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Etiology of Children's Diarrhea in Montevideo, Uruguay: Associated Pathogens and Unusual Isolates

M. E. TORRES,[1] M. C. PÍREZ,[1,2] F. SCHELOTTO,[1]* G. VARELA,[1] V. PARODI,[1] F. ALLENDE,[1] E. FALCONI,[1] L. DELL'ACQUA,[1] P. GAIONE,[1] M. V. MÉNDEZ,[2] A. M. FERRARI,[2] A. MONTANO,[1,2] E. ZANETTA,[3] A. M. ACUÑA,[3] H. CHIPARELLI,[1] AND E. INGOLD[1]

Bacteriology and Virology Department, Institute of Hygiene,[1] Pediatric Clinic "A," Children's Hospital "Pereira Rossell,"[2] and Parasitology Department, Institute of Hygiene,[3] School of Medicine, Universidad de la República, Montevideo, Uruguay

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We studied microorganisms associated with infant diarrhea in a group of 256 children admitted to a public pediatric hospital in Montevideo, Uruguay. Diagnostic procedures were updated to optimize detection of potential pathogens, which were found in 63.8% of cases, and to be able to define their characteristics down to molecular or antigenic type. Coinfection with two or more agents was detected in more than one-third of positive studies. *Escherichia coli* enteric virotypes, especially enteropathogenic *E. coli* (EPEC), were shown to be prevalent. Rotavirus, *Cryptosporidium*, *Campylobacter* (mainly *Campylobacter jejuni*), and *Shigella flexneri* were also often identified. Enterotoxigenic *E. coli, Salmonella,* and *Giardia lamblia* were sporadically recognized. Unusual findings included two enteroinvasive *E. coli* strains, one *Shigella dysenteriae* 2 isolate, and a non-O:1 *Vibrio cholerae* culture. EPEC bacteria and *S. flexneri* (but not *Salmonella*) showed unusually frequent antimicrobial resistance, especially towards beta-lactam antibiotics, which is the subject of ongoing work.

Diarrheal disease is a frequent illness in developing countries. It contributes to the deaths of 4.6 million to 6 million children annually in Asia, Africa, and America. Morbidity is also especially important in poor countries: in tropical climates it has been estimated that each child suffers up to 15 to 19 episodes of diarrhea per year. In the United States, reports calculate 1.5 to 1.9 episodes per child per year. In a peripheral zone of Montevideo, we have shown a figure of 4.2 in 1989 (15, 18).

In past decades, diarrhea and malnutrition (closely related pathologies) contributed significantly in Uruguay to infant mortality, which reached a figure of 120 per 1,000 newborn children (1). Accompanying general improvement of the quality of life, the World Health Organization-guided local programs of diarrheal disease control were instituted, including promotion of breast-feeding, oral rehydration therapy, and specific health education. A gradual decrease in the prevalences of these diseases was registered, especially after 1980, thus helping to diminish the global infant death rate, which at present approaches a figure of 15 per 1,000 (16, 17).

Diarrheal illness still stands, however, as an important cause of infectious morbidity in children, exceeded only by respiratory tract infections. Mortality, in this context, is currently associated with cases that evolve without proper feeding or rehydration care, invasive diarrheas with extraintestinal or systemic involvement, or persistent diarrheas that occur especially in infants from low-level socioeconomic groups, who suffer previous deficiencies and develop severe nutritional consequences of enteric infection.

Enteric pathogens have been the subject of extensive work in our laboratories. Lately, however, techniques have been carefully updated, so a wide variety of potentially pathogenic microorganisms can now be investigated in cases of infant diarrhea in hospital or community settings (F. Schelotto, M. C. Pírez, R. Maglione, G. Algorta, A. Montano, G. Garela, E. Zanetta, A. Acuña, H. Chiparelli, and M. Hortal de Peluffo, 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. C73, 1991; G. Varela, F. Schelotto, T. Pais, M. C. Pírez, L. Dell'Acqua, E. Zanetta, R. Maglione, A. Cardozo, E. Alonso, W. Guillén, S. Muñoz, C. Barrenechea, and P. Parada, 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. A43, 1991).

From 1990 to 1994 we studied enteric pathogens in hospitalized children with persistent or acute diarrhea as part of a project in collaboration with Pediatric Clinic "A" of Children's Hospital "Pereira Rossell."

Our objectives were to perform a detailed investigation of all potential pathogens associated with children's diarrhea, identify microorganisms not previously detected, and characterize local strains of these pathogens.

MATERIALS AND METHODS

We studied 224 children 1 to 20 months old who were hospitalized in the "A" ward of Pereira Rossell hospital with a diagnosis of persistent diarrhea (135 children) or acute diarrhea (89 children). Median ages for the two groups were 5 and 4 months, respectively. Thirty-two control children without diarrhea were also studied. The total number of children of that age hospitalized with diarrhea during the same period in the same hospital was 833, so our sample included more than one-fourth of the entire figure.

Five to ten grams (or milliters) of feces for etiologic studies was obtained from nylon diapers with plastic or wood spoons and collected in sterile plastic vials. A cotton swab was rolled and moistened in the fresh sample and included in a tube with Cary-Blair transport medium. Both parts of the sample were submitted to the laboratory in insulated, refrigerated boxes for copromicrobiologic studies, which included investigation of bacteria, viruses, and parasites. The samples were examined less than 12 h after extraction. Seventy-six additional samples were

* Corresponding author. Mailing address: Bacteriology and Virology Department, Institute of Hygiene, Av. Dr. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay. Phone: 598 2 4875795. Fax: 598 2 4873073. E-mail: bacvir@hc.edu.uy.

obtained from 59 children with persistent diarrhea, 1 week after the first sampling or later.

Microscopic examination was done with methylene blue stain and a modified form of Gram's technique (which substitutes Ziehl's fuchsin for safranin), looking for fecal leukocytes and spiral bacteria.

For parasite detection, fresh direct microscopic examination with saline solution and iodine was carried out with recently emitted feces (less than 6 h after collection), allowing the observation of living and moving trophozoite stages of protozoa. All samples were also processed by the Ritchie concentration method of centrifugation and sedimentation with formalin-ether and stained with a modified Ziehl-Neelsen (Kinyoun) procedure for enteric coccidia. Slide staining with Thionine blue (Merck) and Chlorazole black E (Sigma) for protozoon identification was done when needed.

Classic pathogenic *Enterobacteriaceae* were investigated as specified by Ewing (11). Selective and differential media were used for isolating *Salmonella, Shigella, Yersinia* species, *Escherichia coli,* and *Vibrio*: SS (*Salmonella-Shigella*), MacConkey, Sorbitol MacConkey and thiosulfate-citrate-bile salts agars. The last medium was included since June 1991, after the regional cholera outbreak was recognized.

Campylobacter bacteria were cultured at 42°C in a microaerophilic environment on selective medium prepared with a brucella agar base, hemin, sheep blood, sodium metabisulfite-ferrous sulfate-sodium pyruvate, and Campylosel antibiotic mixture (bioMérieux).

Tetrathionate broth, peptone-sorbitol bile broth, and alkaline peptone water were used as enrichment media for *Salmonella, Yersinia,* and *Vibrio* bacteria, in that order.

Salmonella and *Shigella* strains were identified through standard techniques and antigenically characterized with antisera of our institute's collection. Confirmation of *Shigella dysenteriae* 2 identity was obtained from Central Public Health Laboratories, Colindale, United Kingdom.

Suspected *Yersinia* colonies were selected as lactose-negative or late-positive bacteria from MacConkey primary plates incubated at 28°C or from subculture of 21-day-old enrichment peptone-sorbitol bile broth. Tube biochemical tests and agglutination with locally produced sera ensued.

Identification of enteropathogenic *E. coli (*EPEC) was done by slide agglutination with commercial polyvalent sera (bioMérieux) and tube agglutination with rabbit specific sera prepared in our laboratory. All the EPEC strains were initially identified by seroagglutination tests. Five colonies suspected to be *E. coli* were transferred from MacConkey agar to heart infusion agar, Simmons' citrate, and lysine iron agar. Citrate-negative cultures were tested with polyvalent commercial sera by slide agglutination and further identified with our own monovalent sera by slide and tube agglutination.

Monovalent anti-EPEC OB and O sera were prepared in our laboratory using New Zealand White rabbits inoculated in the ear marginal vein with a bacterial suspension that was tested and prepared as described in Centers for Disease Control procedures (10). The Roschka method was followed; the lipopolysaccharide bacterial antigen was treated with alcohol and acetone previous to drying, and the final bacterial immunizing suspension was prepared with sodium phosphate, potassium alum, and formaldehyde. A brief immunization schedule was developed, as recommended in the above-cited standard technique.

Reference strains for preparing and testing sera were obtained from the Centers for Disease Control and from LREC, Lugo, Spain (J. Blanco).

A few strains were further examined with DNA probes for detection of enteroadherence factor, cytotoxin production was investigated with Vero cells, adherence patterns were studied with HEp-2 cells (7), and ribotyping (restriction fragment length polymorphism of ribosomal DNA, revealed with cold probes) was performed by one of us at Institut Pasteur (14).

Enteroinvasive *E. coli* (EIEC) candidate strains were selected by biochemical tests. Lactose-positive or -negative, lysine-negative colonies were evaluated as potential EIEC isolates, and EIEC antigens were identified with polyvalent and monovalent rabbit antisera produced in our institute. Specific O polyvalent and monovalent antisera were prepared with the same procedures used to produce anti-EPEC O agglutinating sera. Invasiveness was confirmed in guinea pigs' eyes through the Sérény test, and PCR for the *ial* (invasion-associated locus) sequence was performed (25).

Nonfermenting *E. coli* colonies on sorbitol MacConkey plates were tested with anti-O:157 serum to investigate STEC (Shiga toxin-producing *E. coli).*

Six to eight additional suspected *E. coli* colonies were recovered as stab cultures and later checked for labile toxin (LT) and stable toxin (ST) production in enzyme immunoassays. No previous serotype selection was made, but positive cultures were finally serogrouped. *E. coli* enterotoxins were investigated in a spun pool culture of six to eight colonies per sample. A GM1 receptor enzyme-linked immunosorbent assay was performed to test for LT production (5), and a commercial competitive STEIA (Oxoid) was used for detecting ST production. Individual colonies were later analyzed, and confirmed positive cultures were serogrouped in a reference *E. coli* laboratory of Lugo, Spain (Jesús and Jorge Blanco).

Campylobacter isolates were further characterized by means of mobility, catalase, oxidase, nalidixic acid susceptibility, H_2S production, and hippurate hydrolysis tests. Samples were confirmed as positive for *Campylobacter* only when macroscopic growth was obtained and colonies could be characterized.

A single non-O:1 *Vibrio cholerae* isolate was confirmed as such with galerie PAPI 100 of Institut Pasteur, and cholera toxin production capacity was studied through GM1 enzyme-linked immunosorbent assay and PCR for toxin coding genes *ctxA* and *ctxB* (13, 21). The *zot* (zona occludens toxin) gene was also explored.

Rotavirus investigation was performed through commercial enzyme immunoassay kits that detect group A antigens of internal capsid. Viral RNA fragments were characterized in 10 positive samples by means of polyacrylamide gel electrophoresis (PAGE), revealing bands with silver staining.

In Fig. 1, a flow chart of procedures summarizes the methods that were used.

Disk susceptibility tests of *Shigella*, *Salmonella,* and EPEC strains were done by a standard agar diffusion technique, following National Committee for Clinical Laboratory Standards guidelines (20). We included antimicrobials intended for use, but most of them were tested in order to obtain epidemiological information about susceptibility and resistance.

RESULTS

We found potential pathogens in 143 out of 224 children with diarrhea (63.8%); 138 of these positive results were obtained with the first sample (61.6% of 224). Only five additional positive cultures were made in second studies.

On the other hand, only 3 positive cultures came out from 32 control children without diarrhea (9.4%).

Fifty-eight children (close to 4 of 10 positive cases) showed more than one and up to five associated pathogens. Pathogenic *E. coli* was the most frequent etiologic agent identified in the group of 224 diarrheic children: it was present in 88 of 143 positive cases; in 80 of them, EPEC strains were isolated, alone or associated with enterotoxigenic *E. coli* (ETEC) cultures (9) or EIEC bacteria (2). Rotavirus followed, being detected in 42 cases. *Campylobacter* (mainly *C. jejuni*)*, Cryptosporidium,* and *Shigella* (mostly *S. flexneri*) were also often seen.

Table 1 presents these data in detail, including identification down to the species, virotype, and serogroup or serotype level of each causative agent of diarrhea that was recovered.

Ten EPEC strains (three of the O:111 serogroup, three of O:119, three of O:55, and one of O:142) were further studied as previously described and were shown to be nonverotoxic, to possess enteroadherence factor plasmid, and to produce localized adherence. The O:119 and O:55 cultures had identical ribotype patterns; O:111 strains showed the same number of DNA bands, but their distribution pattern was different and was not identical in all three. Extensive investigation of virulence traits is being done with 50 EPEC strains of this series, and a preliminary report of results has already been presented (J. Blanco, M. Blanco G. Varela, and F. Schelotto, 16th Congr. Spanish Soc. Microbiol., Book of Congress, abstr. 152, p. 123, 1997).

ETEC cultures recovered pertained to serogroups O:159, O:8, O:39, O:6, and O:114 (one of each) or were nontypeable.

Two EIEC strains but no *E. coli* O:157 cultures were recovered.

High counts of fecal leukocytes were seen in samples of children with *Shigella* or EIEC, and lower numbers were found in cases associated with *Salmonella* or *Campylobacter.*

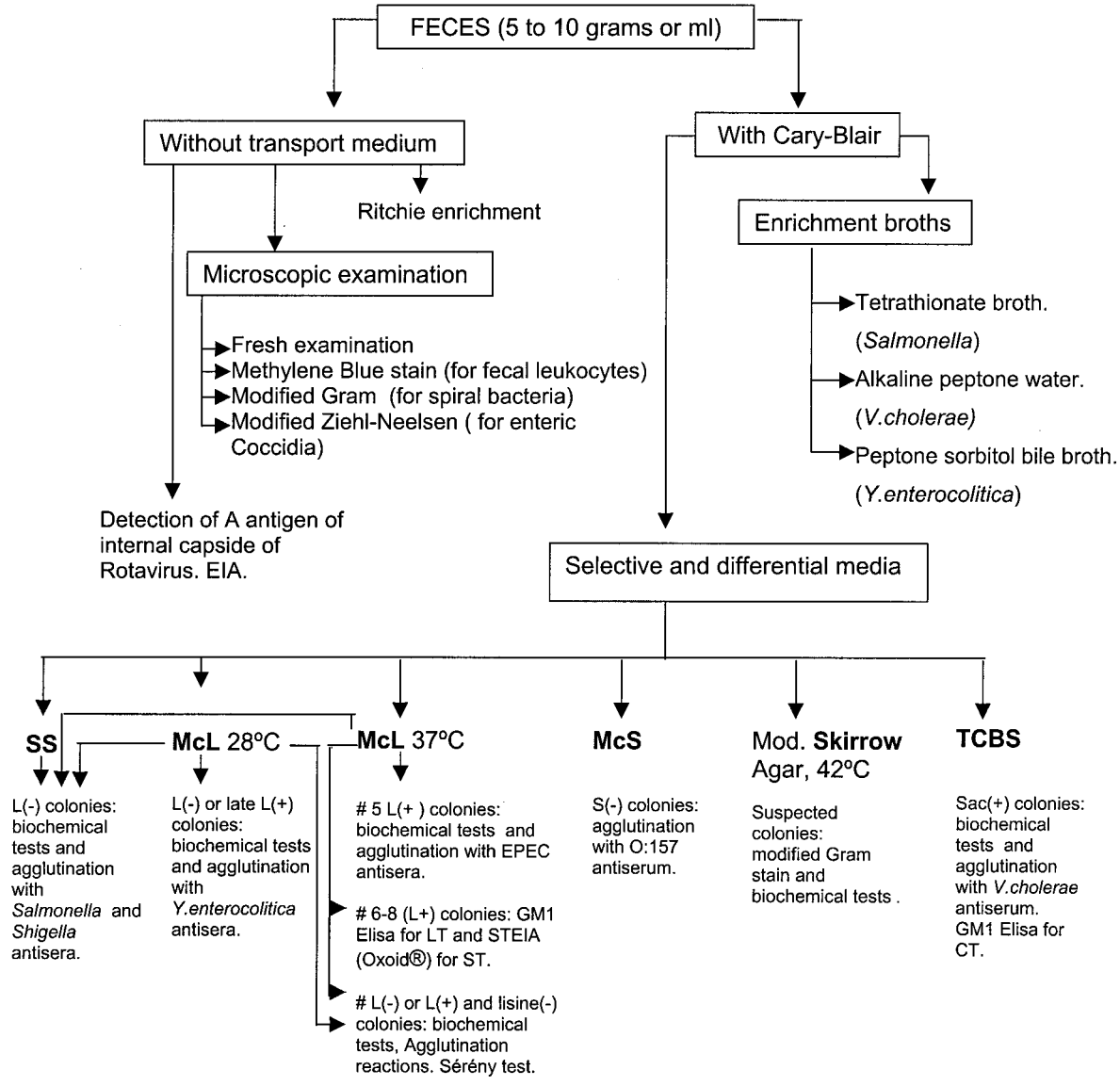

FIG. 1. Flow diagram of the methodology used for the investigation of potential pathogens in feces.

Salmonella strains obtained from feces were *Salmonella enterica* subsp. *enterica* serotypes Agona (two cultures), Derby, Montevideo, Muenchen, Panama, and Corvallis (one strain each).

A single *V. cholerae* isolate was shown to lack O:1 antigen. It was nontoxigenic. PCR results were negative for the *ctxA, ctxB,* and *zot* genes.

PAGE performed on 15 Rotavirus-positive samples yielded identical long migration electropherotypes in all of them.

The parasites observed in association with diarrheic feces were *Cryptosporidium* sp. in 19 cases, *Giardia lamblia* for eight children, *Pentatrichomonas hominis* for three, and *Chilomastix mesnili* once.

Almost all EPEC cultures tested (80 strains) were resistant to ampicillin (Table 2), and many of them were resistant to cephalothin and other beta-lactams, including expanded-spectrum cephalosporins. They were variably resistant to aminoglycosides, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole and were always susceptible to polymyxin B and quinolones. Similar results were obtained with ETEC isolates. Roughly half of the *Shigella* strains studied were resistant to ampicillin or to trimethoprim-sulfamethoxazole. All *Salmonella* isolates were amply susceptible to the antimicrobial agents tested.

DISCUSSION

Our work shows clearly that *E. coli* pathogenic virotypes, and especially EPEC, were the microorganisms most frequently associated with diarrhea of infants from low-income families admitted to the public pediatric hospital "Pereira Rossell" in Montevideo, Uruguay. This was true for both acute and persistent cases of diarrhea. The same observation was previously made for diarrheic children in a poor community setting (Sche-

TABLE 1. Pathogenic microorganisms associated with infant diarrhea in Montevideo, Uruguay, 1990 to 1994[a]

Identified pathogen	Virotype	Serogroup	No. of strains recovered	No. of children carrying:	
				Microbial type	Enteropathogenic microorganism[b]
E. coli					88
	EPEC	All	92	80	
		O:111	39		
		O:119	30		
		O:55	17		
		Others[c]	6		
	ETEC		9	9	
	LT^+		4		
	ST^+		4		
	LT^+ ST^+		1		
	EIEC		2	2	
		O:29	1		
		O:124	1		
Rotavirus					42
Campylobacter					19
C. jejuni				14	
C. coli				2	
NS[d]				3	
Cryptosporidium					19
Shigella					16
S. flexneri				13	
S. sonnei				2	
S. dysenteriae				1	
G. lamblia					8
Salmonella					7
V. cholerae		Non-O:1			1
Other parasites[e]					4

[a] Summary of etiologic results. The total number of affected children studied was 224. Potential pathogens (204) were identified in the feces of 143 of those children with diarrhea.
[b] Some children carried more than one type or group of the same pathogen.
[c] O:142 (two), O:126, O:127, O:125, and O:26 (one each).
[d] NS, nonspecified.
[e] *P. hominis, C. mesnili.*

lotto et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. C73, 1991). EPEC strains have also been frequently isolated from children of similar origin with bloody diarrhea, but their etiologic role was not clear (Varela et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. A43, 1991). Prevalent serogroups of EPEC in cases of children's diarrhea were O:111, O:119, and O:55, as has been locally the case for at least 25 years (3). EPEC is not a frequent cause of diarrhea in developed countries, but it is very commonly associated with enteric disease in developing areas, including close Brazilian regions (4, 9,26, 28). These facts have driven our special attention and motivated our detailed work on local EPEC strains to determine their phenotypic and genetic characteristics.

ETEC bacteria have not been investigated in Uruguay as a cause of diarrhea until 1989. This report shows that with a careful diagnostic search, they can be recognized in association with cases of enteritis, but less frequently than the EPEC virotype. This seems to be the rule in our region of South America. PCR tests or DNA probes may further improve diagnostic yields. Reports from São Paulo, Brazil, have revealed that ST^+ or LT^+ ST^+ strains are frequently recovered from diarrhea cases, whereas LT^+ cultures are found equally often in normal children and in sick children (23). In this series, we recovered both types of ETEC from children with diarrhea (9 of 224; see Table 1 and Fig. 1) and one LT^+ strain from control samples (1 of 32).

We have isolated EIEC strains from feces only twice in this study. Anyway, these unusual findings validate efforts made in our laboratory to prepare, titrate, and use polyvalent and monovalent EIEC O rabbit antisera in slide and tube agglutination reactions. PCR assays confirmed phenotypic identification.

E. coli O:157 was not isolated from these children. This observation, which was also made in previous surveys (Schelotto et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. C73, 1991; Varela et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. A43, 1991), does not exclude the presence of other STEC serogroups, which are still being further investigated, nor does it discard the possibility of a greater prevalence of STEC in higher socioeconomic groups. Indirect evidence (through investigation of fecal cytotoxin) of the association of Shiga toxin-producing microorganisms with hemolytic uremic syndrome cases has been shown (Varela et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. A43, 1991). It has been postulated that wide diffusion of EPEC strains and human immune response to their antigens may contribute to limit the circulation of STEC bacteria in the same population (19, 22). STEC and EPEC strains carry similar virulence components responsible for attaching and effacing effects, but hemolytic uremic syndrome cases occur principally in well-nourished children, and EPEC strains prevail in poor social groups.

Enteroaggregative *E. coli*, exhibiting aggregative adherence on cell cultures) was not investigated with these samples due to a lack of adequate diagnostic laboratory tools. Detection of strains belonging to that *E. coli* virotype would have been of interest for this study, since enteroaggregative *E. coli* has been reported to be associated with persistent diarrhea in children (8, 12). We have now prepared a specific, digoxigenin-labeled DNA probe, which will enable us to further identify these bacteria and to review past isolates that we keep as heart infusion agar stab culture tubes.

S. flexneri is the prevalent species of this pathogen in Uruguay and the leading cause of bloody diarrhea in children. However, other species are recovered, such as *Shigella sonnei*, which is second in frequency, and also, rarely, *Shigella boydii* and *S. dysenteriae.* It is important for microbiologists to re-

TABLE 2. Antimicrobial susceptibilities of EPEC and *Shigella* isolates[a]

Antimicrobial agent	Results							
	Shigella (n = 16)				EPEC (n = 80)			
	No. S	No. R	No. I	No. NT	No. S	No. R	No. I	No. NT
Ampicillin	7	9				77	1	2
Cephalothin	11		4	1	15	50	8	7
Trimethoprim-sulfamethoxazole	9	7			43	32	3	2
Chloramphenicol	16				51	19		10
Tetracycline	15		1		44	13	13	10
Gentamicin	14			2	61	15	1	3
Polymyxin B	16				75			5
Ciprofloxacin	16				75			5

[a] Disk susceptibility to antimicrobials was evaluated through standard agar diffusion tests. S, susceptible to indicated antibiotic; R, resistant; I, intermediate; NT, not tested.

member the extended and late-lactose-positive appearance of colonies of *S. sonnei* to avoid missing its presence. *S. boydii* was not isolated from this group of children but was previously recovered from a child with bloody diarrhea (Varel et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. A43, 1991). *S. dysenteriae* strains are sporadically found, and they belong to subtype 2: they are not toxigenic Shiga bacilli type 1. The identification of these isolates has been confirmed in Central Public Health Laboratories.

It should be noted that *Cryptosporidium* and *Campylobacter* were more frequently recognized than *Shigella* in association with diarrheal diseases of this group of children. Most *Campylobacter* strains were diagnosed as *C. jejuni*, but two *Campylobacter coli* cultures could be identified.

Cryptosporidium sp. was first described in Uruguay in 1986 (29) in association with cases of acute diarrhea in children. This agent is responsible for 11% of acute diarrhea cases in the community (Schelotto et al., 11th Lat.-Am. Congr. Microbiol., Book of Congress, abstr. C73, 1991) and has a seasonal presentation in our country (end of summer and autumn). *G. lamblia* is the enteric protozoan parasite most frequently isolated in Uruguay (2).

Salmonella was found in these children with a low frequency, as is common in Uruguay for this age group in the last two decades. A variety of serotypes were identified. Neither *Salmonella enterica* serovar Typhimurium, which used to be prevalent, nor epidemic *Salmonella enterica* serovar Enteritidis, which diffused after 1994 to 1995, was present in these samples. All strains were susceptible to antibiotics, contrasting with strains that circulated at the time in Argentina (6).

The only confirmed *Yersinia* isolate was shown to be *Yersinia frederiksenii*, which was recognized after cold enrichment of the fecal sample of a girl with acute diarrhea from whom no other pathogen was recovered. Virulence plasmid was not found to be present in this strain when it was comparatively analyzed with other local and reference cultures. This finding had no pathological significance. *Yersinia enterocolitica* is not a frequent cause of diarrhea in children in Uruguay.

Detection of *V. cholerae* in February 1992 (in feces taken from a child with acute diarrhea who later developed persistent illness) caused immediate alarm, in view of the epidemic outbreak that was then occurring in most North, South, and Central American countries. However, the strain proved to be nonepidemic. Repeated isolation of this type of strain was later reported in Argentina (24). We have included thiosulfate-citrate-bile salts and alkaline peptone water media in all stool cultures performed in our laboratory since June 1991, and we obtained negative results. This is a systematic sentinel survey that contributes to confirming the absence of diffusion of this pathogen in our country. A complementary serum survey yielding similar conclusions was performed with an adult local population (27).

Rotavirus is being identified in the feces of diarrheic children with increasing frequency in Montevideo. The relative incidence seems to be higher in children from middle-income social groups (Y. Ramírez, J. Pastorini, J. C. Russi, and A. M. Ferrari, Acute diarrheal illness: characteristics of the population attended at CASMU, Montevideo, from April 1997 to April 1998, 22nd Uruguayan Congr. Pediatr., Book of Congress, p. 101). Further studies are needed, including systematic molecular epidemiology studies through PAGE characterization.

Antimicrobial resistance of enteropathogenic *E. coli* and *S. flexneri* was unusually frequent, especially towards beta-lactam antibiotics. It was thought to reflect the prevalence of resistance traits in gram-negative bowel bacteria due to misuse of antibiotics in the health care system, which explains the occurrence of invasive infections with multiresistant bacilli. These facts are being carefully examined, leading to epidemiologic and molecular studies that are ongoing in our laboratory, regarding resistance mechanisms of gram-negative bacilli (E. Ingold, F. Schelotto, P. Gadea, G. Varela, A. Sirok, C. Arenas, R. Vignoli, E. Calvelo, M. N. Tanzi, and A. Del Monte, 9th Int. Congr. Infect. Dis., Book of Congress, p. 89, abstr. 43.022, 2000; R. Vignoli, E. Calvelo, A. Del Monte, E. Ingold, P. Power, M. Radice, A. Quintana, F. Schelotto, G. Gutkind, and J. Ayala, 9th Int. Congr. Infect. Dis., Book of Congress, abstr. 43.021, p. 89, 2000).

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