Escherichia coli O157:H7 Infections: Discordance between Filterable Fecal Shiga Toxin and Disease Outcome

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Shiga toxin (Stx)–producing *Escherichia coli* O157:H7 are the most common cause of hemolytic uremic syndrome (HUS). We detected free fecal Stx in 48%, 40%, and 17% of infected children with uncomplicated diarrhea, children who subsequently developed HUS, and children with HUS, respectively. Vero cell assay detected Stx more frequently than did a commercial Stx enzyme immunoassay. In children's stool samples obtained on or before day 4 of illness, each 10-fold decrease in titer was, paradoxically, associated with 3.8-fold increased odds of developing HUS ($P = .03$; 95% confidence interval, 0.77–19.7). The fecal Stx type did not correlate with the Stx expressed by bacteria grown in vitro and was not related to bacterial titer in the studied samples. These data suggest that therapeutic and diagnostic strategies directed toward binding or identifying intraintestinal fecal Stx may have limited success.

In 2 nearly simultaneous reports in 1983, the etiologic roles of *Escherichia coli* O157:H7 in hemorrhagic colitis [1] and in the hemolytic uremic syndrome (HUS) during childhood [2] were proposed. Soon thereafter, *E. coli* O157:H7 were shown to express an extracellular toxin for Vero and other eukaryotic cells that could be neutralized by antibodies to Shiga toxin (Stx) [3, 4]. Presumably, Stx was the filterable fecal cytotoxin in the stools of children with HUS whose stools contained *E. coli* O157:H7 and other Stx-producing *E. coli* (STEC) [2, 5]. On the basis of these observations, it has been assumed that, when Stx is absorbed from the gut during *E. coli* O157:H7 infection (which is almost never bacteremic), microangiopathic sequelae result, which lead to HUS in a subset of infected patients.

The ability of Stx to injure the infected host has been the central tenet of our understanding as to how STEC cause HUS. Filterable fecal cytotoxin is, therefore, an attractive candidate target for diagnostic and therapeutic strategies in infected humans. Specifically, the targeting of intraintestinal Stx early in infection via administration of substances that bind to the B subunit of Stx1 and 2, thereby neutralizing these toxins, would be a logical intervention to prevent HUS. Examples of such orally administered agents include αGal(1-4)βGal(1-4)βGlc (Pk-trisaccharide) [6], recombinant laboratory *E. coli,* which expresses the terminal sugar on globotriaosylceramide, the glycosphingolipid to which the Stx1 and 2 B subunits bind [7], and synthetic toxin binders such as Starfish [8] and Super Twig [9].

Antitoxin strategies require that infected patients have a reservoir of Stx that is available for neutralization before they develop HUS. Accordingly, we investigated fecal filtrates from children infected with *E. coli* O157:H7, to determine whether Stx was detectable and neutralizable by specific antibodies to Stx1 and Stx2. Such a pool of toxin would provide a logical target for antitoxin therapy in infected patients prior to the development of HUS.

Patients, Materials, and Methods

Patients. Children <10 years old who were infected with *E. coli* O157:H7 were identified by a network of 46 participating laboratories in the Pacific Northwest [10–12]. One of the Seattle investigators was notified when *E. coli* O157:H7 was recovered from a sorbitol-MacConkey agar stool culture. The investigator contacted the child's physician and asked him or her to approach the child's family for permission to discuss the study. Then the family was approached by one of the investigators, and consent to enroll the child in this prospective study was obtained in writing. If appropriate, assent was also obtained from the child. A standardized questionnaire was administered to determine the time of onset of the illness relative to enrollment and to the collection of the study stool specimen. The first day of diarrhea was considered to be the first day of illness.

After enrollment, children underwent daily laboratory tests until HUS developed and resolved or until it was apparent that this complication did not ensue. Infected children were classified as having HUS if, during the 2 weeks after enrollment, they had hemolytic anemia (hematocrit <30%, with smear evidence of in-

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travascular erythrocyte destruction), thrombocytopenia (platelet count <150,000 cells/mm^3), and renal insufficiency (serum creatinine concentration greater than the upper limit of normal for age). The uncomplicated group comprised infected children whose stools were analyzed and who did not develop HUS. The pre-HUS group comprised patients whose stools were studied and who subsequently developed HUS. The HUS group comprised children whose stools were studied after they developed HUS. Some of the children in the HUS group were also studied in the pre-HUS group, and the remainder of the subjects in the HUS group were children admitted to the Children's Hospital and Regional Medical Center (Seattle) with a diagnosis of HUS whose stools yielded *E. coli* O157:H7 and were of sufficient volume to study but who had not been entered into the study during the pre-HUS phase of their illness.

Stool processing. After enrollment, freshly passed stool was obtained and immediately placed on ice. The stool was transported to the laboratory of one of the authors (P.I.T.) at the Children's Hospital and Regional Medical Center or to a temporary work area in a participating institution. Nine parts of sterile PBS were added to 1 part stool in a 15-mL polypropylene tube and mixed thoroughly by inversion. Brief vortexing was used to suspend the stool in the PBS if necessary. The suspension then was centrifuged (2000 *g* for 45 s), and the supernatant was filtered through a 0.45-μm filter and stored in 0.2–0.4-mL aliquots at −70°C. Only stools that were filtered and frozen within 2 h of production were studied. If possible, stools were also diluted in sterile PBS and plated on sorbitol MacConkey agar to determine the concentration of *E. coli* O157:H7 that they contained. Ten non–sorbitol-fermenting colonies were subjected to latex particle agglutination (Oxoid Limited) to confirm expression of the O157 lipopolysaccharide antigen.

Cytotoxicity assay. Stool supernatants were thawed and added to a monolayer of Vero cells, as described elsewhere [13, 14]. After 48 h of incubation (37°C), the titer plates were stained, and the optical density (595 nm) was determined. The titer was recorded as the highest dilution that killed 50% of the Vero cells. The sensitivity of the assay is ~5 pg of purified Stx/well. All positive samples were neutralized by use of bovine polyclonal antibody against Stx1 [15] and a monoclonal antibody against Stx2 (11E10), when sample volume permitted [16]. In cases of low sample volume, the filtrate was neutralized using a single bovine polyclonal antibody against both Stx1 and Stx2 [13]. Fetal bovine serum (FBS) was used as a negative neutralization control. Several samples nonspecifically reacted with FBS, and these samples were neutralized with a monoclonal antibody against Stx1 (13C4) [17] rather than the bovine polyclonal antibody. Samples were considered to be neutralized if the titer was reduced ⩾4-fold [18].

Cell-associated toxin preparations. *E. coli* O157:H7 were grown overnight in 50 mL of trypticase soy broth (TSB) and pelleted by centrifugation (5500 *g*). They were then resuspended in 1.25 mL of PBS, and polymyxin B was added (final concentration, 0.5 mg/mL). This suspension was then incubated (4°C for 2 h) before pelletting (22,000 *g*).

The proteins in the resulting supernatant were precipitated by adding ammonium sulfate to a final concentration of 36.1 g/100

Table 1. Characteristics of patients, fecal samples, and infecting isolates, by patient groups.

Characteristic	Uncomplicated group (n = 52)	Pre-HUS group (n = 10)	HUS group (n = 6)[a]
Sex, male:female	32:20	7:3	3:3
Age, mean years (SD)	4.8 (2.6)	3.8 (1.9)	5.0 (2.2)
Race or ethnic group			
White	43 (83)	10 (100)	5 (83)
Hispanic	4 (7)	0	1 (17)
Black	1 (2)	0	0
Asian or Pacific Islander	3 (6)	0	0
Native American	1 (2)	0	0
Bloody diarrhea	46 (88)	9 (90)	6 (100)
Day of illness sample obtained, mean (SD)[b]	5 (2)	5 (1)	8 (1)
Positive Vero cell assay[c]	25 (48)	4 (40)	1 (17)
Positive filtrates, geometric mean (range)[d]	1689 (160–40,960)	380 (160–1280)	640[e]
Positive EIA	8 (15)	1 (10)	1 (17)
Bacteriological culture, no. positive/no. performed when patient enrolled in study (%)	20/27 (74)	2/2 (100)	1/4 (25)
Quantitative bacteriological culture, mean (range)[f] [no. of stools subjected to quantitative cultures]	6.2 (4.0–9.0) [27]	7.3 (6.9–7.6) [2]	5.0 (4.0–8.0) [4][g]
stx genotype of cognate isolates			
stx1$^+$/*stx2*$^-$	0 (0)	0 (0)	1 (17)
stx1$^-$/*stx2*$^+$	17 (33)	3 (30)	3 (50)
stx1$^+$/*stx2*$^+$	35 (67)[h]	7 (70)	2 (33)

NOTE. Data are no. (%) of patients, unless otherwise indicated. HUS, hemolytic uremic syndrome.

[a] Two patients in the HUS group were also analyzed in the pre-HUS group.

[b] The onset of diarrhea was considered to be the first day of illness.

[c] Titer ⩾160 was considered to be positive.

[d] Expressed as reciprocal of titer.

[e] No range is given because only 1 value was obtained in the HUS group.

[f] Expressed as $\log_{10}$ cfu/g of stool with a sensitivity of detection of ⩾4 $\log_{10}$ cfu/g of stool.

[g] Values ⩽4.0 were considered to be 4.0 for purposes of averaging.

[h] For 2 patients, a sibling's isolate's Shiga toxin genotype was counted as their cognate isolate Shiga toxin genotype, and an outbreak isolate's Shiga toxin genotype was counted as 1 patient's cognate isolate Shiga toxin genotype.

mL of solution. The precipitate was pelleted by centrifugation (22,000 *g* at 4°C for 15 min) and then resuspended in 2 mL of PBS. This pellet was then dialyzed against PBS by use of a membrane with a molecular-weight cutoff of 10,000 before testing on Vero cells for neutralizable toxicity.

Spiking experiments. To determine whether the fecal filtrates of children from whom *E. coli* O157:H7 was isolated, but in which filterable fecal toxin was not detected, contained Stx-neutralizing activity, an in vitro spiking experiment was conducted that used several filtrates that were available in excess. The cognate *E. coli* O157:H7 strains were grown in TSB (overnight at 37°C with shaking). After centrifugation (20,200 *g* for 5 min), the supernatant was filtered through a 0.22-μm filter and added to selected fecal filtrates. These "spiked" samples were incubated for 24 h at 37°C. Stx titers in the spiked fecal samples were determined immediately after spiking and then again after incubation. Samples were neutralized using bovine polyclonal antibody to Stx.

Stx EIA. Stx antigen was sought in freshly thawed filtrates by use of a commercial EIA (EHEC Premier Test; Meridian Biosciences) and read visually, according to the manufacturer's instructions.

stx *genotypes.* DNA samples from the infecting strains from 66 individual patients were digested with *Bam*HI, electrophoretically separated in 1% agarose in 0.5× Tris-borate EDTA [19], and transferred to a Magnacharge nylon membrane (Osmonics). The cognate isolate was not available from 3 outbreak-related cases, so analyses were performed on *E. coli* O157:H7 isolated from a simultaneous case in the household (a sibling) or an outbreak (a school lunch). The immobilized DNA then was probed with the inserts of plasmids pJN37-19 (specific for *stx1*) and pNN111-19 (specific for *stx2*) [20], labeled with the Megaprime DNA system (Amersham Pharmacia Biotech) and [α-^{32}P]dATP (Perkin Elmer Life Sciences). Membranes and probes were incubated overnight at 65°C, washed twice (15 min each wash at 65°C) in 2× standard saline citrate (SSC) [19] that contained 0.1% SDS, and twice again (15 min at 65°C) in 0.2× SSC/0.1% SDS. The washed membranes were then exposed to x-ray film in the presence of intensifying screens (−70°C).

Statistical methods. Proportions in the uncomplicated and pre-HUS groups were compared by Fisher's exact test, and medians were compared by the Wilcoxon rank sum test. Logistic regression was used to test whether the titer in filterable fecal toxin was a statistically significant factor in explaining the probability of developing HUS. The relationship between titer and colony-forming units of bacteria per gram was assessed by linear regression on the logarithmic transformation of both variables.

Results

Patients enrolled. Between May 1997 and August 2000, we enrolled 128 infected children. Sixty-six of these children produced stools in circumstances that permitted filtrates to be frozen within 2 h, as described above. Two subjects produced stools prior to the development of HUS and again when HUS ensued. A summary of patient characteristics is provided in table 1.

Cytotoxin testing. The ages of patients, days of collection of the study stool, results of cytotoxicity assay, EIA, the results of the simultaneous microbiology evaluation, and the *stx* genotype of the infecting *E. coli* O157:H7 are summarized in table 1, by stage of illness during which specimen were obtained. The Vero cell assay was considered to be positive if the filtrate had a specific neutralizable titer of ⩾1:16 (corresponding to a titer of 1:160 in the stool before dilution). Observed individual values, by group, are provided in table 2.

Filterable Stx was detected in the stools of 48% (25/52) and 40% (4/10) of the children in the uncomplicated and pre-HUS groups, respectively (figure 1) ($P = .74$). The geometric means of the fecal toxin titer of the uncomplicated and pre-HUS groups were 1689 (range, 160–40,960) and 380 (range, 160–1280), respectively. Only 1 of 6 stools obtained from children with HUS, a disorder that is often accompanied by diminished fecal output, had detectable filterable fecal Stx. EIA was positive for 10 stool filtrates, including 1 that was negative by Vero cell assay, but was negative in 21 of 30 filtrates with neutralizable toxin in the Vero cell assay. The mean and median days of illness on which the specimens were obtained were similar for the children with positive and negative filtrates (by Vero cell assay) (table 3).

There were 29 samples that underwent both Stx titer analysis and quantitative bacteriology in the uncomplicated and pre-HUS group. Within this subset of samples, there was no significant correlation between the concentration of fecal *E. coli* O157:H7 and the titer of fecal Stx ($P = .11$, linear regression analysis).

In vitro Stx titers were determined for 23 strains. Titers ranged between 1:16,384 and 1:1,048,576, with most (22/23) strains producing titers of 10^{4-5} CD_{50} units. There was no correlation between the free fecal Stx titers in patients and in vitro Stx production from the cognate strain (data not shown).

We examined 24 stools obtained within the first 4 days of illness to determine whether, during the early phase of illness, the presence or absence of filterable fecal toxin and the titer were associated with outcome (HUS or uncomplicated course). This interval was chosen for focused analysis, to avoid studying children who were already developing HUS [12] and because a subgroup analysis of children administered Pk-trisaccharide suggested that oral toxin binding was most effective early during illness [21]. Logistic regression was performed, with the response variable being whether the child developed HUS and the explanatory variable being the titer, while adjusting for days of illness. The distribution of the titer was skewed, and 2 transformations were tried: a binary transformation (reciprocal titers <160 vs. ⩾160) and a logarithmic transformation of the observed data. The binary transformation of titer was not statistically significant when it was the single variable in the model ($P = .09$). However, the logarithmic transformation of the titer values was significant ($P = .03$). Specifically, a decrease in titer of 1 $\log_{10}$ unit was associated with an increase in the odds of developing HUS by a factor of 3.8 (95% confidence interval, 0.77–19.7).

Table 2. Individual patient profiles.

Group, patient	Day of illness sample was obtained	Filterable fecal Stx			Log_{10} cfu/g[b]	*stx* genotype of cognate isolate	In vitro Stx detected
		Vero cell titer	Antibody neutralization[a]	EIA			
Uncomplicated							
U-1	2	20,480	1, 2	Pos	8.1	1, 2	ND
U-2	3	20	Neg	Neg	ND	2	ND
U-3	3	40	Neg	Neg	<4.0	1, 2	ND
U-4	3	5120	Pos	Pos	7.8	1, 2	ND
U-5	3	40	Neg	Neg	ND	2	ND
U-6	3	<20	Neg	Neg	8.4	1, 2	ND
U-7	4	<20	Neg	Neg	ND	2	ND
U-8	4	<20	Neg	Neg	<4.0	1, 2	ND
U-9	4	160	Pos	Neg	7.0	1, 2	1, 2
U-10	4	40,960	2	Pos	ND	1, 2	2
U-11	4	1280	Pos	Neg	ND	2	2
U-12	4	80	Neg	Neg	6.5	1, 2	ND
U-13	4	2560	Pos	Pos	ND	1, 2	ND
U-14	4	2560	Pos	Neg	ND	1, 2	ND
U-15	4	1280	1, 2	Neg	ND	1, 2	ND
U-16	4	320	2	Neg	ND	2	ND
U-17	4	10,240	2	Neg	ND	2	ND
U-18	4	<20	Neg	Neg	9.0	2	ND
U-19	5	1280	1, 2	Neg	<4.0	1, 2	ND
U-20	5	20	Neg	Neg	6.5	2	2
U-21	5	2560	2	Pos	ND	1, 2	1, 2
U-22	5	20,480	1	Neg	ND	1, 2	1, 2
U-23	5	640	1	Neg	ND	1, 2	2
U-24	5	1280	2	Neg	ND	2	2
U-25	5	40	Neg	Neg	ND	1, 2[c]	ND
U-26	5	40,960	1, 2	Pos	ND	1, 2	1, 2
U-27	5	80	Neg	Neg	6.9	1, 2	ND
U-28	6	<20	Neg	Neg	ND	1, 2[c]	ND
U-29	6	<20	Neg	Neg	7.3	2	ND
U-30	6	<20	Neg	Neg	5.7	1, 2	ND
U-31	6	<20	Neg	Neg	8.0	1, 2	ND
U-32	6	40	Neg	Neg	5.7	2	2
U-33	6	<20	Neg	Neg	4.7	2	2
U-34	6	320	1	Neg	ND	1, 2	1, 2
U-35	6	160	Pos	Neg	5.0	1, 2[c]	ND
U-36	6	1280	Pos	Neg	7.7	1, 2	ND
U-37	6	320	1, 2	Neg	8.0	1, 2	ND
U-38	6	<20	Neg	Neg	6.0	1, 2	ND
U-39	7	20	Neg	Neg	ND	2	ND
U-40	7	640	1, 2	Neg	6.6	1, 2	ND
U-41	7	1280	1	Pos	ND	1, 2	1, 2
U-42	7	20	Neg	Neg	ND	2	2
U-43	7	<20	Neg	Neg	<4.0	1, 2	2
U-44	7	2560	1, 2	Pos	ND	1, 2	2
U-45	7	<20	Neg	Neg	<4.0	2	ND
U-46	7	<20	Neg	Neg	ND	1, 2	ND
U-47	7	40	Neg	Neg	ND	1, 2	ND
U-48	8	640	1, 2	Neg	7.1	1, 2	ND
U-49	8	40	Neg	Neg	<4.0	1, 2	ND
U-50	8	<20	Neg	Neg	<4.0	1, 2	1, 2
U-51	8	320	2	Neg	ND	2	ND
U-52	9	<20	Neg	Neg	8.7	2	ND
Pre-HUS							
P-1[d]	3	80	Neg	Neg	ND	1, 2	ND
P-2[d]	4	160	Pos	Neg	ND	1, 2	ND
P-3	4	20	Neg	Neg	ND	2	2
P-4	4	<20	Neg	Neg	ND	1, 2	ND
P-5	4	20	Neg	Pos	ND	1, 2	ND
P-6	4	40	Neg	Neg	ND	1, 2	2
P-7	5	<20	Neg	Neg	ND	2	2
P-8	5	160	2	Neg	ND	2	ND
P-9	6	1280	2	Neg	7.6	1, 2	2
P-10	6	640	Pos	Neg	6.9	1, 2	ND

(continued)

Table 2. *(Continued.)*

Group, patient	Day of illness sample was obtained	Filterable fecal Stx: Vero cell titer	Filterable fecal Stx: Antibody neutralization[a]	Filterable fecal Stx: EIA	Log_{10} cfu/g[b]	*stx* genotype of cognate isolate	In vitro Stx detected
HUS							
H-1	6	<20	Neg	Neg	ND	2	ND
H-2	6	80	Neg	Neg	8.0	1, 2	ND
H-3	8	<20	Neg	Neg	<4.0	1, 2	ND
H-4	8	640	2	Pos	<4.0	2	2
H-5	8	20	Neg	Neg	<4.0	2	2
H-6	9	20	Neg	Neg	ND	1	ND

NOTE. HUS, hemolytic uremic syndrome; ND, not done; Neg, negative; Pos, positive; Stx, Shiga toxin.

[a] Fecal filtrates were neutralized with specific antiserum when sample volume permitted. Nos. represent Stx type specifically neutralized. In cases of low sample volume, the filtrate was neutralized with Stx1/Stx2 polyclonal antiserum. If toxin was neutralizable, the filtrate was categorized as positive, although toxin type was not determined.

[b] Bacterial titer sensitivity was ≥ 4 log_{10} cfu/g of stool.

[c] Cognate isolate was not available, but the *stx* genotype of an isolate from a symptomatic sibling or contact was shown.

[d] Patients P-1 and P-2 correspond to patients H-2 and H-3, respectively.

Filterable fecal toxin, toxin production, and stx *genotype of the cognate strain.* The specific Stx in the fecal filtrates, as inferred by neutralization testing, did not correspond to the cognate isolate's genotype (table 2). Seven of 23 fecal samples in this study that were obtained from patients infected with *stx1*$^-$/*stx2*$^+$ *E. coli* O157:H7 contained neutralizable Stx2. Twenty-three of 44 stools in this study that were obtained from patients infected with *stx1*$^+$/*stx2*$^+$ *E. coli* O157:H7 contained neutralizable cytotoxin, and the filtrates of 15 of these contained cytotoxin that could be phenotyped. Of these 15 filtrates, 4 had detectable Stx1 but not Stx2, 3 had detectable Stx2 but not Stx1, and 8 had detectable Stx1 and Stx2. The cognate isolates from the 7 patients in which the Stx in the fecal filtrate did not correspond to the isolates' genotype were examined further. Stx1 and Stx2 were each detected in culture supernatants from 4 of these strains. Stx2, but not Stx1, was detected in the culture supernatants and cell-associated toxin preparations (polymyxin extracts) of the cell pellets from the remaining 3 strains. Because the Stx phenotype was inferred by the neutralization of the toxin by specific antibodies, it is possible that Stx1 may have been produced by these strains but was masked by a significantly greater production of Stx2. Additional experiments that do not rely on neutralization to phenotype Stx are currently in progress.

Spiking experiments. The titer of Stx added to 6 fecal filtrates did not decrease after incubation at 37°C for 24 h (table 4).

Discussion

Vascular and extraintestinal damage secondary to the absorption of Stx has been assumed to be crucial to the development of HUS after *E. coli* O157:H7 infection. The paucity of detectable filterable fecal cytotoxin in children infected with *E. coli* O157:H7, both prior to the development of HUS and after HUS develops (although the numbers in this latter group were small), and the inverse relation between the presence and the concentration of this toxin and frequency of development of HUS, were, therefore, unexpected. However, recent studies have raised additional questions about the concordance between filterable fecal cytotoxin and the pathogenesis of extraintestinal host injury during STEC infection. For example, in pigs infected with *E. coli* that produce Stx2e, the toxin associated with edema disease, an increase in fecal Stx titer generally corresponded to a higher incidence of neurological abnormalities [22]. However, individual pigs with relatively low fecal titers of Stx developed systemic disease, and individual pigs with high fecal titers of Stx remained asymptomatic. In addition, *E. coli* O157:H− and *E. coli* O157:H7 that lack *stx* genes have been

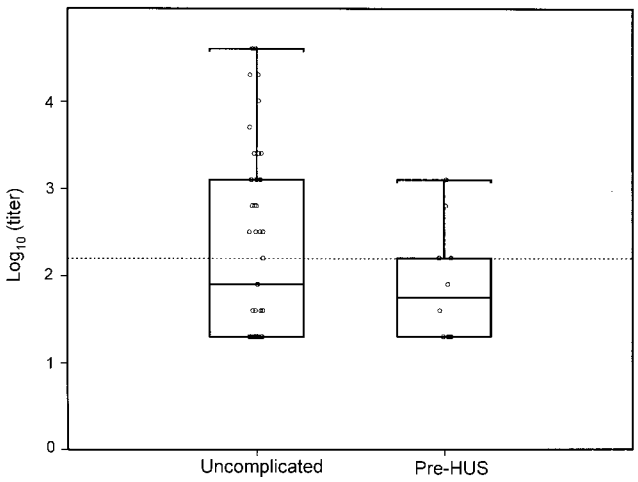

Figure 1. Box plot depiction of log_{10} toxin titer in fecal filtrates obtained from children infected with *Escherichia coli* O157:H7 in the uncomplicated and the pre–hemolytic uremic syndrome (HUS) groups. Horizontal lines within the boxes represent the medians. The lower and upper borders of the boxes represent the 25th and the 75th percentiles, respectively. The extensions beyond the boxes represent the differences between the upper and lower borders times 1.5. The horizontal broken line across the figure represents the upper limit of the titer of the negative samples. The open circles represent the individual titers for each patient.

Table 3. Summary of filterable fecal Shiga toxin cytotoxicities, by group of children.

Day of illness sample was obtained	Uncomplicated		Pre-HUS		HUS	
	Positive (n = 25)	Negative (n = 27)	Positive (n = 4)	Negative (n = 6)	Positive (n = 1)[a]	Negative (n = 5)
Mean (SD)	5 (2)	6 (2)	5 (1)	4 (1)	8	7 (1)
Median (range)	5 (2–8)	6 (3–9)	6 (4–6)	4 (3–5)	8	8 (6–9)

NOTE. HUS, hemolytic uremic syndrome.
[a] Mean, SD, median, and range are not provided because only a single determination was made.

recovered from children with hemorrhagic colitis [23, 24] and with HUS. Stools from some of these children were studied and did not have filterable fecal cytotoxin.

Several possible explanations could justify a role for Stx in the pathogenesis of HUS and of hemorrhagic colitis, despite our inability to find filterable fecal cytotoxin in the stools of many of the study patients. For example, Stx is probably delivered at the mucosal surface, by use of the close attachment mechanisms of *E. coli* O157:H7, a process that is mediated by intimin [25]. Indeed, gnotobiotic pigs challenged with *E. coli* O157:H7 with an intact close attaching phenotype sustain more severe neurologic injury than those challenged with a mutant in *eae,* the gene that encodes intimin [26].

In addition, it is possible that the toxin that causes extraintestinal injury might be absorbed from small bowel, and not from colonic, contents. Along these lines, some children whose *E. coli* O157:H7 infections do not progress to HUS have evidence of shearing of von Willebrand factor [11] and prothrombotic coagulation abnormalities [12], and thrombi have been found in colonic biopsy specimens obtained from adults [27] who did not develop HUS. These findings, in combination with our inability to find Stx in the stools of most infected children, suggest that the prominent colitis that is characteristic of most *E. coli* O157:H7 infections might actually represent vascular injury and not intraluminal effects of toxin on colonocytes. Moreover, primates challenged with intravenous Stx develop colonic lesions [28]. These findings raise the possibility that Stx was produced in greater quantities earlier in the illness and absorbed at that time, before the subjects were enrolled in the study or even before medical attention was sought. Thus, stool might be the wrong substance in which to assess the intraintestinal Stx burden.

Table 4. Shiga toxin (Stx) titer of in vitro–spiked fecal samples.

Patient filtrate	Reciprocal Stx titer immediately after addition of Stx	Reciprocal Stx titer after 24 h
U-50	2048	4096
U-42	768	768
H-5	512	512
U-32	256	256
U-20	128	128
U-18	128	128

Most children with *E. coli* O157:H7–associated HUS have at least one physician contact before developing this complication. As Paton et al. [7] noted, it seems intuitive that this interval provides an opportunity for antitoxin intervention, but our data suggest that toxin production might not be constitutive at all phases of infection. The failure of Pk-trisaccharide to prevent HUS when administered to children infected with *E. coli* O157:H7 [21, 29], a recent report that demonstrated that antibiotic usage in children infected with *E. coli* O157:H7 increases their risk of developing HUS [10], and the data presented above all suggest that it will be difficult to use antibacterial or oral antitoxin therapies to prevent the development of HUS in children infected with *E. coli* O157:H7. Again, it is apparent that the best way to prevent HUS after *E. coli* O157:H7 infection is to prevent primary human infection with this pathogen.

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