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Characterization of enterotoxigenic *Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and community settings

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Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of diarrhoea among young children in developing countries. ETEC vaccines offer promise in reducing the burden of ETEC disease, but the development of these vaccines relies on the characterization of ETEC isolates from a variety of settings. To best reflect the full spectrum of ETEC disease in León, Nicaragua, the aim of this study was to characterize ETEC strains isolated from children with diarrhoea attending different settings (hospital, primary care clinics and in the community) and children from different age groups. We characterized ETEC isolates in terms of their colonization factors (CFs) and enterotoxins, and determined whether these factors varied with setting and age group. Diarrhoeal stool samples were obtained from children under the age of 60 months from: (1) the regional public hospital, (2) four public primary care clinics, and (3) a population-based cohort. In total, 58 ETEC-positive isolates were analysed by multiplex-PCR assays for the identification of CFs (CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS13, CS14, CS15, CS17, CS18, CS19, CS20, CS21, CS22 and CFA/I), and enterotoxins [heat-labile toxin (LT) and heat-stable variants STh and STp]. The frequency of CFs and enterotoxins was compared among the three settings and for different age groups, using Fisher's exact test or a χ^2 test. At least one CF was detected among one-half of samples; CS19 was detected among all strains in which a CF was identified, either alone or in combination with another CF. Among all CFs detected, 91.7 % were identified as members of the class 5 fimbrial family. CFs were detected more commonly among samples from infants captured in the health facility setting compared with the community setting. Overall, LT was detected among 67.2 % of samples, STh was detected among 20.7 % and both enterotoxins were detected among 12.1 %. The enterotoxin STh was detected more commonly among cases in the community, whilst a combination of STh and LT was detected more commonly among cases treated in health facilities. Our results suggest that, to protect against diarrhoeal cases associated with this *E. coli* pathotype in León, Nicaragua, an ETEC vaccine that effectively targets the archeotype CFA/I of the class 5 fimbrial family would be the most effective in this setting.

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of diarrhoea among young children in the developing world (Qadri *et al.*, 2007; Paniagua *et al.*, 1997). Each year, ETEC is responsible for an estimated 280 million diarrhoea episodes and 300 000–500 000 deaths (Wennerås & Erling, 2004; World Health Organization, 2006). In Nicaragua, the different types of diarrhoeagenic *E. coli* have been investigated. However, ETEC and enterohaemorrhagic *E. coli* have been the only types statistically associated with diarrhoea among young children (Paniagua *et al.*, 1997; Vilchez *et al.*, 2009). The results of Vilchez *et al.*

Abbreviations: CF, colonization factor; ESBL, extended-spectrum β-lactamase; ETEC, enterotoxigenic *Escherichia coli*; LT, heat-labile toxin; ST, heat-stable toxin.

(2009) suggest that ETEC as the only pathogen is associated with 11.5 % (44/381) of all diarrhoea cases investigated.

ETEC disease follows the ingestion of contaminated water or food; the resulting watery diarrhoea can last for several days and may result in dehydration and malnutrition in children. This pathotype is thought to cause disease by attaching to the small intestinal epithelium with hair-like fimbriae, and then producing toxins that induce watery diarrhoea. Over 25 specific antigens, called colonization factors (CFs), have been identified in strains isolated from humans (Isidean, *et al.*, 2011). However, in the literature, there are a significant number of reports where ETEC strains are negative for these antigens, suggesting the presence of additional adhesins in clinical ETEC isolates; for example, Del Canto *et al.* (2012) described the identification of a novel adhesion CS23 in the ETEC strain 1766a. In addition, two enterotoxins have been identified in ETEC infections, heat-labile toxins (LTs) and heat-stable (ST) variants (STh and STp). ST variants bind to guanylylcyclase, causing an increase in intracellular GMP levels in the enterocyte. LT, a protein sharing many features with cholera toxin, binds to intracellular adenylcyclase, leading to an increase in cyclic AMP levels. The diagnosis of ETEC infection requires the detection of one or both of these two toxins (Kaper *et al.*, 2004).

The development of ETEC vaccines is based on the acquisition of natural immunity to ETEC. As the LT enterotoxin and CFs have been shown to be immunogenic (Steinsland *et al.*, 2003), most vaccine candidates to date have included these antigens. However, the most recent data by Kotloff *et al.* (2013) highlighted the importance of ST ETEC strains, as their results from a Global Enteric Multicenter Study suggested that these ETEC variants are within the five pathogens that should be addressed in interventions to reduce the global burden of diarrhoea. Nevertheless, in order to develop an effective ETEC vaccine, the characterization of these antigens in different endemic countries is necessary. In addition to varying global regions, the characterization of ETEC isolates from different settings, such as hospitals, primary care clinics and the community, will best reflect the full spectrum of ETEC disease. Moreover, an insight into antimicrobial resistance patterns in different countries is important for controlling the spread of antibiotic-resistant diarrhoeagenic bacteria.

The aims of this study were to identify the CFs and enterotoxins present among ETEC isolated from Nicaraguan children in hospital, primary care clinics and the community, and to investigate the antimicrobial resistance patterns of these ETEC strains. These findings may help with the development of an effective ETEC vaccine and guide the clinical management of ETEC.

METHODS

Setting. Nicaragua is a lower-to-middle income nation in Central America. Despite improvements in child mortality in Nicaragua in recent decades, diarrhoea continues to be among the most common causes of child mortality (Nicaraguan Ministry of Health, 2012; Pan American Health Organization, 2007). The current study was conducted in León, Nicaragua's second largest city in the Pacific region of the country.

Sample collection. The 58 ETEC isolates analysed in this study from children with diarrhoea under the age of 60 months were recovered from two different studies. The first was a surveillance study of 381 children with diarrhoeal episodes, from the regional public hospital and four public primary care clinics, between March 2005 and September 2006 (Vilchez *et al.* 2009). From this study, we investigated 13 ETEC from children hospitalized for diarrhoea with severe dehydration, and 22 strains from children evaluated for diarrhoea in a primary care clinic. The second study was a population-based cohort of children selected from the Health and Demographic Surveillance Site, León (Peña, *et al.*, 2008; Becker-Dreps, *et al.*, 2013), who were followed in their households for diarrhoea episodes between January 2010 and January 2011. From this community cohort, 339 stool samples were screened from 222 children with diarrhoea. Here, we present the analysis of 23 ETEC isolates.

Case definition. Diarrhoea in the above-mentioned studies was defined as three or more loose, liquid or watery stools within a 24 h period. Clinical and demographic information was obtained from the children's medical records (hospital and primary care clinics only) and from questionnaires (community cohort). The severity of diarrhoea episodes in children at the hospital and primary care settings were defined as: 1, mild, without vomiting and with good toleration of oral rehydration therapy at home; 2, moderate, with fever and/or vomiting and with toleration of oral rehydration at a health facility; and 3, severe, an episode with fever and vomiting, requiring intravenous rehydration and hospitalization (World Health Organization, 2000). Whilst children in the community cohort were not examined by a clinician for dehydration levels, basic clinical data were collected using questionnaires, such as the frequency of stools and the presence of vomiting, fever or bloody stools. The studies were approved by the Institutional Review Boards of the National Autonomous University of Nicaragua, León (UNAN-León) and the University of North Carolina at Chapel Hill, NC, USA.

Laboratory analysis. ETEC strains were cultured on MacConkey agar with overnight incubation at 37 °C. All ETEC-positive isolates were reanalysed by PCR as described by Vilchez *et al.* (2009) for the detection of the virulence markers for the enterotoxins LT and STh, and by Rodas *et al.* (2009) for enterotoxin STp, to confirm their ETEC status prior to CF analyses.

Identification of CFs. A PCR assay for the CFs CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS13, CS14, CS15, CS18, CS20, CS21, CS22, CFA/I, CS17 and CS19 was performed as described by Rodas *et al.* (2009), with some modifications. Additionally, to confirm the PCR analysis, some of the amplicons were analysed by sequencing the forward and reverse strands, and parallel PCR assays for CS17 and CS19 were performed using primers described by Del Canto *et al.* (2011). A 25 µl reaction mixture with pureTaq Ready-To-Go PCR Beads (GE Healthcare) was organized in four panels of reactions. Each reaction mixture contained 2 µl bacterial lysate (obtained from a homogenized suspension of bacteria adjusted to a density of a MacFarland 4 standard), from an ETEC-positive strain or an ETEC reference strain (ETEC 091207, ETEC ATCC 35401 or *E. coli* ATCC 11775), 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each dNTP, 2.5 U pureTaq DNA polymerase (GE Healthcare) and 0.2 mM each primer. PCR was performed 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 2.0 % (w/v) agarose gel at 120 mV for 45 min. The DNA bands were visualized and photographed under UV light after staining with ethidium bromide. Each result from the multiplex-PCR was also tested independently by PCR with a primer specific for a suspected CF.

Antibiotic susceptibility testing. The *E. coli* isolates were phenotypically screened for antibiotic resistance to ampicillin, trimethoprim-sulfamethoxazole, ceftazidime, ceftriaxone, ciprofloxacin, chloramphenicol, gentamicin and imipenem by a disk diffusion test. Extended-spectrum β-lactamase (ESBL) production was confirmed using a disk diffusion test with the following antibiotics: amoxicillin-clavulanic acid, ceftazidime, cefotaxime and cefepime. Laboratory protocols used were consistent with the Clinical Laboratory and Standards Institute guidelines (CLSI, 2012).

Data analysis. The frequency of the type of enterotoxin (STh and/or LT) and CFs detected was calculated and compared among the different settings of specimen collection (hospital, primary care clinics and the community) and different age groups using Chi-squared or Fisher's exact test where applicable.

RESULTS

Characteristics of participants

ETEC-positive stool samples were provided by 58 children with diarrhoea. Thirteen were obtained from the hospital, 22 from the primary care clinics and 23 from the community cohort. The mean ages of children providing samples in the hospital, primary care and community settings were 10.6, 16.4 and 27.5 months, respectively.

Clinical characteristics of ETEC

The values for the mean number of stools within a 24 h period and the incidence of breastfeeding, vomiting and fever were 6.1, 46.2 %, 69.2 % and 53.8 % in the hospital; 4.8, 63.6 %, 22.7 % and 18.2 % in the primary care clinics; and 4.0, 34.8 %, 21.7 % and 34.8 % in the community. All 13 children with ETEC in the hospital were characterized as severe. Among the 22 with ETEC in the primary care setting, 90.9 % (20/22) were characterized as mild and 9.1 % (2/22) were moderate. Among the 23 children with ETEC at the community level, 82.6 % (19/23) were mild and 17.4 % (4/23) were moderate.

Identification of ETEC CFs

Overall, we detected at least one CF in 50.0 % (29/58) of the ETEC strains investigated. CS19 was detected in all strains in which a CF was detected, either alone or in combination with one or more other CF (Table 1).

The distribution of CFs and enterotoxins varied by setting. At least one CF was detected among 53.8 % of ETEC isolates from children in the hospital and 90.9 % in the primary care clinic, compared with 8.7 % in the community ($P=0.005$ for comparison of hospital vs community, and $P<0.001$ for comparison of primary care vs community.) Also, STh was more commonly detected among strains from children in the community (47.8 %) compared with those receiving care in a primary care clinic (0.0 %) ($P<0.001$), and a combination of STh and LT was more commonly detected among strains from children receiving care in a primary care clinic (27.3 %) compared with those in the community (0.0 %) ($P=0.009$). Whilst the enterotoxins did not vary by age group, CFs tended to be more commonly detected in strains obtained from young children (57.1 %) compared with those from older children (31.3 %) ($P=0.078$ for comparison of children 0–23 months vs children 24–59 months). Surprisingly, no ETEC STp strains were detected.

Antibiotic resistance

Among the 58 ETEC isolates, 44.8 % were resistant to trimethoprim-sulfamethoxazole and 24.1 % were resistant to ampicillin. Only one ETEC isolate was shown to produce ESBL.

DISCUSSION

Using a PCR-based method on ETEC isolates collected from children with diarrhoea in hospital, primary care and community settings, we found that 50.0 % of investigated isolates were positive for at least one of the CFs tested, with CS19 the most commonly identified. CFs were detected more commonly in ETEC isolates from diarrhoea cases treated in health facilities and from young children compared with those from the community and from older children, which were generally mild. Understanding the types and distribution of CFs in various settings and age groups is important because of their promise as ETEC vaccine antigens, based on evidence from several epidemiological, immunological and challenge studies (Sommerfelt *et al.*, 1996; Vidal *et al.*, 2009). To our knowledge, this is the second Central American report describing ETEC strains characterization in terms of CF detection from the same geographical site in Nicaragua.

Our findings of CF detection from health facilities are comparable to other studies, reporting frequencies between 23 and 94 % (Isidean *et al.*, 2011; Girón *et al.*, 1995). In addition, using PCR-based methods, Rodas *et al.* (2011) detected at least one CF among 65 % of ETEC strains isolated from Bolivian children hospitalized for diarrhoea. Using mAb detection for CFs in Bangladesh, Qadri *et al.* (2007) found that 56 % of ETEC strains from the hospital setting were positive for a CF. However, we detected a lower prevalence of CFs among ETEC strains from the community compared with two other prior studies based in the community (Vidal *et al.*, 2009; Paniagua *et al.*, 1997).

It is worth noting the findings by Del Canto *et al.* (2011), where CS21 was detected in 71.8 % of ETEC strains isolated in children from Chile, either alone or in combination with others, and as a common factor, as in the present study for CS19. CS19 was the most common CF detected, either

Table 1. Characterization of ETEC isolates from Nicaraguan children with diarrhoea

Isolates	Sample origin	Clinical severity	Age (months)	Enterotoxin	CF
1	Hospital	Severe	1	LT	
2	Hospital	Severe	3	LT	CS19
3	Hospital	Severe	4	LT-STh	
4	Hospital	Severe	7	LT	
5	Hospital	Severe	8	LT	CS19
6	Hospital	Severe	10	LT	
7	Hospital	Severe	10	LT	CS19
8	Hospital	Severe	11	LT	
9	Hospital	Severe	12	LT	CS19
10	Hospital	Severe	13	LT	CS19
11	Hospital	Severe	17	STh	
12	Hospital	Severe	21	LT	CS19
13	Hospital	Severe	21	LT	CS19
14	Primary care	Mild	1	LT	CS19
15	Primary care	Mild	4	LT	CS19
16	Primary care	Mild	4	LT	CS19
17	Primary care	Mild	5	LT	CS19
18	Primary care	Mild	7	LT	CS19
19	Primary care	Mild	7	LT	CS19
20	Primary care	Mild	10	LT	CS19
21	Primary care	Mild	10	LT	
22	Primary care	Mild	11	LT	CS19
23	Primary care	Mild	12	LT	CS19 and CS6
24	Primary care	Mild	13	LT	CS19
25	Primary care	Mild	13	LT-STh	CS19, CS2 and CS21
26	Primary care	Mild	15	LT	CS19
27	Primary care	Mild	16	LT-STh	CS19, CS21 and CFA/I
28	Primary care	Mild	16	LT	
29	Primary care	Mild	17	LT-STh	CS19
30	Primary care	Mild	17	LT-STh	CS19 and CS1
31	Primary care	Mild	27	LT	CS19 and CFA/I
32	Primary care	Mild	35	LT	CS19
33	Primary care	Mild	53	LT-STh	CS19
34	Primary care	Moderate	19	LT	CS19
35	Primary care	Moderate	48	LT-STh	CS19
36	Community	Mild	7	LT	
37	Community	Mild	7	STh	
38	Community	Mild	9	STh	
39	Community	Mild	13	LT	
40	Community	Mild	16	LT	
41	Community	Mild	16	LT	
42	Community	Mild	16	STh	
43	Community	Mild	17	STh	CS19
44	Community	Mild	18	STh	
45	Community	Mild	23	LT	
46	Community	Mild	29	LT	
47	Community	Mild	34	STh	
48	Community	Mild	34	LT	
49	Community	Mild	35	STh	
50	Community	Mild	36	LT	
51	Community	Mild	40	LT	
52	Community	Mild	42	LT	
53	Community	Mild	46	STh	
54	Community	Mild	51	LT	
55	Community	Moderate	15	LT	
56	Community	Moderate	26	STh	

Table 1. cont.

Isolates	Sample origin	Clinical severity	Age (months)	Enterotoxin	CF
57	Community	Moderate	46	STh	CS19
58	Community	Moderate	56	STh	

alone or in combination with one or more other CFs. CS19 is a member of the class 5 fimbrial family, which includes CS1, CS2, CS4, CS14, CS17, CS19, PCFO71 and CFA/I (family archeotype) (Anantha *et al.*, 2004). The earlier study in Nicaragua by Paniagua *et al.* (1997) found that 36.4 % of the ETEC strains were positive for CFA/I. In Bolivia, Rodas *et al.* (2011) found the following CF frequencies: CFA/I, 18.6 %; CS17, 18.6 %; CS1 + CS3, 6.9 %; CS2 + CS3, 6.9 %; CS12, 6.9 %; and CS7, 4.7 %. Thus, in these studies and the present study, the class 5 family of fimbriae is important among strains from Latin America and would be important to include in an ETEC vaccine candidate. This is supported by evidence of cross-protection between different homologous CFs; for example, in a study of Bangladeshi children with symptomatic or asymptomatic infections by CFA/I, CS1 + CS3, CS2 + CS3 or CS5 + CS6 strains, a repeat episode of diarrhoea or infection by the homologous CF type was uncommon (Qadri *et al.*, 2007).

Finally, our study provides evidence of antibiotic resistance among ETEC strains. Generally, the use of antibiotics for the treatment of diarrhoea in areas where ETEC is endemic is not recommended due to the lack of routine diagnosis of ETEC (Qadri *et al.*, 2005). However, if antimicrobial therapy is given, it should be guided by the local susceptibility patterns of the pathogens. In spite of this, a publication by den Engelsen *et al.* (2009) showed that outpatients with diarrhoea of presumed bacterial origin at the emergency department of the University Hospital of León, Nicaragua, received antibiotics such as trimethoprim-sulfamethoxazole to shorten the course of the disease. In our study, 44.8 % of ETEC strains were resistant to trimethoprim-sulfamethoxazole and 24.1 % were resistant to ampicillin. Therefore, the ETEC strains in León show resistance to the most commonly used antibiotics, which are on the national formulary and are readily available in most pharmacies. In contrast, we found little resistance to chloramphenicol, gentamicin, ciprofloxacin, ceftazidime and ceftriaxone (<12 %), yet the production of ESBL was confirmed in the ETEC strains resistant to ceftazidime and ceftriaxone. In comparison, among 43 ETEC strains isolated from hospitalized children with diarrhoea in Bolivia, higher resistance to ampicillin (53.5 %) and chloramphenicol (14.0 %), and lower resistance to trimethoprim-sulfamethoxazole (32.5 %), was found (Rodas *et al.*, 2011). An ETEC vaccine that effectively reduces cases of ETEC diarrhoea may reduce the need for antibiotics and prevent the spread of antibiotic resistance.

One strength of this study is that we detected CFs in a variety of ETEC isolates collected from children with diarrhoea from different settings. However, limitations of the study are that we only studied one region and only 58 strains were analysed. Also, we cannot rule out the possibility that the difference in CF prevalence observed in the various settings could reflect unidentified CFs such as the non-classical adhesins and the newly described CS23 (Del Canto *et al.*, 2012) that our PCR-based methods did not detect, or differences in CF distribution in the years studied.

Our results suggest that an ETEC vaccine that includes CFA/I in part would afford the most protection against future infections with homologous CF-expressing ETEC strains in León, Nicaragua. This could be particularly beneficial in preventing more severe cases of ETEC diarrhoea requiring health facility visits and cases among young children.

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