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Long-Term Shedding and Clonal Turnover of Enterohemorrhagic *Escherichia coli* O157 in Diarrheal Diseases

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To investigate the length of time that Shiga-like toxin-producing *Escherichia coli* O157 is excreted after the onset of diarrhea, 456 serial stool specimens were obtained from 53 children. *E. coli* O157 cells were identified by the use of DNA probes followed by agglutination with a specific antiserum. Specimens were collected until three consecutive stool samples (collected within 9 days) were negative for *E. coli* O157. The median durations of shedding were 13 days (range, 2 to 62 days) in patients with diarrhea or hemorrhagic colitis and 21 days (range, 5 to 124 days) in patients that developed hemolytic uremic syndrome. In 36 (68%) of the patients, only the first culture was O157 positive, and the three cultures that followed were negative. In 7 (13%) of the patients, *E. coli* O157 cells were shed for more than 32 days after the onset of diarrhea; these long-term shedders were clinically asymptomatic by the end of this period. In 12 patients, one or two serial O157-negative cultures, obtained up to 8 days after a positive culture, were followed by another positive culture. Comparison of the first and last *E. coli* O157 isolates by pulsed-field gel electrophoresis revealed that in three of the seven long-term shedders, pulsed-field gel electrophoresis types varied. In two cases, a Shiga-like toxin gene was apparently lost during infection. The observation of long-term shedding accompanied by genotypic turnover has epidemiological and diagnostic implications.

Escherichia coli O157:H7 is a major cause of epidemics and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome (HUS) in North America and Europe (6, 18, 21). *E. coli* O157:H7 strains and their nonmotile derivatives have a novel mechanism of pathogenesis that involves the expression of Shiga-like toxins (SLTs [verocytotoxins]) (11). Because of the number of serious outbreaks associated with these organisms, they have been the focus of interest for clinical, laboratory, and epidemiological investigations (9). *E. coli* O157 infection has been linked to ingestion of food and water contaminated with fecal material from bovine or human sources (18, 23). In some cases, however, *E. coli* O157 was transmitted directly from person to person. Direct transmission is most likely in institutional settings, such as hospitals (15), nursing homes (20), and day care centers (22).

An important factor in direct transmission is how long an infective person sheds this pathogen. In HUS patients in the Seattle area, the rate of recovery of O157:H7 in stools decreased from 100% within 2 days after onset of diarrhea to 33% if the stool was cultured more than a week after diarrhea began, suggesting that the pathogen is rapidly cleared (24). In a study of children in day care centers in Minnesota, cultures from serial stool samples obtained at 2- to 3-day intervals revealed a median duration of shedding of 17 days (range, 2 to 62 days) (3). The longest carriage (62 days) occurred in a child who was treated with amoxicillin (3) 26 days after the onset of illness. In an outbreak of diarrheal illness in Nevada, *E. coli* O157 was detected in a stool of one patient 38 days after onset of diarrhea (6).

In this study, we monitored the shedding of *E. coli* O157 in children not treated with antibiotics after onset of diarrheal disease to determine the potential role of long-term carriage in infection spread. Isolates were characterized by pulsed-field gel electrophoresis (PFGE) and SLT genotyping to determine clonal turnover in the course of infection.

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MATERIALS AND METHODS

Patients and analysis of stool samples. Stool samples were collected between March 1988 and December 1993 from different pediatric centers in Germany and sent for analysis. The median age of the patients was 3.6 years (range, 7 months to 9 years). Only patients who had not received any antibiotic treatment were included in the study. Physicians were asked to submit serial stool samples collected at 2- to 4-day intervals.

For the 28 patients with diarrhea or hemorrhagic colitis only, the first stool samples were collected 1 to 6 days after onset of diarrhea (median, 3 days). For the 25 HUS patients, stools were collected during the acute phases of HUS (range, 7 to 17 days after onset of diarrhea; median, 9 days).

A total of 456 serial stool specimens from 53 patients were analyzed. About 1 g of the stool samples was suspended in 1 ml of phosphate-buffered saline. Samples of this suspension and 100 μl of each serial 10-fold dilution were spread on MacConkey agar plates. Plates with 100 to 200 well-separated colonies were replicated, and the colonies were probed as described previously (13). The relationship between SLT probe-positive colonies and total coliforms was calculated in order to estimate the number of *E. coli* O157 CFU per gram of stool. The duration of shedding was defined as the interval from onset of diarrhea to the last positive sample followed by three negative stool cultures. All original stool suspensions were conserved by adding glycerol to a final concentration of 50% and were stored at −80°C.

Phenotyping of the *E. coli* O157 isolates. Up to 16 probe-positive colonies from each sample were identified biochemically and serotyped by the method of Aleksic et al. (1).

Genotyping of the isolates. PFGE of genomic DNA was carried out with 8 to 16 colonies from the primary isolation medium and with the same number of colonies from the convalescent-phase cultures from seven patients who shed this organism for the longest period of time. The same colonies were serotyped as described above. Restriction enzyme digestion and PFGE were carried out as described previously (5). PCR and restriction enzyme analyses for subtyping *slt* genes were performed as described by Rüssmann et al. (19).

Statistical methods. The mean duration of shedding was analyzed by the U test of Mann and Whitney.

RESULTS

Recovery of *E. coli* O157 in consecutive stool cultures. Analysis of serial stool samples revealed that in 36 (68%) of the patients, only the first culture was O157 positive, and the three cultures that followed were negative. The mean duration of shedding was significantly longer in patients with HUS (21 days; range, 5 to 124 days) than in those with diarrhea or hemorrhagic colitis (13 days; range, 2 to 62 days) as determined with the U test ($P < 0.001$). In four patients with diarrhea (Fig. 1A) and three patients with HUS (Fig. 1B), *E. coli* O157 was detected longer than 32 days. In these patients, the first and last isolates were subjected to further analysis (described below).

Intermittent shedding of *E. coli* O157. Intermittent shedding of *E. coli* O157 over a 28-day period is demonstrated for five patients with diarrhea in Fig. 2A and for seven patients in which diarrhea was followed by HUS in Fig. 2B.

***E. coli* O157 colony numbers.** There were seven patients in which *E. coli* O157 was recovered more than 32 days after the onset of diarrhea (Table 1). Notably, in two patients (no. 1 and no. 53), the density of *E. coli* O157 CFU was greater by an order of magnitude at the end of the shedding period (Table 1).

Genotyping of *E. coli* O157 isolates from long-term shedders. To establish whether the initial culture had heterogeneity in genotypes of *E. coli* O157 and whether the genotype in the convalescent-phase culture differed from that in the primary culture, multiple colonies of *E. coli* O157 were analyzed by PFGE and toxin profile (Table 1). A mixture of O157 genotypes, as detected by PFGE, was observed in neither the primary cultures of an individual nor among the colonies from the convalescent-phase stools. However, three individuals shed one PFGE genotype in the initial stool and a different one with two to three fragment bands of different sizes in the convalescent-phase stool (Table 1).

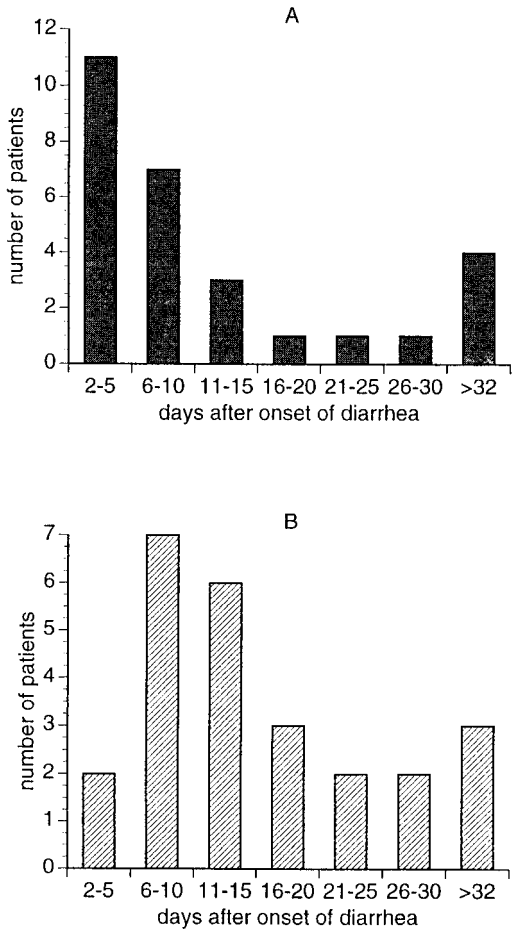

FIG. 1. Recovery of *E. coli* O157 in stool samples from patients with diarrhea (A) and HUS (B). The duration of shedding was estimated as the interval from onset of diarrhea to the last O157-positive culture followed by three negative stool cultures collected at 2- to 4-day intervals.

DISCUSSION

In all long-term shedders, the concentration of *E. coli* O157 was greater than 10^6 CFU/g of stool. The fact that concentrations of less than 10^6 CFU/g of stool were never found in convalescent-phase stools might be due to the low sensitivity of the colony hybridization method used here. To obtain clearly separated colonies which can be subsequently hybridized, several dilutions of stool must be plated (21). The majority of the diarrheal stools yielded between 10^5 and 10^6 CFU of background flora on MacConkey agar plates, whereas 10^7 to 10^8 CFU were present in the convalescent-phase stools (12). Thus, the high concentration of background flora present in convalescent-phase stools may be the reason for a detection limit of 10^5 to 10^6 *E. coli* O157 CFU/g of stool in convalescent-phase stools. This, however, does not rule out the presence of smaller numbers of O157 CFU which were undetected by this method. Because culture on a conventional sorbitol-MacConkey agar does not affect the growth of commensal *E. coli* (16), this method is not perceived as being more sensitive than the method we used. Moreover, Smith et al. (21) demonstrated several cases in which O157 isolates were detected by colony hybridization but not by plating on sorbitol-MacConkey agar followed by testing with a specific O157 antiserum. In addition, growth on sorbitol-MacConkey agar is not selective for the sorbitol-fermenting non-O157 SLT-producing *E. coli* and the sorbitol-fermenting *E. coli* O157, which are frequently the cause of HUS and hemorrhagic colitis in Germany (10). Inclusion of the cephalosporin antibiotic cefixime in sorbitol-MacConkey agar was shown to improve its selectivity for O157 by inhibition of *Proteus* spp. (7). Unfortunately, the sorbitol-fermenting *E. coli* O157 strains are extremely sensitive to cefixime and are missed if this medium is used (12). The question which then arises is whether the use of PCR or stool toxin testing might be more sensitive than culture and subsequent colony hybridization in establishing the infectious or noninfectious state of a patient. In general, detection of DNA and cytotoxic activity do not indicate whether the O157 cells are viable. With the culture method and subsequent colony hybridization, the presence of viable and potentially infectious organisms can be demonstrated. Because the cytotoxicity assay and the PCR were not consistently used with all samples, a direct comparison with the colony hybridization is unfortunately not possible. However, it has been demonstrated that the cytoxicity assay applied to convalescent-phase stool samples might be still positive when culture and colony hybridization are negative (4). Immunomagnetic separation of *E. coli* O157 appears to be a promising alternative for the future and may resolve some of the problems associated with the methods described above (8). This technique enables isolation of *E. coli* O157 cells in the presence of background flora and can further isolate them from the sample environment, which might be inhibitory to PCR and cytotoxicity assays (8). Because of the high sensitivity

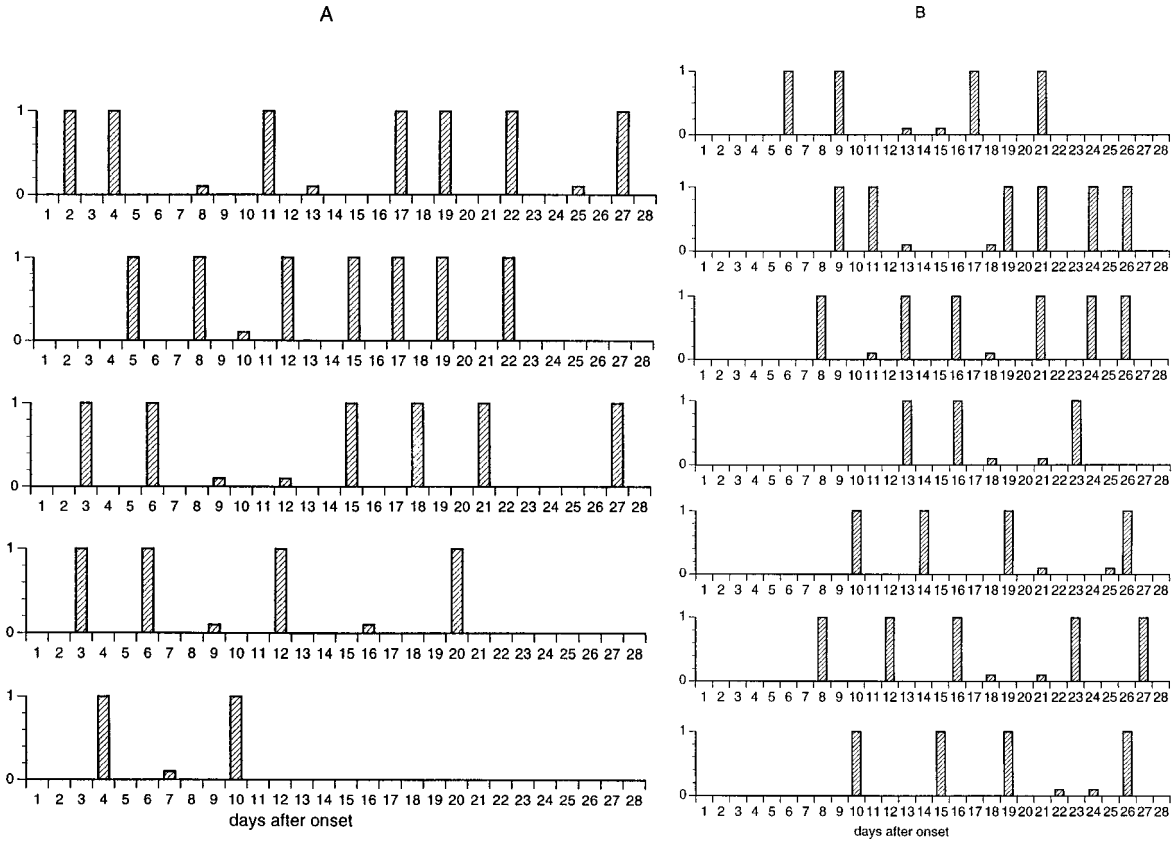

FIG. 2. Intermittent fecal shedding of *E. coli* O157 over a 28-day period in patients with diarrhea (A) and HUS (B). Bars with a size of 1 are culture positive; bars with a size of 0.1 are culture negative.

of this method, it may be shown that *E. coli* O157, even in smaller numbers, can be shed over an even longer interval than shown in this study.

It appears from other studies that the infective dose for O157 is low (3, 6, 17). Belongia et al. (3) have shown that there was no evidence of person-to-person transmission at day care facilities when children with diarrhea were allowed to return to the facility after two consecutive O157-negative stool cultures.

TABLE 1. Comparison of *E. coli* O157 strains isolated from the first and last O157-positive stool samples from long-term shedders

Patient no.	First (top) and last (bottom) day of O157-positive culture	CFU of *E. coli* O157/g of stool	No. of colonies analyzed	Characteristics of multiple colonies from primary- and convalescent-phase stools		
				Serotype	SLT genotype	PFGE type[a]
1	2	1×10^7	8	O157:H7	II	A
	39	4×10^8	8	O157:H7	II	A
12	2	3×10^7	12	O157:H$^-$	II/IIc	B
	42	2×10^7	12	O157:H$^-$	IIc	C
19	2	1×10^6	16	O157:H7	IIc	D
	62	3×10^7	16	O157:H7	IIc	D
22	4	4×10^8	14	O157:H7	II/IIc	E
	33	6×10^7	14	O157:H7	II/IIc	E
39	7	1×10^8	16	SF[b] O157:H$^-$	II	F
	62	1×10^8	16	SF O157:H$^-$	II	G
47	10	2×10^6	16	O157:H7	II/IIc	H
	58	1×10^6	16	O157:H7	II	I
53	10	2×10^6	16	O157:H7	I	J
	124	2×10^7	16	O157:H7	I	J

[a] Different letters represent distinct fragment patterns.
[b] SF, sorbitol fermenting.

As shown in the present study, because of the possibility of intermittent shedding, the standard for noninfectivity might require three negative stool samples collected at 3-day intervals within 9 days. Because most of the patients in our study were preschool children and hygiene practices are difficult to ensure in this age group, we recommend that children excreting *E. coli* O157 should not be allowed to attend day care facilities as long as they are shown to shed O157. The largest *E. coli* O157 outbreak recognized to date in Germany occurred in a day care center and involved 39 children and 2 adults (17). Three of the patients developed HUS, and one child died. It was demonstrated that person-to-person contact was the primary mode of transmission. This outbreak emphasizes the importance of prompt case reporting as well as follow-up testing for *E. coli* O157 in stool cultures. Moreover, future epidemiological studies must determine the optimal detection method for O157, and which tests best establish the infective state.

In three patients, PFGE patterns showed a shift in pattern between the first and last isolates, suggesting three possibilities. Either there was reinfection or a double infection with another strain, or there was a genetic change and clonal replacement within the patient. This latter hypothesis is supported by loss of the *slt*-II gene in O157 isolates from two patients, which could result in a detectable change in the PFGE type. Loss of *slt* genes occurs frequently in non-O157 *E. coli* during laboratory culture (14). Because we have not observed spontaneous loss of *slt* genes within our large collection of *E. coli* O157 strains (12), it is more likely that changes in PFGE type resulted from temporal change in the genotypic composition of the *E. coli* O157 flora of an individual host. We refer to this phenomenon as clonal turnover, which refers to dramatic changes in clonal composition characterized by the appearance of new clonal genotypes and loss of old clones. Clonal turnover can be a result of immigration and local extinction or periodic selection for a mutant that arises in situ.

Change of the O157 genotypes within individual patients has two important implications for epidemiology. First, long-term shedders of O157 strains may spread different genotypes, depending on when in the course of infection the O157 organisms are transmitted. With clonal turnover, a host may shed one PFGE type in the first week of infection and a different genotype, possibly with a different toxin profile, after 2 or 3 weeks of infection. Thus, a single long-term shedder could change the O157 genotypic characteristics in an outbreak. Second, the distinction between sporadic and outbreak cases in O157 epidemics becomes difficult when PFGE profiles change as a result of clonal turnover. Thus, studies such as those performed by Barrett et al. (2) may underestimate the number of outbreak cases when cases are classified primarily by the PFGE profiles of O157 strains. Our results call for further study of the stability of O157 PFGE types within individual patients and during the courses of outbreaks.

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