Prevalence of diarrhoeagenic *Escherichia coli* in children from León, Nicaragua

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Diarrhoeal disease is a public health problem worldwide, mostly affecting children in developing countries. In Nicaragua, diarrhoea is the second greatest cause of infant mortality. During the period March 2005 to September 2006, a total of 526 faecal samples from children aged 0–60 months (381 with and 145 without diarrhoea) from León, Nicaragua, were studied. In order to detect five different diarrhoeagenic *Escherichia coli* pathotypes simultaneously [enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC)], a mixture of eight primer pairs was used in a single PCR. At least one diarrhoeagenic *E. coli* pathotype was detected in 205 samples (53.8 %) of the diarrhoea group and in 77 samples (53.1 %) in the non-diarrhoea group. ETEC was detected significantly more often in children with diarrhoea (20.5 %) than in children without diarrhoea (8.3 %) ($P=0.001$). Atypical EPEC, EIEC and EAEC were detected with slightly lower frequencies in children with (16.0, 0.8 and 27.8 %, respectively) than in children without (20.7, 1.4 and 33.1 %, respectively) diarrhoea. EHEC was only detected in children with diarrhoea (2.1 %). In conclusion, ETEC continues to be an important agent associated with diarrhoea in children from León, Nicaragua. Although not very frequent, the only findings that were 100 % associated with diarrhoea were ETEC *estA* (4.7 %) and EHEC (2.1 %). Nevertheless, EAEC and EPEC were also frequent pathotypes in the population under study. In children with severe diarrhoea, more than half had EAEC, ETEC or EPEC, and EAEC was the most prevalent pathotype.

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INTRODUCTION

Diarrhoeal disease continues to be one of the foremost public health problems worldwide, with over 1.5 million deaths occurring each year, mostly in children under 5 years in developing countries (Bryce *et al.*, 2005). From 1985 to the present, diarrhoea has continued to be the second most common cause of infant mortality in Nicaragua. In 2004, the Ministry of Health in Nicaragua reported that, among the total number of diarrhoea cases, 71 % were in children younger than 5 years of age, with a mortality rate of 11 per 10 000 inhabitants. However, the morbidity rate for children of less than 1 year and of

1–4 years of age was 3058 and 1157 per 10 000 inhabitants, respectively (MINSANIC, 2004).

A broad range of recognized micro-organisms such as viruses, parasites and bacteria are associated with diarrhoea. Among the bacteria, diarrhoeagenic *Escherichia coli* (DEC) are some of the most frequently detected pathogens worldwide. There are six pathotypes of DEC: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or verocytotoxin-producing *E. coli*, enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (Bischoff *et al.*, 2005; Kaper *et al.*, 2004; Matar *et al.*, 2002; Nataro & Kaper, 1998; Paniagua *et al.*, 1997).

The identification of DEC types requires differentiation from non-pathogenic members of the human normal

Abbreviations: DEC, diarrhoeagenic *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.

intestinal flora. Molecular identification and classification of DEC is based on the presence of different chromosomal and/or plasmid-encoded virulence genes that are absent in commensal *E. coli*. Moreover, the prevalence and other epidemiological features of these pathogens as causative agents of diarrhoea vary in the world from region to region, and even between and within countries in the same geographical area (Al-Gallas *et al.*, 2007; Kaper *et al.*, 2004; Nataro *et al.*, 2006; Nguyen *et al.*, 2005; Paniagua *et al.*, 1997; Vernacchio *et al.*, 2006). In Nicaragua, EPEC and ETEC have been investigated and associated with secretory diarrhoea among children younger than 6 years, with children less than 2 years being the most affected (Mayatepek *et al.*, 1993; Paniagua *et al.*, 1997). In a study by Mayatepek *et al.* (1993) carried out in Esteli (northern zone), EPEC was found to be significantly more frequent in children with diarrhoea (16 %) than in healthy children (4 %). Nevertheless, ETEC rates were not statistically significantly different (28 % of cases and 16 % of controls). However, in a study carried out in León (north-western pacific side) by Paniagua *et al.* (1997), ETEC was found in 38 % of diarrhoeal episodes and in 19 % of asymptomatic controls. Furthermore, the majority of these children had two to three episodes of ETEC during the first 18 months of life. These two studies clearly exemplify the differences in prevalence of two DEC types within the same country. In this study, we aimed to determine the relative prevalence of five DEC types in children younger than 5 years of age with and without diarrhoea in León, Nicaragua.

METHODS

Clinical specimens. Between March 2005 and September 2006, 526 stool samples from children who were younger than 60 months of age were investigated for DEC. This included 381 children with and 145 without diarrhoea attending four different health care centres and the main hospital in León. Information on some clinical and epidemiological features was obtained through questionnaires. Patients were enrolled in the study if they had diarrhoea, characterized by the occurrence of three or more loose, liquid or watery stools or at least one bloody loose stool in a 24 h period (WHO, 2000). Control subjects were healthy children with no history of diarrhoea for at least 1 month. The sample collection was performed with the consent of the children's parent or guardian, and was approved by the local bioethical committee of the Faculty of Medical Sciences, National Autonomous University of Nicaragua, León.

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 toleration of oral rehydration at a health care centre; and 3, severe, episode with fever and vomiting, requiring intravenous rehydration and needing hospitalization (WHO, 2000).

Microbiological tests. Stool samples collected in sterile plastic containers were transported at 4 °C to the Microbiology Department of the National Autonomous University of Nicaragua, where primary microbiological examinations were conducted. Samples were cultured on MacConkey agar for the selection of *E. coli* isolates and on other media, such as deoxycholate citrate agar (for the selection of *Shigella* and *Salmonella*), with overnight incubation at 37 °C. All samples were

tested for *E. coli*, *Shigella* and *Salmonella* by colony morphology and conventional biochemical tests as described previously (Gillespie & Hawkey, 2006). In addition, samples were analysed for the presence of *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium* and *Trichuris trichiura* using conventional microscopy and staining methods as described previously (Leiva *et al.*, 2005; Tellez *et al.*, 2003). The presence of rotavirus and norovirus was also investigated by ELISA and RT-PCR as described previously (Bucardo *et al.*, 2007, 2008). For *E. coli*, a full loop from each cultured plate suspected of being *E. coli* was stored at -70 °C in brain heart infusion broth containing 15 % (v/v) glycerol for further analysis. All samples were sent to the Department of Laboratory Medicine, Karolinska University Hospital, Huddinge, Stockholm, Sweden, where further characterization was performed. Occurrences of DEC were the main focus of this study.

Multiplex PCR. All *E. coli*-positive samples were analysed essentially as described by Nguyen *et al.* (2005) with some modifications. A brief description is detailed below.

DNA templates for PCR. A smear of bacteria from the first area of a MacConkey plate was suspended in 1 ml PBS to a density of a 4 McFarland standard (1×10^9 – 5×10^9 bacteria ml⁻¹). The bacterial suspension was boiled for 20 min, followed by centrifugation at 2500 g for 10 min to pellet the cell debris. The supernatant was used for PCR.

Multiplex PCR assay. Two microlitres of the lysate from the reference strains ETEC ATCC 35401, EHEC ATCC 43890, EHEC ATCC 43889, EPEC ATCC 43887, EIEC ATCC 43893, EAEC strain 97R harbouring pCVD432 and the *E. coli* negative control ATCC 11775, and from *E. coli*-positive samples, was subjected to multiplex PCR with specific primers (Table 1) for detection of the following virulence markers: *eaeA* (structural gene for intimin of EHEC and EPEC), *bfpA* (structural gene for the bundle-forming pilus of EPEC), *vt1* and/or *vt2* (verocytotoxin 1 and 2 of EHEC), *eltB* and/or *estA* (enterotoxins LT and ST of ETEC), *ial* (invasion-associated locus of the invasion plasmid found in EIEC and *Shigella*) and pCVD (the nucleotide sequence of the *EcoRI*–*PstI* DNA fragment of pCVD432 of EAEC). The primers used for amplification matched the corresponding sequences of the genes for EAEC, EHEC, EIEC, EPEC and ETEC in GenBank. The minimum criteria for determination of DEC were defined as follows: the presence of *eltB* and/or *estA* for ETEC; the presence of *vt1* and/or *vt2* for EHEC (the additional presence of *eaeA* confirmed the detection of a typical EHEC isolate); the presence of *bfpA* and *eaeA* for typical EPEC (but the presence of only *eaeA* for atypical EPEC); the presence of *ial* for EIEC and *Shigella*; and the presence of pCVD432 for EAEC. Additionally, to confirm the PCR analysis, amplicons were analysed by sequencing the forward and reverse strands (data not shown) and by taking another smear of the same area cultured on a fresh MacConkey plate to get separate colonies. After incubation at 37 °C overnight, five to eight colonies with typical *E. coli* morphology were streaked onto fresh plates. Each colony was tested independently by PCR with a primer specific for a suspected DEC isolate from the multiplex PCR.

PCRs were performed in a 25 µl reaction mixture with a pureTaq Ready-To-Go PCR Bead (GE Healthcare UK). Each reaction mixture consisted of 2 µl bacterial lysate, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 2.5 U pureTaq DNA polymerase and 0.2 µM each primer (from Interactiva Biotechnologie GmbH), except for primer VT1, which was used at a concentration of 0.4 µM (determined as the optimal concentration after standardizing the PCR assay). PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) with the following thermocycling conditions: 96 °C for 4 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and a final 7 min extension at 72 °C. PCR products (10 µl) were evaluated on a 1.5 % (w/v) agarose gel (UltraPure Agarose;

Table 1. Primers used in the multiplex PCR for the detection of DEC types

DEC type	Target gene	Primer	Primer sequences (5'→3')	Fragment size (bp)	GenBank accession no.
ETEC	<i>eltB</i>	LT	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	322	S60731
	<i>estA</i>	ST	GTCAAACCAAGTA(G/A)GGTCTTCAAAA CCCGGTACA(G/A)GGAGGATTACAACA	147	M34916
EHEC	<i>vt1</i>	VT1	GAAGAGTCCGTGGGATTAC AGCGATGCAGCTATTAATAA	130	AF461172
	<i>vt2</i>	VT2	ACCGTTTTTCAGATTTT(G/A)CACATA TACACAGGAGCAGTTTCAGACAGT	298	AY143337
EPEC	<i>eaeA</i>	eae	CACACGAATAAACTGACTAAAATG AAAAACGCTGACCCGCACCTAAAT	376	AE005595
	<i>bfpA</i>	bfpA	TTCTTGCTGCTTGGTGTCTTTT TTTTGTTTGTGTATCTTTGTAA	367	U27184
EIEC	<i>ial</i>	SHIG	CTGGTAGGTATGGTGAGG CCAGGCCAACAAATTATTTCC	320	AY167049
EAEC	pCVD432	EA	CTGGCGAAAGACTGTATCAT AAATGTATAGAAATCCGCTGTT	630	X81423

Invitrogen Life Technologies) at 120 mV for 30 min. A molecular marker (TrackIt 100 bp DNA Ladder; Invitrogen Life Technologies) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel had been stained with ethidium bromide.

Data analysis. A χ^2 test or Fisher's exact test was used to determine the statistical significance of the data. A *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Clinical specimens

A total of 526 stool samples from 381 children with diarrhoea and 145 children without diarrhoea were collected. Among the total samples, 280 (53.2%) children were younger than 12 months, 160 (30.4%) were 13–24 months and 86 (16.4%) were 25–60 months. The overall sex distribution was 320 (60.8%) male and 206 (39.2%) female.

Detection of DEC

In this study, in order to detect the five different categories of DEC simultaneously, a mixture of eight primer pairs specific for the target genes was used in a single PCR. Samples with each target sequence of the five different categories of DEC were tested by the multiplex PCR. Fig. 1 shows that the multiplex PCR could detect one or more DEC strains in a single sample. Due to close similarities in band sizes, it was difficult to distinguish individual bands in some cases; as a result, it was necessary to run a primer-specific PCR on single colonies to verify the detection of the suspected DEC isolate.

From the 526 samples, at least one DEC type was detected in 282 (53.6%) of the samples. In the diarrhoea group, 205

(53.8%) samples were positive, and 77 (53.1%) were positive in the non-diarrhoea group. However, overall, 348 DEC were isolated from the 526 samples: 256 (67.2%) were identified in the samples from children with diarrhoea, and 92 (63.4%) in children without diarrhoea (Table 2). Forty-eight children (12.6%) from the diarrhoea group and 15 (10.3%) from the non-diarrhoea group were colonized with more than one DEC pathotype. EAEC was the most prevalent pathotype identified, followed by atypical EPEC (only *eaeA*), ETEC, EHEC and EIEC. No typical EPEC (*eaeA* + *bfpA*) was detected in this study.

The ETEC pathotype was isolated significantly more often in children with (20.5%) than in children without (8.3%) diarrhoea (*P* < 0.001) (Table 2). Atypical EPEC, EIEC and EAEC were isolated with slightly lower frequencies in children with (16.0, 0.8 and 27.8%, respectively) than in children without (20.7, 1.4 and 33.1%, respectively) diarrhoea. EHEC was only detected in children with diarrhoea (2.1%).

The ETEC pathotype was detected in all age groups, but a slightly higher prevalence was seen in children under 12 months of age (Table 3), where a significant difference (*P* < 0.001) in frequencies between children with and without diarrhoea was also seen. Atypical EPEC, EIEC, EHEC and EAEC were detected in all age groups, but no statistically significant differences were seen in any of the groups. Of the 78 ETEC strains isolated in the diarrhoea group, 60 were *eltB*, four were *estA* and 14 were *eltB/estA*. ETEC was identified in 12 of the non-diarrhoea group children. However, these strains were positive only for *eltB*, a finding that clearly associates *estA* (*P* = 0.008) with diarrhoeal disease. Thus ETEC appears to cause secretory diarrhoea early in life, well before 1 year of age, after which a high carriage rate is seen.

In this study, isolation of one or more DEC types alone or in combination with another enteric pathogen or patho-

Fig. 1. Multiplex PCR amplification of reference strains and clinical samples of DEC. Lanes: 1, molecular marker (TrackIt 100 bp DNA Ladder; Invitrogen Life Technologies); 2, EAEC 97R; 3, ETEC ATCC 35401; 4, EHEC ATCC 43889; 5, EHEC ATCC 43890; 6, *E. coli* ATCC 11775; 7, EPEC ATCC 43887; 8, EIEC ATCC 43893; 9–18, clinical samples. Numbers on the left denote fragment sizes (bp).

gens was common: 81 (21.3 %) and 16 (11.0 %) samples in the diarrhoea group and the non-diarrhoea group ($P=0.007$), respectively (Table 4). The most frequent combinations were EAEC + EPEC (3.9 %), EAEC + ETEC (2.9 %), EAEC + norovirus (2.4 %), ETEC + EPEC (1.3 %), ETEC + norovirus (1.8 %) and EPEC + norovirus (1.3 %). However, statistically significant differences were seen only for EAEC + norovirus co-infection ($P=0.05$), and as an overall result.

Clinical symptoms from the children with diarrhoea such as fever, vomiting, nausea, loss of appetite, abdominal pain, stool properties and the number of episodes per day were recorded. In general, the frequencies of symptoms were similar regardless of the isolated DEC pathotype (Table 5). This suggests that, based on these clinical findings, an infection with, for example, ETEC could not be distinguished from an infection with atypical EPEC, EIEC, EAEC or EHEC.

The majority of the diarrhoeal cases were classified as mild (269; 70.6 %), followed by severe (68; 17.9 %) and moderate (44; 11.5 %). However, when a DEC type was identified, 139 cases (67.8 %) were mild, 42 (20.5 %) severe and 24 (11.7 %) moderate. Twenty-two EAEC (32.4 %)

were identified in severe cases, followed by 19 ETEC (27.9 %) and 14 atypical EPEC (20.6 %), either alone or in combination (Table 5).

Our results highlight the importance of ETEC as a cause of childhood diarrhoea in the studied region of Nicaragua. Unlike a study in Vietnam (Nguyen *et al.*, 2005), our study shows that the prevalence of ETEC in the diarrhoea group was significantly higher in children younger than 1 year of age than in older children, although ETEC was detected in all age groups. Additionally, our results support data from a previous prospective cohort study on the incidence of infection with ETEC in infants from Nicaragua, where it was shown that the highest incidence of ETEC diarrhoeal illness occurred during the first year of life (Paniagua *et al.*, 1997).

EAEC is an emerging pathogen associated with diarrhoea. It has been identified in travellers, children in the developing world and human immunodeficiency virus-infected patients with diarrhoea (Adachi *et al.*, 2002; Kaper *et al.*, 2004; Keskimäki *et al.*, 2000; Nguyen *et al.*, 2005; Nishikawa *et al.*, 2002). In the present study, EAEC strains were the most frequently isolated pathotype of *E. coli*; however, the isolation rate as a total was slightly higher in

Table 2. DEC among children with and without diarrhoea from León, Nicaragua

DEC type	PCR type	No. (%) of children with and without diarrhoea		Odds ratio (95 % CI)*	P value (χ^2)
		Diarrhoea group (<i>n</i> =381)	Non-diarrhoea group (<i>n</i> =145)		
ETEC	All	78 (20.5)	12 (8.3)	2.4 (1.4–4.4)	0.001
	<i>eltB</i>	60 (15.7)	12 (8.3)	1.9 (1.1–3.4)	0.026
	<i>estA</i>	4 (1.0)	0 (0.0)	NA	0.216
	<i>eltB/estA</i>	14 (3.7)	0 (0.0)	NA	0.019
Atypical EPEC	<i>eaeA</i>	61 (16.0)	30 (20.7)	0.7 (0.5–1.1)	0.205
EIEC	<i>ial</i>	3 (0.8)	2 (1.4)	NA	0.532
EAEC	pCVD432	106 (27.8)	48 (33.1)	0.8 (0.6–1.1)	0.234
EHEC	All	8 (2.1)	0 (0.0)	NA	0.079
	<i>vt1</i>	1 (0.3)	0 (0.0)	NA	0.537
	<i>vt2</i>	6 (1.6)	0 (0.0)	NA	0.129
	<i>vt2/eaeA</i>	1 (0.3)	0 (0.0)	NA	0.537

*CI, Confidence interval; NA, not applicable.

Table 3. Prevalence of DEC among children less than 1 year of age

DEC type	PCR type	No. (%) of positive children (0–12 months)		Odds ratio (95 % CI)*	P value (χ^2)
		Diarrhoea group (n=192)	Non-diarrhoea group (n=88)		
ETEC	All	44 (22.9)	5 (5.7)	4.0 (1.7–9.8)	<0.0001
	<i>eltB</i>	36 (18.8)	5 (5.7)	3.3 (1.3–8.1)	0.03
	<i>estA</i>	1 (0.52)	0 (0.0)	NA	0.498
	<i>eltB/estA</i>	7 (3.6)	0 (0.0)	NA	0.070
Atypical EPEC	<i>eaeA</i>	33 (17.2)	18 (20.5)	0.8 (0.5–1.4)	0.511
EIEC	<i>ial</i>	2 (1.0)	1 (1.1)	NA	0.943
EAEC	pCVD432	64 (33.3)	29 (33.0)	1.1 (0.7–1.5)	0.950
EHEC	All	1 (0.5)	0 (0.0)	NA	0.498
	<i>vt1</i>	0 (0.0)	0 (0.0)	NA	NA
	<i>vt2</i>	1 (0.5)	0 (0.0)	NA	0.498
	<i>vt2/eaeA</i>	0 (0.0)	0 (0.0)	NA	NA

NA, Not applicable.

*CI, Confidence interval.

Table 4. Frequency of co-infections when a DEC type was identified, among the diarrhoeal and non-diarrhoeal group

Diarrhoeagenic <i>E. coli</i> + diarrhoeal pathogen	No. (%) of positive samples	
	Diarrhoea group (n=381)	Non-diarrhoea group (n=145)
EAEC + aEPEC	15 (3.9)	11 (7.6)
EAEC + ETEC	11 (2.9)	2 (1.4)
ETEC + aEPEC	5 (1.3)	0 (0.0)
EAEC + aEPEC + ETEC	3 (0.8)	0 (0.0)
EAEC + EHEC	1 (0.3)	0 (0.0)
aEPEC + EHEC	1 (0.3)	0 (0.0)
ETEC + EIEC	1 (0.3)	0 (0.0)
ETEC + <i>Entamoeba histolytica</i>	1 (0.3)	0 (0.0)
ETEC + <i>G. lamblia</i>	2 (0.5)	1 (0.7)
aEPEC + <i>G. lamblia</i>	1 (0.3)	0 (0.0)
EAEC + <i>G. lamblia</i>	2 (0.5)	0 (0.0)
EAEC + aEPEC + <i>G. lamblia</i>	0 (0.0)	1 (0.7)
EAEC + ETEC + <i>G. lamblia</i>	1 (0.3)	0 (0.0)
EAEC + EHEC + <i>G. lamblia</i>	1 (0.3)	0 (0.0)
EAEC + aEPEC + <i>Cryptosporidium</i>	0 (0.0)	1 (0.7)
EAEC + <i>T. trichiura</i>	1 (0.3)	0 (0.0)
ETEC + norovirus	7 (1.8)	0 (0.0)
EIEC + norovirus	1 (0.3)	0 (0.0)
aEPEC + norovirus	5 (1.3)	0 (0.0)
EAEC + norovirus	9 (2.4)	0 (0.0)*
EAEC + aEPEC + norovirus	3 (0.8)	0 (0.0)
EAEC + ETEC + norovirus	3 (0.8)	0 (0.0)
ETEC + aEPEC + norovirus	2 (0.5)	0 (0.0)
EAEC + EHEC + norovirus	1 (0.3)	0 (0.0)
ETEC + rotavirus	1 (0.3)	0 (0.0)
aEPEC + rotavirus	1 (0.3)	0 (0.0)
ETEC + <i>Shigella</i> spp.	1 (0.3)	0 (0.0)
aEPEC + <i>Shigella</i> spp.	1 (0.3)	0 (0.0)
Total	81 (21.3)	16 (11.0)†

*P < 0.05.

†P = 0.007.

Table 5. Clinical symptoms in children with diarrhoea where a DEC pathotype was identified

Properties	ETEC (78)	EPEC (61)	EAEC (106)	EHEC (8)	EIEC (3)
Vomiting (n, %)	25 (32.0)	18 (29.5)	34 (32.0)	2 (25.0)	1 (33.3)
Fever $\geq 38^{\circ}\text{C}$ (n, %)	26 (33.3)	17 (27.9)	42 (39.6)	3 (37.5)	1 (33.3)
Nausea (n, %)	8 (10.3)	5 (8.2)	10 (9.4)	1 (12.5)	0 (0.0)
Loss of appetite (n, %)	52 (66.7)	42 (68.9)	73 (68.9)	7 (87.5)	2 (66.7)
Abdominal pain (n, %)	25 (32.1)	22 (36.1)	29 (27.4)	5 (62.5)	1 (33.3)
Stool properties (n, %)					
Watery	51 (65.3)	41 (67.2)	66 (62.2)	5 (62.5)	2 (66.7)
Semi-solid	15 (19.3)	8 (13.1)	22 (20.8)	2 (25.0)	0 (0.0)
Watery + mucous	10 (12.8)	10 (16.4)	12 (11.3)	0 (0.0)	1 (33.3)
Semi-solid + mucous	2 (2.6)	2 (3.3)	6 (5.7)	1 (12.5)	0 (0.0)
Episodes per day (mean)	5.5	5.3	5.6	4.9	4.7
Severity					
Mild	49 (62.8)	42 (68.9)	70 (66.0)	6 (75.0)	3 (100.0)
Moderate	10 (12.8)	5 (8.2)	14 (13.2)	0 (0.0)	0 (0.0)
Severe	19 (24.4)	14 (22.9)	22 (20.8)	2 (25.0)	0 (0.0)

the non-diarrhoeal group than in the diarrhoeal group. An even higher isolation rate in the non-diarrhoeal group compared with the diarrhoeal group in children aged 12–24 (34.0 vs 23.3 %) and 25–60 (34.0 vs 20.5 %) months was correspondingly seen (data not shown). However, in children less than 1 year of age, similar isolation rates were observed in the diarrhoea (33.3 %) versus the non-diarrhoea (33.0 %) group (Table 3). These observations are not in disagreement with other studies where EAEC was isolated at higher frequency rates from controls than from children with diarrhoea (Spano *et al.*, 2008; Vernacchio *et al.*, 2006). However, in other studies carried out in children in Vietnam and the USA, EAEC was isolated at higher prevalence in cases (11.6 and 4.5 %, respectively) than in controls (7.2 and 1.7 %, respectively) (Nataro *et al.*, 2006; Nguyen *et al.*, 2005). The fact that EAEC was not statistically associated with diarrhoea in the present study, even though it was the most dominant pathotype isolated from the severe cases, followed by ETEC and EPEC, could be due to a high level of asymptomatic carriers. This lack of association with diarrhoea has been observed in another study and with other well-established enteropathogens (Gomes *et al.*, 1991). It has also been shown that EAEC is a heterogeneous group of *E. coli* (Czczulin *et al.*, 1999; Jenkins *et al.*, 2007) and not all strains are capable of causing diarrhoea. Furthermore, the DNA probe used in this study to detect EAEC was a derivative from the pAA plasmid, and does not discriminate between true virulent strains. A need for future characterization of these EAEC strains is required involving a more detailed molecular characterization of the different putative virulence genes in order to determine their actual role in the studied population (Czczulin *et al.*, 1999; Jenkins *et al.*, 2006, 2007; Jiang *et al.*, 2003).

EPEC is also a very important pathogen in children with diarrhoea. EPEC infection is primarily a disease of infants younger than 2 years of age. Numerous case-control studies in many countries have found that EPEC is more

frequently isolated from children with diarrhoea than from healthy controls (Kaper *et al.*, 2004; Nataro & Kaper, 1998). In a study carried out in a northern city of Nicaragua, EPEC was found to be significantly more frequent in diarrhoeal (16 %) than in healthy (4 %) children (Mayatepek *et al.*, 1993). However, in our study, the isolation rate of EPEC strains from children with diarrhoea was slightly lower than that from children without diarrhoea. This finding has also been seen in another study, where this pathotype was recovered more frequently from non-diarrhoeal cases than from diarrhoeal cases (Echeverria *et al.*, 1989). Our findings highlight the variation in prevalence of one DEC type (EPEC) as a causative agent of diarrhoea within the same country. Additionally, the role of atypical EPEC in diarrhoea has not been established decisively (Kaper *et al.*, 2004; Nataro & Kaper, 1998; Nataro *et al.*, 2006), and this study did not demonstrate a diarrhoeagenic role for this pathotype.

EPEC and EHEC share *eaeA* (the intimin structural gene), but the major virulence factors defining the characteristics of EHEC are the verocytotoxins. In the present study, we identified a few EHEC strains from children with diarrhoea, but none from children without. These results agree with the low prevalence of EHEC infection in developing countries (Brown *et al.*, 1989; Strockbine *et al.*, 1992). It is also evident that the carrier rates of these pathogens are very low (<1 %).

The low frequencies of EHEC and EIEC strains in the studied population were, in general, in agreement with other studies performed in different parts of the world (Gomes *et al.*, 1991; Nguyen *et al.*, 2005).

In conclusion, ETEC continues to be an important agent associated with diarrhoea in children from León, Nicaragua, as reported previously, and the carriage rate of *estA* (the gene for ST) is below 5 %. EAEC and EPEC, on the other hand, were recovered at high rates from children with and without diarrhoea, indicating a wide spread of

these pathotypes in the study population and a high carriage rate after primary infection. Additionally, more than half of the children with severe diarrhoea carried EAEC, ETEC or EPEC.

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