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Analysis of Incidence of Infection with Enterotoxigenic *Escherichia coli* in a Prospective Cohort Study of Infant Diarrhea in Nicaragua

M. PANIAGUA,[1,2] F. ESPINOZA,[1] M. RINGMAN,[2] E. REIZENSTEIN,[2] A.-M. SVENNERHOLM,[3] AND H. HALLANDER[2]*

Department of Microbiology, National Autonomous University (UNAN), León, Nicaragua,[1] and Swedish Institute for Infectious Disease Control (SMI), Stockholm,[2] and University of Göteborg, Göteborg,[3] Sweden

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Diarrheal episodes with enterotoxigenic *Escherichia coli* (ETEC) were prospectively monitored during the first 2 years of life in a cohort of 235 infants from León, Nicaragua. ETEC was an etiological finding in 38% (310 of 808) of diarrheal episodes and in 19% (277 of 1,472) of samples taken as asymptomatic controls at defined age intervals ($P = <0.0001$). The majority of diarrheal episodes (80%) occurred before 12 months of age. The major ETEC type was characterized by colonization factor CFA I and elaboration of both heat-labile enterotoxin and heat-stable enterotoxin (ST). The proportion of *E. coli* strains with CFA I was significantly higher in cases with diarrhea ($P = 0.002$). The second most prevalent type showed putative colonization factor PCFO166 and production of ST. The prevalence of PCFO166 was approximately 20%, higher than reported before. Children with a first CFA I episode contracted a second ETEC CFA I infection 24% of the time, compared with 46% for ETEC strains of any subtype. Most of the ETEC episodes were of moderate severity, and only 5% (15 of 310) were characterized as severe. In conclusion, our results give valuable information for the planning of intervention studies using ETEC vaccines.

Enterotoxigenic *Escherichia coli* (ETEC) strains are an important worldwide cause of diarrheal disease in humans, mainly affecting children in developing countries (3) and travellers going from developed countries to less developed countries (4, 5). Characteristically, these bacteria colonize the small intestine by adhering to the epithelium and induce secretion by elaborating toxins without invasion of or damage to cells. They can elaborate one or more enterotoxins that are either heat stable (ST) or heat labile (LT) (7).

ETEC strains have two major virulence determinants, enterotoxins and colonization factor antigens (CFA), which are almost always encoded by plasmids also encoding ST and/or LT (7). The colonization factors of human ETEC strains are mainly protein fimbriae; the most well characterized are CFA I, CFA II, and CFA IV (previously known as putative colonization factor 8775 [PCF8775]) (9, 10, 33, 38). Several other PCFs, e.g., CFA III (16), CS7 (14), CS17 (25) PCFO9 (13), PCFO20 (41), PCFO148 (17), PCFO159 (37), and PCFO166 (26), have been found in ETEC strains. The roles of PCFs have also been demonstrated recently (34). Although LT type I toxins are closely related to the cholera toxin (32), diarrheal disease caused by ETEC is usually less severe than that caused by cholera, but morbidity and mortality probably exceed those of cholera considerably on a worldwide basis due to the high frequency of ETEC infection. The incidence of ETEC infection is greater than that of rotavirus and is one of the major causes of dehydrating diarrheal disease throughout the developing world (6, 18, 29). In developing countries, children under 2 years of age typically have two or three episodes of diarrhea per year, with ETEC infections representing more than 25% of all diarrheal illnesses (6). Several studies have been carried out to determine the prevalence or incidence of ETEC infection and its colonization factors (2, 8, 12, 20, 39), but few of them have analyzed the situation over time (19, 20).

The aim of the present study was to prospectively analyze the incidence of ETEC infection and its colonization factors related to age in León, Nicaragua. The follow-up design for the first 2 years of life will also make it possible to examine the development of natural immunity.

MATERIALS AND METHODS

Design. This study was carried out in an urban territory of the Perla María Norori health area, which was randomly selected among the three existing health areas in León, the second largest city of Nicaragua. A follow-up, community-based study to be performed in this health area was designed.

Clinical materials and case definition. The size of the study population was 252 children, which corresponds to approximately 10% of the expected number of children born in 1991. The children were recruited as newborns from February 1991 to February 1992 at the University Hospital of Oscar Danilo Rosales and monitored for 2 years. Surveillance covered the period from February 1991 through January 1994. For the purpose of this study, diarrhea was defined as a change in the child's stool pattern, characterized by an increase in frequency (to at least three liquid stools per 24 h) and a watery consistency or the presence of blood and/or mucus, together or not with nausea, vomiting, and loss of appetite. Diarrheal episodes were considered to have commenced when they were preceded by 7 consecutive days without diarrhea and to end when the child was free of diarrheal symptoms for at least 24 h.

Severity was defined as follows: 1, mild, when the episode lasted no longer than 3 days without fever and vomiting and with good toleration of oral rehydration therapy at home; 2, moderate, episode duration of more than 3 days with fever and/or vomiting and with good toleration of oral rehydration at home; and 3, severe, episode with fever, vomiting, signs of severe dehydration, and potential need for hospitalization.

Surveillance. Active surveillance through home visits with trained nurses employed for the project was started from the first week by collecting samples and filling in forms with information regarding the epidemiological and socioeconomic conditions of each child's family. After this, a program of weekly home visits for 2 years was carried out. At each contact, the parents or any relations at home were asked about diarrheal episodes.

Sampling. At each diarrheal episode, stool specimens were obtained in screw-capped plastic tubes with a spoon and without any preservatives or by rectal swabbing for laboratory investigation. The samples were collected most often within 3 days after the onset of diarrhea. On some unusual occasions, there was a delay when the child was taken care of outside the home. Fecal samples were also taken at 0, 1, 3, 6, 9, 12, 18, and 24 months of age if they did not concur with an episode. A total of 847 samples were collected from 222 children with diarrhea, and 1,506 samples were collected during asymptomatic intervals.

* Corresponding author. Mailing address: Swedish Institute for Infectious Disease Control, S-105 21 Stockholm, Sweden. Phone: 46 8 735 13 00. Fax: 46 8 730 32 48.

Laboratory procedures. After collection, samples were transported to the Microbiology Department of the University of León, where microbiological examinations were conducted. The samples were analyzed for the presence of ETEC, enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, *Salmonella*, shigellae, campylobacters, *Giardia lamblia*, *Entamoeba histolytica*, cryptosporidia, and rotavirus. The problems associated with ETEC were the focus of this study.

Primary isolation. For primary isolation, the samples were inoculated on MacConkey, deoxycholate-citrate, and xylose-lysine-deoxycholate agars (BBL, Cockeysville, Md.) and incubated overnight at 37°C. All types of colonies growing were identified by conventional biochemical tests. Independent of the number of *E. coli* colonies found, a swab inoculated with at least five such colonies was kept in nutrient broth with the addition of 15% (wt/vol) glycerol at −70°C for further analysis.

Subculture. For LT and ST determinations, a full loop of bacteria was transferred from blood agar to Trypticase broth with yeast extract (Difco, Detroit Mich.), incubated at 37°C overnight with shaking (200 rpm), and then centrifuged at 3,000 × *g* for 15 min. The supernatant was tested in LT and ST enzyme-linked immunosorbent assays (ELISAs).

Detection of LT, ST, and CFA. When present, five to six *E. coli* colonies were collected from each fecal culture. ETEC screening was performed at the Swedish Institute for Infectious Disease Control, Stockholm, Sweden, by LT GM1 ELISA, ST inhibition ELISA ($n = 657$), or PCR ($n = 1,590$).

CFA testing was done at the Dept. of Medical Microbiology, University of Göteborg, Göteborg, Sweden. Before testing for CFA, the isolates were retested for LT and ST production by the in-house ELISA.

PCR. Strains were analyzed as crude lysates. Approximately 10 μl of colony material was suspended in 0.5 ml of distilled water and boiled for 10 min, and then 5 μl of a 1/50 dilution was amplified.

The detection of LTh, STIa, and STIb genes was modified from the method of Abe and coworkers (1). A 618-bp fragment from nucleotide 433 of LTh *toxA* to nucleotide 272 of LTh *toxB* (1, 42, 43) was amplified with primers LTh1, 5′ TGGTATCGTGTTAATTTTGGTG 3′, and Lth2, 5′ TCCTTCATCCTTTCAA TGGC 3′ (Scandinavian Gene Synthesis AB, Köping, Sweden, or Operon, Alameda, Calif.), in PCR buffer (Perkin-Elmer; manufactured by Roche Molecular Systems, Inc., Branchburg, N.J.) by adding 2 mmol of $MgCl_2$ per liter, 150 μmol (each) of dATP, dTTP, dGTP, and dCTP (Perkin-Elmer) per liter, 0.2 μmol of each primer per liter, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer) in a total volume of 50 μl. PCR was performed with a PE TC1 (Perkin-Elmer) for 32 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a 3-min denaturation step in the first cycle and a final extension step of 5 min, or with a PE 9600 (Perkin-Elmer) for the same cycles but with ramping from 50 to 72°C for 1 min and with a first denaturation step of 2 min, generating results comparable to those with PE TC1. A 240-bp STIa fragment (30) was amplified with primers by the method of Abe et al. (1). A 172-bp STIb fragment, encompassing nucleotides 45 to 216 (27), was amplified with primers STIb1, 5′ TTCACCTTTCCC TCAGGATGC 3′, and STIb2, 5′ ATAGCACCCGGTACAAGCAGG 3′ (Pharmacia Biotech, Sollentuna, Sweden, or Operon). A PCR with both primer pairs was performed as described above with the following modifications: 1.5 mmol of $MgCl_2$ per liter and 50 μmol (each) of dATP, dTTP, dGTP, and dCTP per liter.

Amplified products (10 μl) were analyzed by 3.2% agarose gel electrophoresis (Nusieve GTG; FMC, Rockland, Maine.) To avoid cross-contamination, three separate rooms with entirely separate equipment and solutions were used. Thus, the handling and treatment of samples and adding of template, the handling of DNA-free PCR reagents, and post-PCR work were stringently separated. Positive displacement tips (CP 10; Microman Gilson, Viliers-le-Bel, France) were used for the handling of strains.

(i) Negative controls. Between every fourth specimen, a water sample was processed in parallel with the others during the entire procedure of dilution of crude lysates and PCR. No such controls were positive by LT PCR. However, among 45 ST PCR analyses, two runs included one false-positive control each for STIb. In total, there were 15 strains positive for STIb within these runs. When these strains were retested, 6 of 15 gave negative PCR results. The nine that remained positive were finally considered positive cases.

(ii) Positive controls. Two crude lysates of *E. coli* strains (CCUG 35633 and CCUG 35634 [Culture Collection, University of Göteborg]) and one plasmid preparation (CCUG 35634) were analyzed in three or four dilution steps. Both strains were PCR positive for LT, STIa, and STIb. Suspensions of positive controls were frozen in aliquots and thawed only once for each experiment. At least two of the three positive controls were required to be positive at ≤200 CFU or ≤1 pg of plasmid for acceptance of the LT PCR result. Correspondingly, approval of the STI PCR result required detection of ≤2,000 CFU or ≤10 pg of plasmid, except for strain CCUG 35633, where ≤10^5 CFU were required for STIb.

ETEC ELISA I (Stockholm). (i) LT ELISA. A GM1 ELISA (ganglioside; catalog no. G-7641 [Sigma]) was used for testing undiluted supernatants in duplicate from the test strains. LT binding was detected after reaction with monoclonal human anti-mouse antibodies (R. Möllby, Karolinska Institute, Stockholm, Sweden) and sheep anti-mouse immunoglobulin G (Sigma) labeled with alkaline phosphatase. An optical density of ≥0.1 was considered positive, and an optical density of <0.1 was considered negative.

(ii) ST ELISA. Microtiterplates were coated with STIa (catalog no. E-8633 [Sigma]), and supernatants from the test strains were added undiluted and diluted 1/5 on the plates. Anti-STIa antibodies (R. Möllby, Karolinska Institute) were added, and after incubation, goat anti-rabbit conjugate (Sigma) was added. The percent inhibition was calculated as $1 - a$, where a is the value of the sample divided by the mean of negative samples. The results were considered positive when 2 dilutions produced >60% inhibition.

ETEC ELISA II (Göteborg). All the positive strains detected by ELISA or PCR were reanalyzed for the production of LT and ST by ganglioside GM1 ELISAs (35, 36). Three colonies from each sample were cultured individually in GM1-coated wells containing culture medium by a microplate method at the University of Göteborg as described elsewhere (36). A positive ST result was 50% or more inhibition compared to the absorbance of monoclonal ST mixed with ST negative control cultures. A positive LT result was an absorbance of 0.1 above the background.

In total, 307 samples from symptomatic children and 258 samples from asymptomatic children were analyzed. As there is a regular loss of sensitivity from retesting, the results from Stockholm and Göteborg are presented separately.

CFA. All the *E. coli* strains positive for either LT, ST, or both by either ELISA or PCR were analyzed for different CFA or PCFs by a dot blot test as previously described (21). Single colonies were first inoculated in microplates for enterotoxin assay and then on two CFA-agar plates (Casamino Acids-yeast extract agar [10]) with and without bile (26). To nitrocellulose membranes previously soaked in phosphate-buffered saline and partially dried, samples of 2 μl of suspended bacteria harvested from CFA-agar were added, after which membranes were dried. Some of the CFA better expressed on CFA-agar with bile are, in particular, CS5, PCFO159, PCFO166, CS7, CS17, and CFA III. Strains negative by testing on CFA-agar without bile were therefore also tested on CFA-agar with bile. The nitrocellulose membranes were blocked with 1% bovine serum albumin in 0.05% Tween–phosphate-buffered saline. CFA-expressing strains were detected by separate developing of the nitrocellulose membranes (21) with specific monoclonal antibodies against CFA I, CFA III, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS17, PCF O159, and PCF O166 (22, 23, 40).

Statistical methods. A chi-square test was used for the calculation of differences between diarrheal episodes and control periods.

RESULTS

Data for 235 children, 119 girls and 116 boys, were available for analysis after the 2-year follow-up program of the original 252 children. Only children with completed monitoring protocols over the 2 years were included. At the scheduled contacts, information from anyone related to the household was accepted. There was a dropout rate of 6.7% ($n = 17$). Fourteen were lost due to moving out of the area or to parental rejection. Three deaths occurred, with two due to congenital heart disease and one due to gastrointestinal illness with rotavirus.

Of the 235 children, 222 (94%) presented at least one diarrheal episode. A total of 885 diarrheal episodes were recorded over the 2-year period. ETEC was the etiology in at least one diarrheal episode among 75% of the sick infants (167 of 222). Most of these episodes (80%) occurred between 3 and 12 months of age (Fig. 1) (Table 1).

ETEC strains in symptomatic and asymptomatic children. ETEC analysis was performed with 808 samples associated with diarrheal episodes, as well as with 1,472 samples from asymptomatic controls collected in the cohort group over the same period. ETEC strains were found in a higher proportion among diarrheal samples, 38% (310 of 808), compared to that of samples from asymptomatic controls, 19% (277 of 1,472) ($P < 0.0001$) (Table 1). This difference was statistically significant when diarrheic and asymptomatic samples were analyzed for the age group of 0 to 18 months. In the age group of 0 to 6 months, the proportions were 42 and 20%, respectively ($P < 0.0001$); in the age group of 7 to 12 months, the proportions were 37 and 17%, respectively ($P < 0.0001$). Among children of 13 to 18 months of age, the corresponding figures were 36 and 17% ($P < 0.0001$). On the other hand, the P value was >0.05 for the age group of 19 to 24 months (Table 1). This means that 18% of the cohorts were healthy carriers when the follow-up was finished at 2 years of age.

Prevalence of LT and ST. The concomitant presence of LT and ST was the most common ETEC type isolated from the diarrheal stools of different age groups, representing 16% of all symptomatic samples (131 to 808), while ST alone was the

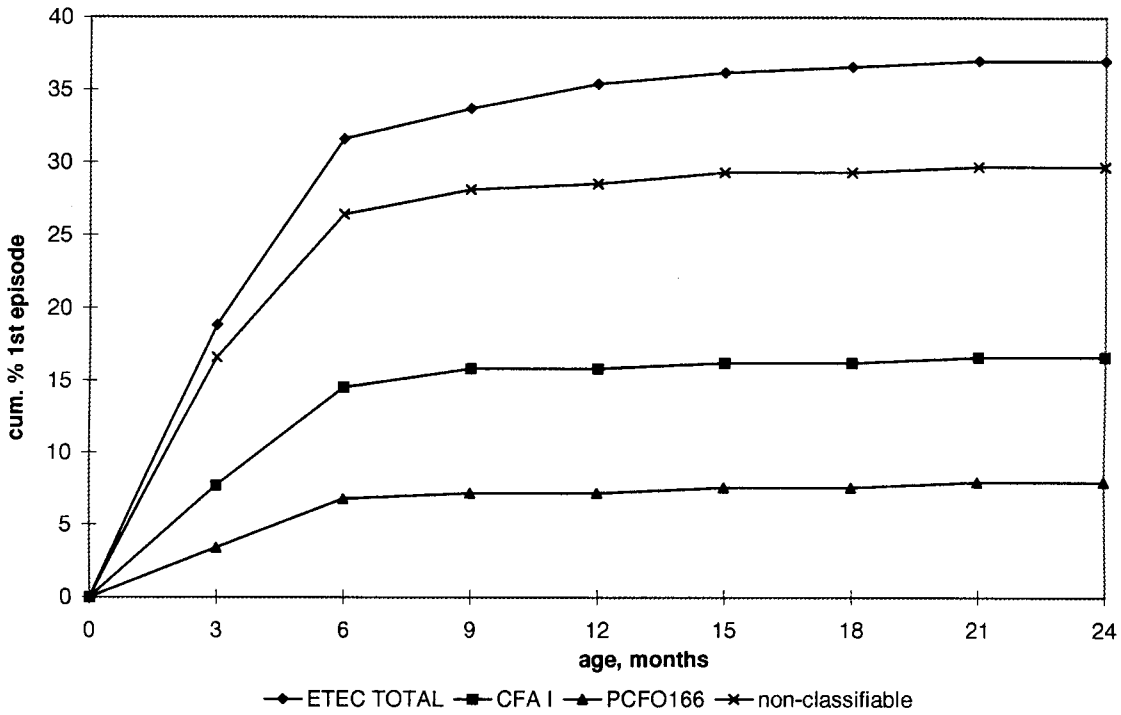

FIG. 1. Cumulative (cum.) percentages of ETEC first diarrhea episodes in a prospectively studied cohort (n = 235).

more frequent ETEC type among the samples from asymptomatic patients, representing 8% of the total samples from this group (122 of 1,472) (Table 1). Among ETEC strains from diarrheal episodes, the proportion producing both enterotoxins was about 50% (Table 2).

CFA in ETEC strains. *E. coli* isolates positive for LT and/or ST by ELISA or PCR at the Swedish Institute for Infectious Disease Control were further examined in Göteborg. By a slightly modified GM1 ELISA, 264 strains from diarrheal episodes and 203 strains from controls were classified as ETEC strains (Table 2). The proportion of toxin-positive strains was 17% lower than that found at the initial examination by ELISA or PCR. In this subsample, the proportion of ETEC strains from infants of less than 12 months of age was 27% (221 of 808) in the diarrheal group and 10% (150 of 1472) in the asymptomatic group, compared to 31% (254 of 808) and 14% (203 to 1,472) in the total material. Thus, the difference between symptomatic and asymptomatic cases shown in Table 1 was almost identical to that in this subset. Among the strains isolated from symptomatic cases, 49% produced LT and ST, 19% produced LT only, and 32% produced ST only. Among the strains from asymptomatic controls, 40% produced ST and LT, 25% produced LT only, and 35% produced ST only.

The expression of CFA on *E. coli* strains verified to produce enterotoxin by subculture in Göteborg is shown in Table 2. A colonization factor (CFA I, CFA IV, PCFO166, or PCFO159) was detected in 61% (160 of 264) of the ETEC strains from children with diarrhea and in 52% (105 of 203) of the ETEC strains from asymptomatic controls. Two main CFA, CFA I (36% of ETEC strains from diarrheal episodes and 23% of strains from asymptomatic control samples) and PCFO166 (19% of ETEC strains from diarrheal episodes and 22% of strains from asymptomatic controls), were found. There was a significantly higher proportion of *E. coli* strains with CFA I among strains from diarrheal episodes (P = 0.002). CFA IV and PCFO159 were less frequent and found in similar proportions in asymptomatic controls and from diarrheal episodes.

Among ETEC strains from both diarrheal cases and asymptomatic controls, CFA I was significantly more prevalent in *E.*

TABLE 1. ETEC distribution by age for children with and without symptoms of diarrhea

Age (months)	No. of ETEC findings among children (%)								P
	With symptoms[a]				Without symptoms[b]				
	LT	ST	LT and ST	Total	LT	ST	LT and ST	Total	
0–6	21 (6)	47 (14)	75 (22)	143 (42)	35 (5)	64 (9)	44 (6)	143 (20)	<0.0001
7–12	35 (12)	39 (13)	37 (12)	111 (37)	20 (6)	26 (7)	14 (4)	60 (17)	<0.0001
13–18	14 (12)	16 (13)	13 (11)	43 (36)	13 (6)	17 (8)	6 (3)	36 (17)	<0.0001
19–24	3 (7)	4 (9)	6 (14)	13 (30)	19 (9)	15 (7)	4 (2)	38 (18)	0.08
Total	73 (9)	106 (13)	131 (16)	310 (38)	87 (6)	122 (8)	68 (5)	277 (19)	<0.0001

[a] The numbers of samples per age group were 341, 303, 121, 43, and 808, respectively.
[b] The numbers of samples per age group were 695, 363, 210, 204, and 1,472, respectively.

TABLE 2. Presence of CFA in relation to toxin production in ETEC strains isolated from symptomatic and asymptomatic children

CFA	No. of strains producing toxin (%)							
	Episodes with diarrhea				Episodes without diarrhea			
	LT	ST	LT and ST	Total	LT	ST	LT and ST	Total
CFA I	1 (0.4)	4 (1.5)	91 (34.5)	96 (36.4)	0	0	46 (22.7)	46 (22.7)
CFA IV	1 (0.4)	2 (0.8)	7 (2.7)	10 (3.8)	1 (0.5)	4 (2.0)	6 (3.0)	11 (5.4)
PCFO166	2 (0.8)	46 (17.5)	3 (1.1)	51 (19.3)	0	44 (21.7)	0	44 (21.7)
PCFO159	3 (1.1)	0	0	3 (1.1)	4 (2.0)	0	0	4 (2.0)
None	43 (16.3)	32 (12.2)	29 (11.0)	104 (39.4)	45 (22.1)	23 (11.3)	30 (14.7)	98 (48.3)
Total	50 (19)	84 (32)	130 (49)	264 (100)	50 (25)	71 (35)	82 (40)	203 (100)

coli strains producing both LT and ST, while PCFO166 was more frequent in *E. coli* strains producing ST only (Table 2).

Repeated ETEC infections. The prospective cohort design of this study made it possible to examine clinical immunity to repeated infections. Of 167 cases with at least one diarrheal ETEC episode, 89 (53%) had a second episode and 38 (23%) had three or more. In the subset tested for CFA in Göteborg, the corresponding proportions were 49 and 16%. The appearance of a second episode also occurred at an early age, with almost all of them before 12 months (Fig. 2).

Two major CFA types, CFA I (LT and ST) and PCFO166 (ST), circulated in the study area together with a group of nontypeable strains. The next step of the analysis was to examine the risk of reinfection in these cohorts separately (Table 3). For this purpose, the ETEC CFA status of the first episode was compared with those of the following ones. Episodes preceded by a control sample with an identical CFA type were excluded. Of children with CFA I in the first episode ($n = 46$), 46% ($n = 21$) had a second symptomatic episode with any ETEC type and 24% ($n = 11$) had a second symptomatic episode with the same CFA type. The illness was characterized as moderate or severe in 96% of the first CFA I-associated episodes and in 64% of the second episodes with the same CFA type (Table 3). Similar or slightly lower incidences of reinfection were found in children whose first symptomatic infection was with PCFO166. It was also possible to examine whether there was evidence of cross-immunity between the CFA I ($n = 46$) and PCFO166 ($n = 22$) groups. A second diarrheal episode with the opposite strain was recorded in one case from each group.

In the more heterogeneous group of children affected by nontypeable strains, there was an opposite tendency, with 60% having a second symptomatic episode.

Among the nonclassifiable strains, the LT and/or ST pattern was rather evenly distributed (23 LT and ST, 17 LT, and 17 ST strains). The type of enterotoxin elaborated from strains isolated from a first ETEC episode did not seem to influence the incidence of second episodes. For strains with LT and ST patterns, there was a second episode in 17 of 23 (74%) children, compared with 7 of 17 (41%) children for strains with ST only.

Severity of the disease. The vast majority of the 808 diarrheal episodes were classified as mild (43%) or moderate (54%). In addition, there were 28 episodes (3.5%) classified

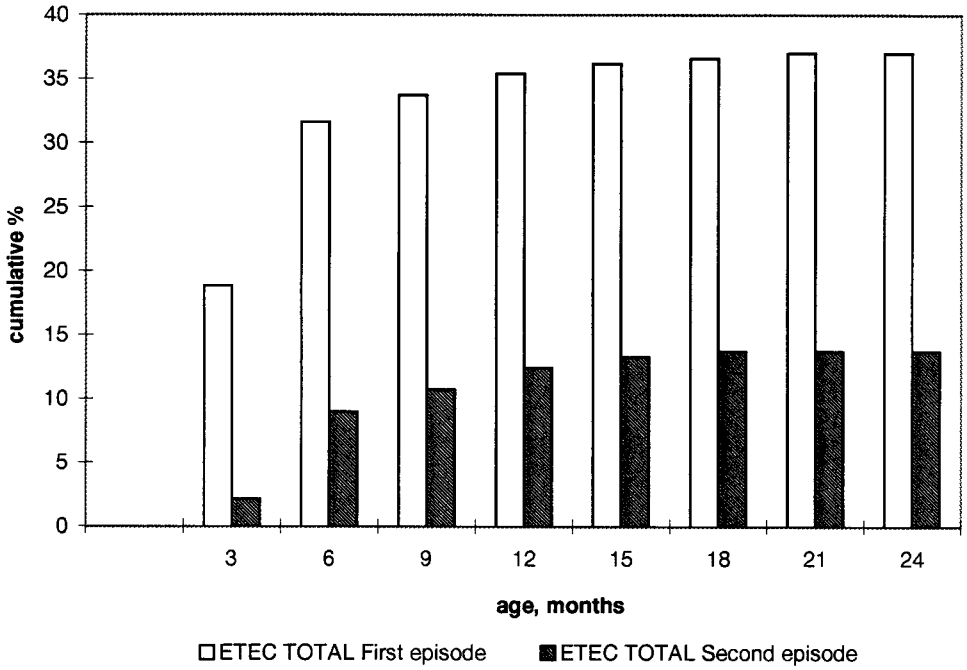

FIG. 2. Cumulative percentages of ETEC first and second diarrhea episodes in a prospectively studied cohort ($n = 235$).

TABLE 3. Repeated diarrheal episodes related to CFA type and severity

CFA	First episode		Second episode			
			Any ETEC type		Same CFA type	
	No. of children	Moderate and severe illness ratio (%)	No. of children/ total no. (%)	Moderate and severe illness ratio (%)	No. of children/ total no. (%)	Moderate and severe illness ratio (%)
CFA I	46	44/46 (96)	21/46 (46)	15/21 (71)	11/46 (24)	7/11 (64)
PCFO166	22	20/22 (91)	7/22 (32)	5/7 (71)	4/22 (18)	3/4 (75)
Nontypeable	57	49/57 (86)	34/57 (60)	24/34 (71)	NT[a]	
Others	9	7/9 (78)	5/9 (56)	4/5 (80)	NT	
Total	134	120/134 (90)	65/134 (49)	47/65 (72)	15/68 (22)	10/15 (67)

[a] NT, not tested.

with a severity of 3. All moderate and severe cases were examined by physicians, and instructions were given for treatment. Thirteen of the 28 children with more severe dehydration were hospitalized. The remaining ones had medical attention at home because their parents rejected hospitalization.

Of the 310 episodes associated with ETEC infection, 15 (4.8%) were severe and 8 were treated at a hospital. The proportion of moderate episodes was 78% in this part of the material, compared to 54% in the total material. Seven of the 15 cases with severe illness had a combination with other microorganisms as follows: 4 with *G. lamblia*, 1 with *E. histolytica*, 1 with *Salmonella*, and one with campylobacters. In those children contracting a second episode, the majority showed a similar clinical picture when they were infected with the same CFA type (Table 3).

DISCUSSION

Diarrheal disease is said to kill 4 to 6 million children globally each year, with ETEC as a causative agent in approximately 800,000 cases in children under 5 years of age (11). In León, Nicaragua, the mortality rate from acute diarrheal disease in children under 1 year of age was 2.55 per 1,000 in 1994 and 1.73 per 1,000 in 1995 according to official sources (24a).

In this 2-year prospective cohort study (including 235 newborns), many of the most common pathogens were recognized. The proportion of diarrheal episodes related to ETEC strains was 39% during the first year of life (254 to 644), sometimes together with other pathogens. ETEC strains were also isolated from 19% (203 of 1,058) of the control samples collected during asymptomatic periods, but the difference was statistically significant ($P < 0.0001$). At the end of the study, 18% of the children studied (38 of 204) were healthy carriers. During the last 6 months, there was no difference between the groups ($P = 0.08$). This is in agreement with a prevalence study carried out in León from 1984 to 1986 (28). In material mostly from children of above 2 years of age, there was no statistically significant difference for LT positivity between the diarrheal and control groups, in contrast to ST findings. Similar results were obtained for ETEC strains in a study from Esteli, Nicaragua (24), with 100 children of between 5 months and 6 years of age. Therefore, one conclusion is that the first ETEC diarrheal episode is contracted early in infancy, in most cases before 1 year of age.

In a study from Chile (19), ETEC strains were associated with 12.3% of cases overall. The rate of ETEC isolation was significantly greater than that from controls (12.3 versus 7%; $P = 0.000039$). The highest incidence of ETEC diarrheal illness occurred in the first year of life.

Similar to the results of other studies (11), approximately 50% of the ETEC strains produced both enterotoxins. Strains positive for LT or ST were further examined with regard to CFA. This analysis showed that strains producing LT and ST as well as expressing CFA I constituted a dominant subgroup. The proportion in samples from infants with diarrhea was also significantly higher than that of controls. In contrast, the second most common subgroup, strains expressing PCFO166 and producing ST only, occurred as often in controls as in diarrheal samples. Nevertheless, when they occurred as a primary infection, these organisms were associated with illness as often as were ETEC strains producing LT and ST. For nontypeable strains, the proportion was even higher among control samples.

All *E. coli* strains positive for LT and/or ST in Stockholm were retested in Göteborg as part of the local laboratory routines for CFA testing. In Göteborg, the strains were subcultured twice; after that, three colonies were picked and tested for phenotype. In most cases, only one or two colonies were positive by ELISA, indicating that the plasmid coding for enterotoxin may have been lost during the process. It is a well-known fact that plasmids coding for enterotoxin are easily lost by repeated subculture so it was not surprising that 17% of the strains positive in Stockholm were negative in Göteborg. A contributing factor may be that a streak of bacteria was used in Stockholm for the analysis of LT and ST, increasing the sensitivity. Although the total proportion of ETEC positives was reduced at retesting, the relation of positivity between samples collected from symptomatic and asymptomatic children did not change.

One specific aim of this study was to examine protection against reinfection. Of the children shown to have a CFA I (LT and ST) strain in their first episode, 24% presented the same subtype in a second diarrheal episode. The number of episodes classified as severe or moderate was smaller among second episodes with the same subtype, 64 and 96%, respectively. In comparison with total material, a second ETEC episode was recorded for 53% of the children. More than two ETEC-related episodes were seen in 23% of cases for the whole material and in 16% of cases for the type-specific CFA groups.

It was not possible to draw any certain conclusions about cross-immunity between different subtypes. However, a first infection with a CFA I-type strain rarely resulted in a second infection with a PCFO166-type strain and vice versa. It is of interest that the LT and ST pattern of the strain from the initial episode did not seem to influence the disposition for reinfection. Considering the severity of ETEC disease, most cases were characterized as moderately ill. Altogether, only eight cases needed hospitalization. Seven of the 15 cases with severe symptoms had a combination with other microorgan-

isms. In other studies, the most severe forms have been seen in about 5% of the cases (11).

The materials were collected during 3 years, and throughout that period, CFA I and PCFO166 were the dominating subtypes. CFA I and CS1 through CS6 have been found most frequently in studies of ETEC diarrhea (11). PCFO166, however, has not been reported at such a high prevalence before. From a study in North India, a prevalence of 7% was reported (31). Possibly certain clones have been circulating in León, Nicaragua, offering a stable epidemiological situation for vaccine trials. As specific vaccines are now available, there are plans to compare the efficacies of a Swedish oral ETEC vaccine and a Swedish oral cholera vaccine (15) in a double-blind, placebo-controlled study in León. The oral ETEC vaccine has a potential to cover most of the diarrhetic strains circulating in the area.

The strategy is to modify the severity of the first ETEC episode to a mild or subclinical infection. Subsequent booster infections with circulating wild strains would strengthen immunity.

The conclusion from this study is that the first ETEC infection appears early in life and that natural immunity develops within the first year in most cases, meaning that an intervention program should be implemented early in infancy.

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