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Enterohemorrhagic *Escherichia coli* Associated with Hemolytic-Uremic Syndrome in Chilean Children

ALVARO CORDOVÉZ,[1] VALERIA PRADO,[1]* LEONARDO MAGGI,[1] JAIME CORDERO,[2] JUAN MARTINEZ,[1] ALFREDO MISRAJI,[3] RICHARD RIOS,[4] GUILLERMO SOZA,[5] ALICIA OJEDA,[1] AND MYRON M. LEVINE[6]

Microbiology Unit, Faculty of Medicine, University of Chile,[1] Luis Calvo Mackenna Children's Hospital,[2] and Robero del Rio Children's Hospital,[3] Santiago, and Hospital Regional, Instituto de Pediatria, Universidad Austral, Valdivia,[4] and Hospital Regional, Temuco,[5] Chile, and Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201[6]

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A clinicoepidemiological study was undertaken to determine if enterohemorrhagic *Escherichia coli* (EHEC) was associated with hemolytic-uremic syndrome (HUS) in children in Santiago, Valdivia, and Temuco, Chile. Prospective surveillance detected 20 hospitalized cases of HUS in children less than 4 years of age in these cities from March 1988 to March 1989. Each HUS patient was matched (by sex and age) with two control children (hospitalized elective-surgery patients). To detect EHEC, DNA from stool culture isolates of *E. coli* was detected by hybridization with biotin-labelled DNA probes specific for the EHEC virulence plasmid, Shiga-like toxin I (SLT-I) or SLT-II. Stool cultures from 6 of 20 cases (30%) and from 2 of 38 controls (5.3%) yielded EHEC ($P = 0.0158$). EHEC isolates from all HUS cases hybridized with the EHEC plasmid probe and with probes for SLT-I or -II (or both). The serogroups of the isolates included O157, O26, and O111. EHEC causes HUS in Chile, and the biotinylated gene probes are practical diagnostic tools for epidemiologic studies.

Hemolytic-uremic syndrome (HUS), which is characterized by acute renal insufficiency, microangiopathic hemolytic anemia, and thrombocytopenia (5, 7–10), constitutes an important clinical problem in pediatric populations in Chile, Argentina, and Uruguay (4, 5, 9, 22, 36). In Argentina and Chile, HUS is the most common cause of acute renal insufficiency in children less than 4 years of age, and a proportion of affected children die of this illness (4, 5, 9, 22, 36). As experience accumulated worldwide, it became apparent that cases of HUS typically are preceded by an episode of (often bloody) diarrhea (7–10), suggesting that an infectious agent was responsible. In the 1980s, this proved to be correct when a novel category of diarrheagenic *Escherichia coli*, so-called enterohemorrhagic *E. coli* (EHEC), that was causally associated with the syndrome of hemorrhagic colitis (1, 4) and subsequently with HUS was discovered (1, 12, 16–18, 25, 26, 33). While the prototype EHEC strain is of serotype O157:H7, a number of other serotypes, such as O26:H11, O111:H8, and O145:NM, are also recognized (20).

The virulence properties manifested by EHEC include the elaboration of Shiga-like toxin I (SLT-I) or SLT-II (or both) encoded by bacteriophages (32, 34), carriage of a plasmid associated with the expression of a novel variety of fimbriae that favors the attachment of EHEC to epithelial cells (14), and the expression of a protein (encoded by a chromosomal gene) that results in intimate attachment to intestinal cells (36, 37). The terms Verotoxin I and II (32) are synonymous with SLT-I and -II, respectively. The DNA sequence of SLT-I is identical to Shiga toxin elaborated by *Shigella dysenteriae* 1, while there is 58% sequence homology between antigenically distinct SLT-I and SLT-II (13).

Several diagnostic tools have been developed on the basis of the virulence properties of EHEC, particularly O157:H7. These include a DNA probe to detect the 60-MDa plasmid of EHEC (21), as well as probes to detect the genes that encode SLT-I and -II and the phages that carry the SLT structural genes (27). The use of serologic tests to measure significant rises in the serum titer of antitoxin or of O157 antibody or of immunoassays to detect SLTs directly in stools increases the sensitivity of detecting EHEC infection since these assays are positive in a proportion of patients with negative cultures (17, 19, 23). Although HUS is a well-studied clinical entity and an important public health problem in Argentina and Chile (4, 5, 9, 10, 22), etiologic studies have been few and limited (21, 23, 31). In the clinicoepidemiologic study described herein, we used DNA probes to identify EHEC in stool cultures of children hospitalized with HUS and in age-matched control children without diarrhea or renal disease.

MATERIALS AND METHODS

Definitions of HUS patients and controls. The isolation of EHEC from HUS patients and that from age- and sex-matched hospitalized control children without diarrhea or HUS were compared. HUS patients were defined as hospitalized children less than 4 years of age with a clinical diagnosis of HUS and whose clinical picture included an acute onset of microangiopathic hemolytic anemia and renal insufficiency. Surveillance to detect such HUS patients was maintained in the several pediatric hospitals of metropolitan Santiago and in Valdivia and Temuco during the period

* Corresponding author.

TABLE 1. Comparison of the demographic and clinical characteristics of and isolation of EHEC from two study groups, children hospitalized with HUS and healthy control children

Group	No. of children	Mean age (mo)	% Males	No. receiving recent antibiotic therapy	No. (%) from which EHEC was isolated
HUS patients	20	13	50	12	6 (30)[a]
Controls	38	12.1	47	0	2 (4.5)[a]

[a] HUS cases versus controls, $P = 0.0158$.

March 1988 to March 1989. For each HUS patient, two controls were sought who were matched by age (within 3 months) and sex. The controls consisted of children admitted to the hospital for elective surgery who did not have diarrhea, HUS, or renal disease.

Calculation of sample sizes. Sample sizes for the HUS and healthy child control groups were generated by estimating that 1.8% of healthy control children would have EHEC in their stool cultures (on the basis of unpublished surveillance data of a pediatric cohort in Santiago) and that the rate of isolation of EHEC from HUS patients would be at least 25%. To detect a significant difference (alpha = 0.05, beta = 0.2) between the groups (single-tail hypothesis), it was calculated that 19 HUS patients and 38 healthy controls would suffice.

Collection of specimens and bacteriologic methods. A sample of stool on a cotton-tipped applicator stick or a rectal swab (if stool was not available) was obtained from each of the HUS patients as close as possible to the time of admission to the hospital and then inserted into Cary Blair transport medium. Stool cultures were collected from 17 HUS patients within 24 h of admission and from the remaining 3 patients during the second day of hospitalization. Of the 13 HUS patients who had diarrhea on admission to the hospital, all had stool cultures taken while they still had diarrhea. Samples from controls were identically transported in Cary Blair medium. The swabs were plated onto MacConkey agar and incubated at 37°C for 18 to 24 h. Lactose-negative colonies were investigated to identify *Shigella*, *Salmonella*, and *Yersinia* spp. Ten to 20 lactose-positive coliform colonies from each plate were selected and confirmed as *E. coli* by using standard biochemical methods (6).

Detection of EHEC by DNA probes. Confirmed *E. coli* colonies from the coproculture of each subject were transferred to Whatman filter papers for colony blot hybridization with biotinylated probes that detect the EHEC plasmid associated with fimbrial expression (21), SLT-I, and SLT-II (27). The methods to prepare the filters and to prepare and use the biotinylated probes have been previously described in detail (11). Briefly, the filters were treated with NaOH and heat to release DNA from the bacterial colonies, render it single stranded, and fix it to the solid phase. The filters were then exposed to a solution containing 1.5 mg of lysozyme per 100 ml and 25 g of sucrose per 100 ml for 30 min, washed, and then treated with a proteinase K solution (250 μg/ml) for 30 min at 37°C. The pCVD 419 EHEC probe is a 3.4-kb fragment of the EHEC plasmid of prototype strain 933 that serves as a highly sensitive and specific probe to detect EHEC. The SLT-I probe consists of a 1.1-kb phage-derived fragment that encodes 98% of the A subunit and the entire B subunit (27). The SLT-II probe consists of an 0.84-kb fragment that encodes 95% of the A subunit (27). For the purposes of this study, EHEC strains were defined as strains that hybridized with at least one of the probes. *E. coli* isolates were serogrouped by agglutination with O antisera obtained from ProBac, Sao Paulo, Brazil.

RESULTS

Characteristics of the patients and equivalence of the study groups. A comparison of the relevant demographic and clinical aspects of the two study groups is summarized in Table 1. A total of 38 controls were matched to the 20 HUS patients (for two cases, only a single control was obtained, whereas for one case, the only available controls were more than 3 months apart in age). Thirteen of the 20 cases of HUS occurred during the summer months of December to March. All 20 HUS patients presented a prodrome of diarrheal disease, 13 of which experienced bloody diarrhea; the mean duration of the diarrheal illness (by history) was 4.6 days. Twelve HUS patients had a history of having received antibiotics during the prodromal diarrheal illness; four patients received amoxicillin or ampicillin, three got trimethoprim-sulfamethoxazole, three received furazolidone, one got neomycin (in a kaolin suspension), and one received chloramphenicol. At the time of admission to the hospital, 13 HUS patients were still suffering from diarrhea, while in the other HUS patients, diarrhea had ceased 1 to 7 days earlier. Sixteen of the HUS patients presented to hospital with oliguria or anuria, and 10 were obtunded.

The hematological examinations in all patients revealed schistocytes and other abnormal morphologic forms of erythrocytes; hematocrits ranged from 14 to 29%, while hemoglobin counts were between 3.4 and 9.3 g%. Platelet counts were significantly diminished in 14 children, with a mean value of 41,571 per mm^3. Blood urea nitrogen and creatinine levels were elevated, reaching means of 86 and 4.2 mg/100 ml, respectively.

Isolation of EHEC. Colony blot hybridization with the DNA probes detected EHEC in 6 of 20 HUS patients (30%) versus 2 of the 38 matched control children (5.3%) ($P <$

TABLE 2. Profile of virulence properties of EHEC isolates from HUS patients and control children

Source of isolates and isolate no.	EHEC plasmid	SLT-I	SLT-II	Serogroup
HUS patients[a]				
1	+[b]	+[b]	+[b]	O157
2	+	+	+	O157
3	+	+	+	O26
4	+	+	+	O26
5	+	+	+	O111
6	+	+	−	O157
7	+	+	−	O26
8	+	+	−	O111
Controls				
1	+	+	−	O26
2	+	+	−	O26

[a] Isolates 1 to 6 came from six patients in the case-control study, while isolates 7 and 8 came from two sporadic cases of HUS.
[b] Detected by DNA probe.

TABLE 3. Comparison of the rates of isolation of EHEC from HUS patients in North America, Europe, and South America

Geographic site	Isolation rate of EHEC[a]		P	Source or reference
	HUS patients	Controls		
Canada	12/40 (30)	0/40 (0)	0.00019	17
Argentina	4/51 (8)	0/25 (0)	0.30	23
United Kingdom	52/196 (26.5)	2/51 (3.9)	0.00012	19
United States	7/14 (50)	—	—	35
Germany	8/21 (38)	—	—	2
Chile	6/20 (30)	2/38 (5.3)	0.0158	This study

[a] Number of children with EHEC/number of children with HUS whose stool cultures yielded *E. coli* (%).

0.0158). Three of the eight HUS patients who did not receive antibiotics yielded EHEC (38%). There was a suggestion that the detection of EHEC may be related to the interval between the onset of the prodromal diarrheal illness and the time of obtaining stool cultures. For example, EHEC strains were detected in 3 of 4 children (75%) who had diarrhea for 1 to 4 days before culture, from 3 of 14 children (21%) who had diarrhea for 5 to 8 days before culture, and from 0 of 3 children who had onset of their diarrhea 9 to 11 days before stool cultures were taken.

Virulence properties of the EHEC strains. The genotypes of the strains isolated from the study participants, including the six HUS patients and the two controls, are summarized in Table 1. Five additional cases of HUS were detected in Santiago in October 1989 after completion of the case-control study. These patients were also cultured, and EHEC strains were isolated from two of these patients. These two EHEC strains were characterized along with strains from the case-control study. As shown in Table 1, all strains hybridized with at least one of the toxin probes and with the EHEC plasmid probe (Table 2). The most common pattern (seen in strains from five of eight HUS patients) revealed positivity with all three probes. EHEC strains from the patients with HUS included serogroups O157 (three patients), O26 (three patients), and O111 (two patients). EHEC strains from the two control patients were both of serogroup O26 (Table 2).

Antibiotic sensitivity of the EHEC strains. The EHEC isolates from all HUS patients and from the controls were examined for susceptibility to antibiotics by the disk method. In total, 65% of the strains were resistant to ampicillin, 41% were resistant to trimethoprim-sulfamethoxazole, 29% were resistant to tetracycline, and 6% were resistant to chloramphenicol.

DISCUSSION

Observations made in this study in Chile show striking similarities to reports of HUS in North America and Europe and to the few reports from Argentina. As was the case in North America, HUS cases were more common in the summer. In Chile, 30% of the HUS patients yielded EHEC strains in their stool cultures, a significant difference from the 5.3% recovery rate in healthy control children (P = 0.0158). Worldwide, there have been only a few prospective studies in which stool cultures from patients with HUS were systematically tested for *E. coli* that produce SLTs and even fewer in which matched control children were sampled. These studies differ from one another in the methodologies used to identify EHEC (in this context defined as any SLT-producing strain). Nevertheless, the rate of isolation of EHEC from Chilean children with HUS is similar to the rates of isolation from HUS patients in North America (17, 25), the United Kingdom (19), and Germany (2) (Table 3). As in other studies, the isolation of EHEC from controls was low.

A characteristic feature of EHEC infection in patients with hemorrhagic colitis and HUS is the short duration of fecal shedding of the organisms, which leads to an underestimation of the magnitude of the EHEC problem (35). The use of serologic tests to measure significant rises in serum titer of antitoxin or of O157 antibody or of immunoassays that detect SLTs in stools increases the sensitivity of detecting EHEC infection since these assays are positive in a proportion of patients with negative cultures (17, 19, 23). It is likely that if these additional methods had been employed in this study, the proportion of HUS cases judged to be due to EHEC would be even higher.

A slightly higher rate of recovery (3 of 8 [38%]) of EHEC was found in the Chilean HUS patients who had not received antibiotics during the prodromal illness. On the other hand, some clinicoepidemiological studies suggest that prior antibiotic therapy may be a risk factor for development of HUS (3, 24, 28). A possible explanation for this may lie in results of microbiological studies that show that the expression and release of SLTs are increased when EHEC bacteria are exposed to sublethal concentrations of certain antimicrobial agents such as trimethoprim-sulfamethoxazole (15).

As is the case in North America, Europe, and neighboring Argentina, circa 90% of Chilean HUS patients experience a prodrome of diarrhea (4, 5), which is bloody in approximately one-half of the patients. HUS is well recognized to occur as a complication in a few percent of patients during outbreaks of diarrheal illness and hemorrhagic colitis due to EHEC (28, 33). Thus, it is now appreciated that the spectrum of EHEC infection includes asymptomatic carriage, nonbloody diarrhea, hemorrhagic colitis, and, at the severe end, HUS.

The eight HUS patients in our study were infected with EHEC serogroups (O157, O26, and O111) that are well recognized in North America and Europe (1, 2, 3, 17, 19, 29, 30); H typing was not carried out. Notably, all strains possessed the EHEC plasmid and at least one SLT; strains from five of the eight HUS patients possessed genes for both SLT-I and SLT-II, while three had only SLT-I. In North America and Europe, EHEC strains from HUS patients most commonly express SLT-II with or without SLT-I (1–3, 19, 27, 32, 34).

Positive features of this clinicoepidemiological study of EHEC were the practicality and ease of use of the three biotinylated gene probes. These advantages suggest that the probes might be quite useful in performing studies that investigate the human, animal, and environmental reservoirs of EHEC, modes of transmission, and risk factors for development of diarrhea, hemorrhagic colitis, and HUS.

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