)	Bloody diarrhea	SLT-I	64	7	0	O73:H34	3
14 (5)	Watery diarrhea	SLT-I	4	3	0	O73:H34	3
15 (1)	Watery diarrhea	SLT-I	6	6	0	O73:H34	3

[a] No colonies were SLT probe positive after subcultivation on TSA-II.

plated on TSA or blood agar. Bacterial lysis fixation of liberated DNA and hybridization and washing conditions were as described elsewhere (6). For some experiments colony blots were prepared by overlaying nitrocellulose filters on bacterial colonies grown on MacConkey agar to obtain replica blots.

The oligonucleotides used as primers in polymerase chain reaction (PCR) experiments were as follows. Primer pair MK1-MK2 was used to amplify an approximately 230-bp fragment from both *slt*-IA and *slt*-IIA (7). Oligonucleotides MK3 (5′ CCA GAA TTC TTA AGG TTG CAG CTC 3′) and MK4 (5′ TTC GAA TTC AAC GAA AAA TAA CTT GGC 3′) were used as primers to amplify the promotor and the coding regions of *slt*-I. Primers MK5 and MK6 were used to amplify an approximately 1.4-kb DNA segment containing the promoters together with the coding regions of the A and B subunits of SLT-II. These primers have been described previously (13).

Amplifications of material from single colonies were performed as described previously (7) in 100-μl reaction mixtures containing 10 μl of a suspension (10^5 bacteria). The samples were incubated at 94°C for 1.5 min to denature the DNA, at 44°C for 2 min to anneal primers MK1 and MK2 (55°C when primer pairs MK3-MK4 and MK5-MK6 were used), and at 72°C for 1 min to extend annealed primers MK1 and MK2 (3 min when primer pairs MK3-MK4 and MK5-MK6 were used). In contrast to this scheme, only the first denaturation step was performed for 10 min in order to ensure complete denaturation of the DNAs being investigated.

Agarose gel electrophoresis, transfer of DNA to Zeta probe membranes (Bio-Rad, Richmond, Calif.), electroblotting, and the procedure used for direct gel hybridization were performed as described previously (7). Phage DNAs were digested with *Eco*RI and analyzed by electrophoresis through 0.9% agarose gels. The gels were dried under vacuum, hybridized with radiolabelled oligonucleotide 772, and reused for hybridization with oligonucleotide 849 after being subjected to denaturation and neutralization as described previously (7). Hybridizations with radiolabelled oligonucleotides 772 and 849 were carried out as described elsewhere (6).

DNA sequencing was accomplished as described previously (13).

Genomic DNA used for pulsed-field gel electrophoresis was isolated and separated as described elsewhere (1). The DNA was cleaved with *Xba*I (New England Biolabs, Beverly, Mass.) as described previously (1). For hybridization of DNA separated by pulsed-field gel electrophoresis, 230-bp fragments of SLT-II-producing *E. coli* C600/933W or SLT-I-producing *E. coli* C600/H19J were obtained by PCR with primer pair MK1-MK2 and were used as hybridization probes after labelling with digoxigenin (Boehringer, Mannheim, Germany) as described previously (1).

The presence of free neutralizable fecal SLT (toxin titers ranging from 80 to 1,280) was demonstrated in 56 of 784 stool samples from patients with gastrointestinal disorders. Only 48 of those 56 samples with neutralizable fecal SLT grew *E. coli* on MacConkey agar plates, and of those, 45 samples yielded *E. coli* that hybridized with SLT-specific DNA probes 772 and 849. Fifteen stool samples had colonies that hybridized only with the SLT-I probe, 24 stool samples contained colonies that hybridized with the SLT-II probe, and 6 stool samples harbored colonies that hybridized with both probes. Eight probe-positive stool samples originated from the 28 patients with ulcerative colitis, and the remaining samples were from patients with bloody or watery diarrhea. We now tested the stability of SLT genes after subcultivation in the 45 DNA probe positive samples. For 30 of 45 strains, all three colonies tested were cytotoxic (titers ranged from about 10^3 to 10^6 50% cytotoxic doses), while for 15 of the 45 strains, all three colonies tested were noncytotoxic after growth in Trypticase soy broth (titers < 10). In addition to the determination of the cytotoxic activity, material from the same colonies was subjected to a second hybridization experiment: colony material from the TSA-I plate was plated onto nitrocellulose and fresh TSA plates (TSA-II). Once again, in these 15 identical cases with no cytotoxic effects, all three colonies tested did not hybridize, remaining negative in subsequent hybridization. As seen from Table 1, these *E. coli* isolates belonged to serotypes O2:H5 (eight isolates), O26:H11 (three isolates), O73:H34 (three isolates), and O100:H32 (one isolate). From seven patients we further tested larger colony numbers by DNA

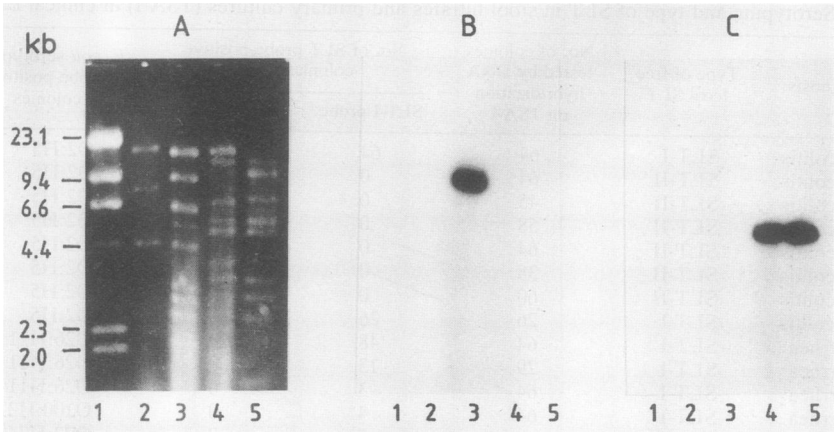

FIG. 1. (A) Agarose gel electrophoresis of *Eco*RI-digested DNAs of phages from *E. coli* E57 (lane 2), C600/H19J (lane 3), C600/933W (lane 4), and Cu-2 (lane 5). Lane 1, DNA molecular weight markers (*Hin*dIII-digested λ DNA; Boehringer). (B and C) Hybridization of the same gel with the SLT-I-specific probe 772 (B) and the SLT-II-specific probe 849 (C).

hybridization to assess the frequency with which SLT genes are lost upon subcultivation. As indicated in the footnote to Table 1, in these cases there are no examples in which colonies tested after subcultivation remain DNA probe positive.

In view of the fact that serotype O2:H5 was most prevalent (Table 1) and many probe-positive colonies were available on MacConkey agar and on TSA-I plates, further investigation on the loss of SLTs was carried out with two O2:H5 strains. Both strains hybridized with the DNA probes on the TSA-I plate but not on the TSA-II plate. One of these strains (referred to as Cu-1) hybridized with the SLT-I probe, while the other (referred to as Cu-2) hybridized with the SLT-II probe. After cultivation, amplification signals were observed from colonies grown on TSA-I plates but not from those on TSA-II plates. This confirmed the hybridization data in that all three colonies from the TSA-II plates were negative by the PCR method. Since there were hybridization signals from colonies grown on TSA-I plates, the amplification products of Cu-1 and Cu-2 as well as from control strains C600/H19J, C600/933W, 4821, 7279, and E57 were sequenced and compared with the sequences described for SLT-I, SLT-II, SLT-IIvhc, and SLT-IIv. These data revealed that the Cu-1 strain harbors SLT-I sequences and the Cu-2 strain harbors SLT-II sequences. The sequences from controls were identical either to SLT-I or SLT-II or to SLT-IIvhc or SLT-IIv. This indicates that the sequence analysis from Cu-1 and Cu-2 was correct and that the sequencing technique accurately distinguishes between the toxin genes. In order to further prove the presence of toxin genes we used the primer pairs MK3-MK4 (*slt*-I specific) and MK5-MK6 (*slt*-II specific) to amplify the complete toxin genes. With strain Cu-1 an amplification product of about 1.4 kb was seen on agarose gels (not shown) when MK3-MK4 was used but not when primer pair MK5-MK6 was used. By contrast, after the PCR was performed with primer pair MK5-MK6, an amplicon of the same size was observed only with DNA from the Cu-2 strain. Colonies grown on TSA-II plates were negative according to this PCR system.

Since we could not detect cytotoxic activity in culture filtrates from overnight cultures of colonies grown on TSA-I plates, we tested material from colonies grown on MacConkey agar. Single colonies from Cu-1 and Cu-2 obtained on MacConkey agar plates were grown in a Trypticase soy broth. Low titers ranging from 80 to 160 were detected in the cytotoxicity assay. However, when we plated material from the broth culture containing low cytotoxic activity onto the TSA, none of the 200 to 300 colonies that had grown overnight on the solid medium hybridized. By contrast, replica blots from the MacConkey agar plate contained pure cultures of DNA probe-positive colonies, indicating that the bacterial cells lost their genes during cultivation in the liquid broth. Here arose the question of whether the material that has been transferred from MacConkey plates to the TSA-I plate was sufficient to give a hybridization and PCR signal. In fact, we did find that this was precisely the case. To preclude the growth of the bacteria on MacConkey agar being responsible for the loss of SLT genes, we plated the stools onto other media (i.e., blood agar and TSA) and came to the conclusion that the loss of SLT genes was not dependent on the media used here.

In addition to DNA hybridization and amplification techniques, the absence of SLT sequences in strains Cu-1 and Cu-2 after subcultivation was shown by pulsed-field gel electrophoresis. Hybridization of DNAs separated by pulsed-field gel electrophoresis revealed specific binding of oligonucleotide 772 to the SLT-I and of oligonucleotide 849 to the SLT-II reference strain but not to the DNAs of Cu-1 and Cu-2 (not shown).

To investigate whether spontaneous induction of lysogenic cells to lysis occurred, material from a single colony grown on the MacConkey agar plate was used to inoculate Trypticase soy broth and the supernatant was concentrated and used to transduce the laboratory strain W3110. Stable lysogens expressing SLT-II were obtained with supernatants from strain Cu-2 but not with supernatants from Cu-1. Neutralization studies confirmed that the transduced laboratory strain expressed SLT-II. Since, in contrast to the finding in the wild-type strain, there was no spontaneous release of phages from the laboratory strain, UV light was used to induce phages. These were then purified, and the DNA was used for hybridization experiments. In Fig. 1A the DNA fragment obtained after restriction with *Eco*RI of the phage from strain Cu-2 is shown in lane 5 and is compared with DNA from phages from control strains E57 (lane 2), C600/H19J (lane 3), and C600/933W (lane 4). Hybridization of the DNA with the SLT-I probe is shown in Fig. 1B, and hybridization with the SLT-II probe is shown in Fig. 1C. On

an *Eco*RI fragment of about 8.5 kb, hybridization occurred only with the phage purified from the SLT-I-producing reference strain. By contrast, hybridization occurred both with the phage from the SLT-II reference strain and with the phage from the Cu-2 strain on 5-kb *Eco*RI fragments.

The loss of toxin genes among SLT-producing *E. coli* seems to be quite common and has been observed in different experimental conditions, for example, after growth in liquid and solid culture media. The mechanism by which this occurs is poorly understood and warrants further analysis of the genetic phenomena governing SLT gene loss. There remains the question of how frequently loss of SLT genes occurs in the intestine, this also being why cytotoxic activity is sometimes observed in the fecal sample but the clinical laboratories are unable to detect cytotoxic colonies from the primary culture (9, 10). Our data may well be important clinically in the diagnosis of SLT-producing *E. coli* infections because wherever the isolates from the patients are subcultured, the toxic organisms can become nontoxigenic, the infection therefore being difficult to diagnose. Since defined conditions to prevent the loss of the SLT genes are not yet available, direct screening methods, such as determination of cytotoxic activity of fecal samples, are absolutely essential. Moreover, the role of SLT-producing *E. coli* as the causal agent of infectious enteritis may well prove to be much greater than was previously assumed.

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