JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1996, p. 516–519 Vol. 34, No. 3
0095-1137/96/$04.00+0
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Isolation of Enterohemorrhagic *Escherichia coli* O157 Strains from Patients with Hemolytic-Uremic Syndrome by Using Immunomagnetic Separation, DNA-Based Methods, and Direct Culture

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Received 12 September 1995/Returned for modification 9 November 1995/Accepted 7 December 1995

We examined 30 children with classical hemolytic-uremic syndrome (HUS) for the presence of enterohemorrhagic *Escherichia coli* (EHEC) strains in stool samples and determined the specific immune response to O157 lipopolysaccharide in acute-phase serum samples from these patients. EHEC O157 strains were isolated from stool samples of 18 (60%) of the patients, and non-O157 EHEC strains were isolated from 5 (17%) of the patients. For O157 strain isolation from stools, we introduced a selective enrichment step using O157-specific antibodies attached to paramagnetic particles (immunomagnetic separation [IMS] method). This procedure allowed the detection of O157 strains at 10^2 CFU/g of stool in the presence of 10^7 coliform background flora organisms. By using IMS followed by plating on sorbitol MacConkey (SMAC) agar and cefixime-tellurite SMAC (CT-SMAC) agar, O157 strains were detected in 18 samples, whereas colony hybridization detected a subset of 12 positive samples and direct culture on CT-SMAC or SMAC agar detected only 7. Three of the 18 O157-positive stools were negative by cytotoxicity assay performed with stool filtrates and by direct PCR with DNA extracted from stools. The IMS technique allowed the isolation of O157 strains from 18 of 20 patients with serological evidence for O157 infection. Apart from the increase in sensitivity in O157 detection compared with that of direct culture, the IMS technique also has the advantage of being less labor-intensive and less time-consuming than the molecular methods. IMS can therefore be considered an efficient method for widespread use in the detection of O157 strains in clinical microbiology laboratories. However, because a significant number of HUS cases were attributable to non-O157 EHEC serogroups, the use of additional methods besides IMS in the bacteriological diagnosis of HUS is necessary.

Enterohemorrhagic *Escherichia coli* (EHEC) O157 is a major cause of serious outbreaks and sporadic cases of hemorrhagic colitis and hemolytic-uremic syndrome (HUS). The infectious dose of *E. coli* O157 is very low, and as a result the organism can be transmitted efficiently not only via contaminated foods but also from person to person (9). Direct transmission within families (12) and in institutional settings such as nursing homes (5) and day care facilities (2) has been reported.

The currently accepted methods for the isolation of O157 strains consist of assays for the detection of Shiga-like toxins (SLTs), either directly or at the genomic level, coupled with direct plating on sorbitol MacConkey (SMAC) agar (14), cefixime-SMAC agar (6), or SMAC agar supplemented with cefixime and tellurite (CT-SMAC) with subsequent serotyping. Accurate diagnosis of EHEC O157 infections requires the isolation of the pathogen to clarify the etiology of disease and the infectiousness of patients as well as to allow subtyping of strains for epidemiological purposes (10). The probability of isolating *E. coli* O157 strains from stool cultures of patients is inversely related to the interval between the onset of diarrhea and the microbiological culture (18). In the majority of patients with HUS, the syndrome occurs about 1 week after the onset of diarrhea, and it is often not possible to isolate the organism (3, 8). This could be because of elimination or excretion of low numbers of O157 organisms not detectable by currently used methods.

In the food microbiology and veterinary sectors, the detection rates for O157 strains have recently been improved by the introduction of a selective enrichment step using O157-specific antibodies attached to paramagnetic particles (immunomagnetic separation [IMS] method) (7, 19). This study was undertaken to clarify whether a similar improvement could be made for human stool samples.

MATERIALS AND METHODS

Stool and serum samples of HUS patients. Stool samples from 30 sporadic-HUS patients, collected between March and August 1995 for a prospective study from different pediatric centers in Germany, were investigated. All patients had diarrhea, and in 21 of the 30 cases (70%) the diarrhea was bloody. The mean age of the patients was 3.7 years (range, 1 year 6 months to 10 years 10 months). Stools only from patients who had not received antibiotic treatment at the time the stool samples were taken were investigated. A single, fresh stool sample obtained from each patient 1 to 3 days after the beginning of HUS (5 to 9 days after the beginning of diarrhea) was analyzed. All tests from one patient's stool were run simultaneously. In addition, a serum sample collected during the same period (before transfusion or dialysis) was investigated for O157 lipopolysaccharide (LPS) immunoglobulin M antibodies by an immunoblot assay as described previously (3).

Cytotoxicity assay. A cytotoxicity assay with Vero cells was carried out with stool samples diluted geometrically from 1:2 to 1:128 in phosphate-buffered saline (PBS) (3).

PCR and colony hybridization. PCR with primer pairs KS7-KS8 and GK5-GK6, specific for *slt-II*B and *slt-I*B, respectively (16), was performed directly with stools which were processed as described by Ramotar et al. (15). Colony hybridization with 100 to 200 well-separated colonies grown on SMAC agar was performed as described elsewhere (11).

Phenotyping, genotyping, and serotyping. Up to 20 sorbitol-negative colonies

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TABLE 1. Comparison of results of direct culture, DNA-based methods, and cytotoxicity assay for stool samples from 18 patients with O157-positive stool cultures

No. of samples	Direct culture on:		Colony hybridization	IMS and culture on:		Direct stool analysis by:	
	SMAC agar	CT-SMAC		SMAC agar	CT-SMAC	PCR	Cytotoxicity assay
5	+	+	+	+	+	+	+
2	−	+	+	+	+	+	+
2	−	−	+	+	+	+	+
2[a]	+	−	+	+	−	+	+
2	−	−	−	+	+	+	+
3	−	−	−	+	+	−	−
1	−	−	−	+	+	−	+
1	−	−	+	−	+	−	+

[a] Sorbitol-fermenting O157:H$^-$ strains.

or, in their absence, sorbitol-positive colonies were screened by slide agglutination with anti-O157 serum (Oxoid, Unipath GmbH, Wesel, Germany). *E. coli* strains confirmed as SLT producers by colony hybridization were biotyped and serotyped as described by Aleksic et al. (1). SLT genotyping was performed as described by Rüssmann et al. (16).

IMS. For IMS, 1-ml portions of the patients' stool samples diluted 1:2 with PBS were inoculated into 10 ml of GN Broth Hajna (Difco, Detroit, Mich.) and incubated at 37°C for 4 h. To 1 ml of preenriched sample was added 20 μl of anti-*E. coli* O157 Dynabeads in a 1.5-ml microcentrifuge tube, and the tubes were incubated for 10 min at room temperature with constant mixing. The tubes were placed in a magnetic separator rack (MPC-M; Dynal, Oslo, Norway), and the magnetic slide was placed in position and left for 3 min. The culture supernatant was removed by aspiration with a Pasteur pipette, the magnetic slide was removed from the rack, the paramagnetic bead-bacterium complex was washed twice by being resuspended in PBS-Tween buffer (0.05% Tween 20) and repeating the magnetic separation step. Finally, the paramagnetic bead-bacterium complex was resuspended in 100 μl of PBS-Tween, and 50 μl of this suspension was plated in parallel on SMAC agar (Oxoid) and CT-SMAC, prepared as described previously (20). Putative O157 colonies were subjected to slide agglutination with anti-O157 serum as described above.

Sensitivities of direct culture, IMS, and DNA-based methods. Three strains of *E. coli* O157:H7 (EDL 933, 4821/87, and 6578/91) and three strains of O157:H$^-$ (E32511, 7576/92, and 6537/91) were each grown overnight at 37°C in Luria broth, and the CFU were estimated by a standard serial dilution method.

Three different human O157-negative stool samples containing 10^7 coliform bacteria per g were each diluted 1:2 with PBS and spiked with different concentrations (10^1 to 10^7 CFU/ml) of the above O157 strains or left untreated (negative control). The following tests were carried out: PCR directly on stool samples, direct plating of serially diluted samples (10-fold dilution steps) onto SMAC agar or CT-SMAC, and colony hybridization from SMAC agar plates. Furthermore, 1-ml samples of the stool suspensions were inoculated into 10 ml of GN Broth Hajna, incubated at 37°C for 4 h, and plated on SMAC agar and CT-SMAC. In addition, IMS and subsequent plating were carried out. To determine the ratio of O157 colonies to the total number of colonies grown on SMAC agar or CT-SMAC at the highest dilution at which detection of O157 strains was still possible, all colonies were counted. Moreover, all non-sorbitol-fermenting colonies at the limiting dilution were tested by agglutination with O157 antiserum. Colonies that gave positive results with this test were further analyzed by colony hybridization with DNA probes for SLT genes as described above.

RESULTS

Isolation of EHEC strains from stool samples of HUS patients. Eighteen of 30 samples (60%) had *E. coli* O157 strains, 5 of 30 (17%) had non-O157, SLT-producing *E. coli* (SLTEC) strains, and 7 of 30 (23%) were negative for all SLTEC strains. The non-O157 EHEC strains were identified by colony hybridization, and for these samples the cytotoxicity assay and PCR were also positive. The non-O157 SLTEC strains were of serotypes O2:H6, O8:H21, O26:H$^-$, O69:H$^-$, and O111:H$^-$. In 18 of the samples, O157 strains were detectable. Only IMS gave a positive result for all 18 samples. With the other detection methods, the rate of detection of O157 strains was lower. The results are summarized in Table 1.

TABLE 2. Correlation between the presence of O157 LPS immunoglobulin M antibodies in acute-phase sera and result of stool culture for EHEC

Result of stool culture for EHEC	No. of serum samples with the indicated O157 antibody test result	
	Positive	Negative
O157		
Positive	18	0
Negative	2	10
Non-O157		
Positive	1	4
Negative	0	6

Phenotypic and genotypic characterization of O157 strains. Additional testing of the O157 strains showed that 17 isolates possessed *slt-II* genes either alone or in combination with *slt-IIc*, one non-sorbitol-fermenting O157:H7 isolate carried *slt-I*, and all 18 strains were toxigenic. Two of the O157 EHEC isolates were nonmotile, sorbitol-fermenting strains. These strains did not grow on CT-SMAC (Table 1).

Detection of anti-O157 LPS antibodies. In 20 of the 30 patient serum specimens, O157 LPS antibodies of the immunoglobulin M class were detected. Table 2 shows the correlation between the presence of anti-O157 antibodies and the culture results. As seen from this table, for all but two of the patients with serological evidence of O157 infection, the organism could be isolated. One patient had non-O157 EHEC organisms in the stool but developed antibodies against the LPS of serogroup O157.

Sensitivity of IMS. The limits of detection of O157 strains with spiked stool samples are shown in Table 3. There were no differences in the results for the three stool samples tested. IMS was at least 100-fold more sensitive than any other tested method. Even at the limiting dilutions, 10 to 15% of the colonies grown on SMAC agar were O157 strains. On CT-SMAC, the ratio of O157 colonies to the total number of colonies was between 80 and 100%.

DISCUSSION

In the past decade, many investigators have confirmed the pioneer work by Karmali et al. (13) on the association of infection with SLTEC and the development of HUS. Because all epidemiological data, case reporting, and surveillance systems rely on strain isolation, the most efficient methods must be determined (10). There are indications (3, 8) that the rate of isolation of O157 strains by currently used techniques is not satisfactory: (i) serological data provide evidence of O157 infections in patients with HUS from whom the organism cannot be isolated, and (ii) cytotoxicity assays applied to stool samples can be positive when direct culture and molecular methods are negative. Since the IMS technique has provided promising results in the isolation of *E. coli* O157 strains from bovine feces and foodstuffs (7, 19), we evaluated this technique with stool samples from HUS patients and compared it with serological and microbiological methods. Using the IMS method, we were able to isolate O157 strains from 90% of the patients with a positive O157 serology. In previous studies, this pathogen was isolated from only 26% (8) or 20% (3) of the HUS patients with serological evidence of O157 infection. The present study provides evidence that *E. coli* O157 is still excreted in stools of the majority of HUS patients and can be isolated by IMS after preenrichment culture. The improvement by IMS over cur-

TABLE 3. Limits of detection of *E. coli* O157 strains in fecal samples containing 10^7 coliforms and 10^1 to 10^7 O157 organisms

Strain no.	Fewest detectable CFU/g of fecal sample by[a]:							
	Direct culture on:		Colony hybridization from SMAC agar	Plating on[b]:		IMS[b] and culture on:		PCR on stool
	SMAC agar	CT-SMAC		SMAC agar	CT-SMAC	SMAC agar	CT-SMAC	
EDL 933	10^5	10^4	10^5	10^4	10^4	10^2	10^2	10^5
E32511	10^5	10^4	10^5	10^5	10^4	10^3	10^2	10^5
4821/87	10^5	10^4	10^5	10^5	10^4	10^3	10^2	10^5
6578/91	10^5	10^4	10^5	10^5	10^4	10^3	10^2	10^5
7576/92	10^6	10^5	10^5	10^5	10^5	10^3	10^2	10^6
6537/91	10^5	10^5	10^5	10^5	10^4	10^3	10^2	10^5

[a] Data are averages of three experiments performed with different stool samples.
[b] After 4 h of preenrichment.

rently used methods will thus allow detection of O157 strains in many HUS patients for whom stool cultures are negative by currently used isolation techniques.

We have shown that direct culture on SMAC agar or CT-SMAC is the least sensitive method for the isolation of O157 strains from HUS patients. This is probably because of the low number of O157 organisms excreted by HUS patients in stools and the high level of background flora (17). After selective enrichment, with IMS, distinct colonies were observed on both SMAC agar and CT-SMAC plates. In many samples, the background flora on CT-SMAC was greatly reduced compared with that on SMAC agar, making it easier to obtain O157 strains in pure culture. However, the two SLT-II-producing, sorbitol-fermenting O157:H$^-$ strains isolated in this study did not grow on CT-SMAC. This finding is at variance with that of Zadik et al. (20), who reported that 314 of 315 toxigenic O157 isolates from human feces grew on CT-SMAC.

Despite the generally accepted high level of sensitivity of molecular methods, O157 strains could not be detected in several IMS-positive patient samples. From our data, approximately 10^5 CFU of EHEC per g of stool is necessary to obtain a positive PCR result. Ramotar et al. (15) reported that their PCR assay was able to detect 10^3 to 10^5 CFU/g of stool spiked with SLT-I-containing laboratory strain *E. coli* C600 (H19J) and 10^8 CFU/g of stool for SLT-II-producing *E. coli* C600 lysogens. Notably, 17 of the 18 O157 strains isolated in our study possessed SLT-II-encoding genes. Moreover, an additional drawback of PCR is that it does not allow isolation of the organism. Stool samples considered O157 culture negative by PCR or colony hybridization may still contain up to 10^5 CFU of *E. coli* O157 per g of stool. Since immunomagnetically selected *E. coli* O157 is detectable on CT-SMAC plates of preenriched samples containing as little as 100 *E. coli* O157 cells per g of stool, this technique seems to be the best approach for establishing O157 infection in a patient.

Apart from the increase in sensitivity shown by IMS, this method also has the advantage of being less sophisticated than the molecular methods with which it has been compared here. IMS can therefore be considered a suitable method for widespread use in the detection of O157 strains in clinical microbiology laboratories. The improvement of O157 strain isolation by IMS will allow detection of O157 strains at lower excretion rates and thus assist further epidemiological investigations, such as clarification of the infection routes and determination of the infectiousness of patients. This in turn may lead to the development of more-effective control and prevention strategies.

Although EHEC O157 was the major pathogen of HUS in this study, a significant number of sporadic HUS cases were attributable to other EHEC serogroups. Since the data presented were restricted to patients who had not received antibiotics at the time the stool samples were taken, the data cannot be extrapolated to draw conclusions about the regional distribution of O157 and non-O157 strains.

Interestingly, one of our patients showed a high anti-O157 antibody titer but yielded a non-O157 EHEC strain in the stool culture. This could be due to a cross-reactivity of O157 LPS with that of the infecting strain or a concomitant infection with two different EHEC strains, as suggested by others (3, 8), or, tentatively, a secondary infection of a host *E. coli* strain with O157 phages.

In 4 of the 10 HUS patients with negative O157 serology, non-O157 EHEC strains were detected. The diagnostic challenge posed by non-O157 EHEC has been reported by other investigators (4, 10, 13, 17) and at present necessitates the use of additional methods besides IMS in the bacteriological diagnosis of HUS. However, since the presently used tests for non-O157 EHEC are not easily adapted for busy clinical laboratories, new sensitive methodological developments, such as extending the range of EHEC serogroups that can be isolated by IMS, are warranted.

ACKNOWLEDGMENTS

We thank Kofitsyo Cudjoe (Dynal, Oslo, Norway) for his help in establishing the IMS technique in our laboratory and Peter Symmons (Dynal, Hamburg, Germany) for help in the study design. We thank Jochen Bockemühl, Hamburg, Germany, and Jürgen Heesemann, Würzburg, Germany, for helpful discussions.

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