Antibiotic resistance patterns of intestinal *Escherichia coli* isolates from Nicaraguan children

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In developing countries, diarrhoeal diseases are one of the major causes of death in children under 5 years of age. It is known that diarrhoeagenic *Escherichia coli* (DEC) is an important aetiological agent of infantile diarrhoea in Nicaragua. However, there are no recent studies on antimicrobial resistance among intestinal *E. coli* isolates in Nicaraguan children. The aim of the present study was to determine the antimicrobial resistance pattern in a collection of 727 intestinal *E. coli* isolates from the faeces of children in León, Nicaragua, between March 2005 and September 2006. All samples had been screened previously for the presence of DEC by multiplex PCR. Three hundred and ninety-five non-DEC isolates (270 from children with diarrhoea and 125 from children without diarrhoea) and 332 DEC isolates (241 from children with diarrhoea and 91 from children without diarrhoea) were analysed in this study. In general, antimicrobial resistance among the 727 intestinal *E. coli* isolates was high for ampicillin (60%), trimethoprim–sulfamethoxazole (64%) and chloramphenicol (11%). Among individual *E. coli* categories, enteroaggregative *E. coli* isolates from children with and without diarrhoea exhibited significantly higher levels of resistance ($P<0.05$) to ampicillin and trimethoprim–sulfamethoxazole compared to the other *E. coli* categories. Resistance to ceftazidime and/or ceftriaxone and a pattern of multi-resistance was related to CTX-M-5- or CTX-M-15-producing *E. coli* isolates. The results suggest that *E. coli* isolates from Nicaraguan children have not reached the high levels of resistance to the most common antibiotics used for diarrhoea treatment as in other countries.

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INTRODUCTION

In developing countries, diarrhoeal diseases are one of the major causes of death in children under 5 years of age (Boschi-Pinto *et al.*, 2008; Bryce *et al.*, 2005). Since the disease is generally self-limiting, antimicrobial agents are not usually recommended for treatment of diarrhoea (Gadewar & Fasano, 2005). However, there are some exceptions, i.e. to prevent diarrhoea in travellers (DuPont, 2005), to shorten the course of the disease when the pathogen has been identified and to prevent transmission

of the disease (Gadewar & Fasano, 2005; O’Ryan *et al.*, 2005).

Diarrhoeagenic *Escherichia coli* (DEC) strains are among the bacteria most frequently associated with diarrhoea in children from developing countries (O’Ryan *et al.*, 2005; Thapar & Sanderson, 2004). 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* (Kaper *et al.*, 2004; Nataro & Kaper, 1998). Reports of antibiotic resistance have increased steadily in DEC isolates affecting children and travellers (Djie-Maletz *et al.*, 2008; Estrada-García *et al.*, 2005; Mendez Arancibia *et al.*, 2009; Nguyen *et al.*, 2005; Ochoa *et al.*, 2009; Vila *et al.*, 2001). Resistance to antibiotics such as ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole is found in DEC isolated from children with diarrhoea in developing

Abbreviations: DEC, diarrhoeagenic *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ESBL, extended-spectrum β -lactamase; ETEC, enterotoxigenic *E. coli*; RAPD, randomly amplified polymorphic DNA.

A supplementary table giving more information on resistance in the *E. coli* isolates is available with the online version of this paper.

countries where the overuse and misuse of antibiotics is common (Djie-Maletz *et al.*, 2008; Nguyen *et al.*, 2005).

Resistance in commensal *E. coli* isolates in children is emerging (Bartoloni *et al.*, 2006; Sepp *et al.*, 2009) and it is well known that commensal bacteria are reservoirs for antimicrobial resistance genes in both the community and hospital settings (Aleksun & Levy, 2006).

Information regarding the prevalence of antimicrobial resistance in pathogens can be used for selecting an optimal treatment when necessary (Okeke *et al.*, 2005). DEC remains an important aetiological agent of infantile diarrhoea in Nicaragua (Mayatepek *et al.*, 1993; Paniagua *et al.*, 1997; Vilchez *et al.*, 2009). There are no recent studies regarding antimicrobial resistance among DEC and non-DEC isolates in Nicaraguan children.

The aim of the present study was to determine the antimicrobial resistance pattern of intestinal *E. coli* isolates with and without DEC virulence markers in children younger than 5 years of age with and without diarrhoea in León, Nicaragua.

METHODS

***E. coli* isolates.** In this study, the susceptibilities to different antimicrobial agents were investigated in 727 *E. coli* isolates identified in a previous study (Vilchez *et al.*, 2009). Three hundred and ninety-five isolates were non-DEC (270 from children with diarrhoea and 125 from children without diarrhoea) and 332 were DEC (241 from children with diarrhoea and 91 from children without diarrhoea). The distribution of the DEC isolates herein analysed was as follows: 203 EAEC isolates, 73 ETEC isolates, 47 EPEC isolates, 8 EHEC isolates and 1 EIEC isolate.

Antibiotics. The following antibiotics were tested: ampicillin (AstraZeneca), amoxicillin-clavulanic acid (Sigma and SmithKline Beecham), ceftazidime (Sigma), ceftriaxone (Sigma), ciprofloxacin (Sigma), chloramphenicol (Sigma), gentamicin (Sigma), imipenem (Merck) and trimethoprim-sulfamethoxazole (Sigma).

Antibiotic susceptibility testing. MICs were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains. The antibiotic susceptibility testing data were analysed using WHONET 5.4 and SPSS 17.0 software. In addition, the data were analysed using the Kruskal-Wallis *H* test for multiple comparison and the Mann-Whitney *U* test for comparing two groups of *E. coli* in terms of antibiotic resistance to each of the tested antimicrobial agents. A *P*-value of <0.05 was considered significant.

Phenotypic detection of extended-spectrum β -lactamases (ESBLs). ESBL production in the *E. coli* isolates that showed resistance to any of the third-generation cephalosporins tested (cefotaxime, ceftriaxone and ceftazidime) was analysed using the Etest system (bioMérieux), cefotaxime/cefotaxime + clavulanic acid, ceftazidime/ceftazidime + clavulanic acid, and cefepime/cefepime + clavulanic acid.

PCR amplification for detection of β -lactamase genes and sequencing analysis. *E. coli* isolates resistant to at least two antibiotics including one β -lactam were screened for the resistance genes encoding SHV, TEM, CTX-M and OXA enzymes by a multiplex PCR assay using universal primers following the procedure described

by Fang *et al.* (2008). Further detection of CTX-M groups 1, 2, 9, 8 and 25 in the *E. coli* isolates positive for CTX-M was performed using a multiplex PCR assay as described by Dallenne *et al.* (2010) and a single PCR assay as described by Pitout *et al.* (2004). PCR amplification was carried out on a GeneAmp PCR system 9700 (Applied Biosystems Division) thermal cycler.

DNA sequencing analysis was performed in *E. coli* isolates that were positive for ESBL production with the Etest system. For TEM and OXA enzymes, sequencing primers described by Fang *et al.* (2008) were used, and for CTX-M enzymes, sequencing primers described by Pitout *et al.* (2004) were used. Amplified PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and bidirectional sequencing was performed. Each sequence was then compared with known β -lactamase gene sequences (<http://www.lahey.org/Studies/>) by multiple-sequence alignment using the BLAST program.

Typing of *E. coli* isolates producing ESBLs by randomly amplified polymorphic DNA (RAPD) analysis. The epidemiological relationships between *E. coli* isolates producing ESBL were analysed intra sample and among the samples by RAPD as described by Touati *et al.* (2007) with some modifications. Total DNA was prepared with a QIAamp DNA mini kit (Qiagen) and used for RAPD typing, which was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare) together with primer 4 (5'-AAGAGCCCGT-3') and primer 5 (5'-AACGCGCAAC-3') (Thermo Fisher Scientific). PCR amplification was carried out as follows: 1 cycle at 94 °C for 5 min, 35 (primer 4) and 31 (primer 5) cycles at 94 °C for 5 s, 42 °C for 30 s and 72 °C for 1 min, with a final extension period at 72 °C for 5 min. After amplification, the banding pattern of randomly amplified DNA was visualized and analysed on a 1.5 % agarose gel in Tris/acetate buffer. A negative control was included in each PCR run with no target DNA. Reproducibility of the amplification results was evaluated in parallel experiments by the repetition of the PCRs three times. Electrophoresed agarose gels were analysed using BioNumerics version 6 software (Applied Maths). Dendrograms based on the Jaccard coefficient and unweighted pair group method using arithmetic averages were generated.

RESULTS AND DISCUSSION

Antibiotic susceptibilities in the *E. coli* isolates

To the best of our knowledge, there are no studies in Latin America that have included antibiotic resistance analysis in both DEC and non-DEC from children with and without diarrhoea. However, there are a few studies that have investigated antibiotic resistance only in *E. coli* isolates positive for a DEC virulence marker (Estrada-García *et al.*, 2005; Ochoa *et al.*, 2009) or only in non-DEC (Bartoloni *et al.*, 2006; Pallecchi *et al.*, 2004, 2007). The results of the antibiotic susceptibility testing performed on 727 *E. coli* isolates (Table 1) showed that resistance to ampicillin was found in 67.7 % (225/332) of the DEC isolates and in 53.2 % (210/395) of the non-DEC isolates, and resistance to trimethoprim-sulfamethoxazole was found in 71.6 % (238/332) of the DEC isolates and 57.7 % (228/395) of the non-DEC isolates. Furthermore, resistance to chloramphenicol was found in 9.3 % (31/332) of the DEC isolates and in 13 % (51/395) of the non-DEC isolates. No resistance to imipenem was observed, and for the other agents the level of resistance was low in all *E. coli* isolates (≤ 2.6 %).

Table 1. Distributions of resistance and MICs (mg l⁻¹) in DEC (*E. coli* isolates positive for diarrhoeagenic virulence markers) and non-DEC isolates from children with and without diarrhoea

Children with diarrhoea					Children without diarrhoea				
<i>E. coli</i> isolate group*	MIC ₅₀	MIC ₉₀	MIC range	n (% R)	<i>E. coli</i> isolate group*	MIC ₅₀	MIC ₉₀	MIC range	n (% R)
DEC (241)					DEC (91)				
AMC	8	16	2–32	5 (2.1)	AMC	4	16	2–32	1 (1.1)
AMP	128	128	2–128	169 (70.1)	AMP	128	128	2–128	56 (61.5)
CAZ	0.25	0.5	0.016–32	2 (0.8)	CAZ	0.25	0.5	0.032–16	3 (3.3)
CHL	4	128	2–128	27 (11.2)	CHL	4	8	2–128	4 (4.4)
CRO	0.064	0.25	0.03–128	2 (0.8)	CRO	0.064	0.125	0.03–128	3 (3.3)
CIP	0.032	0.064	0.016–8	3 (1.2)	CIP	0.032	0.032	0.008–0.5	
GEN	1	1	0.25–32	6 (2.5)	GEN	1	1	0.064–2	
IMP	0.125	0.5	0.064–2		IMP	0.125	0.5	0.032–2	
SXT	4	4	0.032–4	177 (73.4)	SXT	4	4	0.016–4	61 (67)
Non-DEC (270)					Non-DEC (125)				
AMC	8	16	1–32	5 (1.9)	AMC	4	16	1–32	1 (0.8)
AMP	128	128	2–128	152 (56.3)	AMP	8	128	2–128	58 (46.4)
CAZ	0.25	0.5	0.064–32	2 (0.7)	CAZ	0.25	0.5	0.032–16	6 (4.8)
CHL	4	128	2–128	29 (10.7)	CHL	4	128	2–128	22 (17.6)
CRO	0.064	0.064	0.03–128	2 (0.7)	CRO	0.064	0.125	0.03–128	6 (4.8)
CIP	0.032	0.064	0.008–8	5 (1.9)	CIP	0.032	0.064	0.008–8	6 (4.8)
GEN	1	2	0.032–32	7 (2.6)	GEN	0.5	1	0.064–62	7 (5.6)
IMP	0.125	1	0.064–8		IMP	0.25	0.5	0.12–2	
SXT	4	4	0.032–4	171 (63.3)	SXT	0.25	4	0.016–4	57 (45.6)

*AMC, Amoxicillin–clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CRO, ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin; IMP, imipenem; SXT, trimethoprim–sulfamethoxazole.

Differences in the distributions of resistance and MICs were seen for individual antibiotics and for each category of *E. coli* strains. The multiple comparisons showed a significant difference in resistance to amoxicillin–clavulanic acid ($P=0.031$), ampicillin ($P<0.001$) and trimethoprim–sulfamethoxazole ($P<0.001$).

When comparisons of the antibiotic resistance levels of two groups of *E. coli* strains were performed, it was found that the group of EAEC from children with diarrhoea showed the major number of differences in term of resistance to most of the tested antibiotics when compared to other *E. coli* categories isolated from children with and without diarrhoea. For example, this group of *E. coli* strains was significantly more resistant ($P<0.005$) than EHEC and EPEC to ampicillin and trimethoprim–sulfamethoxazole. Interestingly, EAEC from children without diarrhoea was also significantly more resistant ($P<0.005$) than non-DEC from children with and without diarrhoea to amoxicillin–clavulanic acid, ampicillin and trimethoprim–sulfamethoxazole. In addition, EAEC was significantly more resistant ($P=0.021$) than ETEC from children with diarrhoea to amoxicillin (more information about the level of resistance in the other *E. coli* group can be found in Supplementary Table S1 of JMM Online). Other studies carried out in DEC have also shown that EAEC possesses higher levels of antibiotic resistance than other *E. coli* groups. For example,

Estrada-García *et al.* (2005) found in a study carried out in Mexican children that among all of the DEC categories, EAEC was significantly more resistant than EPEC to ampicillin and trimethoprim–sulfamethoxazole. In a recently published study carried out in Peru, the prevalence of resistance to ampicillin, cotrimoxazole, tetracycline and nalidixic acid was significantly higher ($P<0.05$) in EAEC than in EPEC and ETEC (Ochoa *et al.*, 2009). The authors hypothesized that the higher resistance levels in EAEC compared to other groups of DEC could be due to frequent use of antibiotics since these diarrhoeagenic pathotypes often cause persistent diarrhoea and/or are present in asymptomatic carriers. This hypothesis is supported by the findings by Vilchez *et al.* (2009), who showed that EAEC was the most frequently isolated pathotype of *E. coli*, with a high level of asymptomatic carriers. Our data clearly show that, at least in Latin America, EAEC isolated from children possesses high levels of resistance to ampicillin and trimethoprim and could become a serious health problem.

Different resistance patterns were defined in all *E. coli* isolates with and without diarrhoeagenic virulence markers. The two most prevalent multi-resistance patterns (resistance to at least two antibiotics) among the 727 *E. coli* isolates included in this study were (i) ampicillin and trimethoprim–sulfamethoxazole (41 %) and (ii) ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole (7.2 %). Antimicrobial res-

istance patterns in *E. coli* isolates differ among countries. In Peru, Ochoa *et al.* (2009) reported multi-resistance to ampicillin and trimethoprim–sulfamethoxazole as the second most common pattern in DEC, whereas in Vietnam, Nguyen *et al.* (2005) reported that ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole was the most common resistance pattern among DEC.

Although amoxicillin–clavulanic acid, ceftazidime, gentamicin, ceftriaxone and imipenem are not indicated to treat diarrhoea in children, we tested the susceptibilities to these antibiotics in all isolates, since they could be empirically or incidentally used. A recent publication showed that outpatients with diarrhoea of presumed bacterial origin at the emergency department of the University Hospital of León, Nicaragua (50 % being children <6 years of age), received trimethoprim–sulfamethoxazole (18.5 %) and ciprofloxacin (8.5 %) to shorten the course of the disease (den Engelsen *et al.*, 2009). As shown above, some EAEC and non-DEC isolates from children with/without diarrhoea show low level of resistance to amoxicillin–clavulanic acid, ceftazidime and/or ceftriaxone and ciprofloxacin. Resistance to ceftazidime and/or ceftriaxone was related to ESBL production and a pattern of multi-resistance (ampicillin, chloramphenicol, ceftazidime and/or ceftriaxone, gentamicin and ciprofloxacin) was observed in those *E. coli* isolates, whereas resistance to amoxicillin–clavulanic acid could be due to presence of other enzymes such as AmpC (Denton, 2007). Contrary to our results, the studies carried out in Peru and Mexico showed that DEC was sensitive to ceftazidime and/or to ceftriaxone (Estrada-García *et al.*, 2005; Ochoa *et al.*, 2009).

Detection of β -lactamase genes and sequencing analysis

The results from the detection of *E. coli* isolates harbouring genes encoding SHV, TEM, CTX-M and OXA enzymes are

shown in Table 2. The gene encoding TEM enzyme was detected in most isolates, with a higher prevalence in EAEC isolates from children with diarrhoea (12.7 %) and in non-DEC from children without diarrhoea (13 %). The gene CTX-M was more commonly detected in EAEC (4.3 %) and in non-DEC from children without diarrhoea (5.6 %). Among the isolates harbouring the CTX-M gene, 13/13 were resistant to ampicillin, 13/13 to ceftazidime, 10/13 to chloramphenicol, 13/13 to ceftriaxone, 5/13 to ciprofloxacin, 8/13 to gentamicin and 13/13 to trimethoprim–sulfamethoxazole. The gene encoding OXA enzyme was detected in EAEC isolates from children with diarrhoea and in non-DEC from children with/without diarrhoea (Table 2). None of the *E. coli* isolates harboured the gene encoding SHV enzyme.

The *E. coli* isolates positive for ESBLs were selected for sequencing. All were positive in the PCR for CTX-M and TEM, and 1/13 was positive for OXA enzyme. The multiplex PCR for detection of CTX-M-1, 2, 9, 8 and 25 showed that 2/13 *E. coli* isolates were positive for the CTX-M-2 group and 11/13 were positive for the CTX-M-1 group. After sequencing, it was found that TEM-1 and OXA-1/-30 were present in the *E. coli* isolates positive in the PCR assay for TEM or OXA enzymes. For the CTX-M groups, CTX-M-5 was found as the specific enzyme in 2/13 *E. coli* (one EAEC and one non-DEC) isolates positive for the CTX-M-2 group and CTX-M-15 was found as the specific enzyme in 11/13 *E. coli* (four EAEC and seven non-DEC) isolates positive for the CTX-M-1 group.

The CTX-M group of enzymes has become one of the main public health concerns due to their ability to be present in bacteria causing both nosocomial and community-acquired infections. *E. coli* is most often responsible for producing CTX-M β -lactamases and seems to be a true community ESBL pathogen (Pitout & Laupland, 2008). The prevalence of CTX-M enzymes in Latin American

Table 2. Resistance profile and β -lactamase production in *E. coli* isolates

See Table 1 for antibiotic abbreviations.

Organism	Resistance profile [n (%)]		β -Lactamase			
	AMP SXT	AMP CHL SXT	TEM	CTX-M	OXA	Etest ESBL
EAEC* (134)	77 (57)	10 (7)	17 (12.7)	2 (1.5)	2 (1.5)	2 (1.5)
EPEC* (34)	12 (35)	2 (6)	3 (8.8)			
ETEC* (64)	33 (52)	5 (8)	5 (7.8)			
EHEC* (8)	1 (13)	2 (25)				
Non-DEC* (270)	106 (39)	17 (6)	29 (11)	1 (0.3)	4 (1.5)	1 (0.3)
EAEC† (69)	40 (58)	3 (4)	7 (10.1)	3 (4.3)		3 (4.3)
EPEC† (13)	3 (23)					
ETEC† (9)	4 (44)	1 (11)	1 (11)			
Non-DEC† (125)	26 (20)	12 (9.6)	16 (13)	7 (5.6)	1 (0.8)	7 (5.6)

*Children with diarrhoea.

†Children without diarrhoea.

countries is among the highest in the world (Villegas *et al.*, 2008) with CTX-M-1 and -2 groups among the most commonly detected. The production of CTX-M-2, -9, -14, -15, -24 and -56 has been reported in non-DEC strains from healthy children (Pallecchi *et al.*, 2004, 2007), whereas such findings have not been shown for DEC. Our study showed the production of CTX-M-2 and CTX-M-15 in DEC and non-DEC strains from children with and without diarrhoea. β -Lactamase enzymes are the main mechanism of resistance to β -lactam antibiotics in members of the *Enterobacteriaceae* (Paterson, 2006; Torres *et al.*, 2007). Currently, the most widely distributed CTX-M enzyme on a worldwide basis is CTX-M-15, which was first detected in *E. coli* isolated from India during 2001. CTX-M-15 has often been associated with the co-production of other β -lactamases such as TEM-1 and OXA-1 (Peirano & Pitout, 2010; Pitout, 2010). Our results are in accordance with that information, i.e. the production of CTX-M-15 together with the co-production of TEM-1 and OXA-1/30 was found in our study. It is clear that ESBLs have emerged in EAEC and non-DEC from Nicaraguan children with and without diarrhoea.

Typing of ESBL-producing *E. coli* isolates by RAPD analysis

Thirteen *E. coli* isolates producing ESBLs were selected for RAPD analysis. The analysis revealed that these *E. coli* isolates could be separated into five clones (Fig. 1). Among these clones, D1 encompassed all of the EAEC isolates (three of three *E. coli* isolates) and clone D4 most of the non-DEC isolates (four of seven *E. coli* isolates) from children without diarrhoea. In addition, these isolates harboured the enzyme CTX-M-15. Interestingly, clone D5 contained one EAEC and one non-DEC producing CTX-M-5 from children with diarrhoea, which could indicate the transfer of resistance genes from non-pathogenic to pathogenic bacteria.

The problems of antimicrobial resistance require appropriate interventions with special regard to multi-resistant Gram-negative pathogens (Gould, 2008, 2009). The rate of resistance to the most common antibiotics used for diarrhoea treatment is increasing in Latin America. In *E. coli* isolates, the resistance is seen in both pathogenic and non-pathogenic isolates (Estrada-García *et al.*, 2005;

Fig. 1. Clonal similarity of *E. coli* producing ESBLs. EAECa, enteroaggregative *E. coli* from children with diarrhoea; EAECb, enteroaggregative *E. coli* from children without diarrhoea; non-DECa, non-diarrhoeagenic *E. coli* from children with diarrhoea; non-DECb, non-diarrhoeagenic *E. coli* from children without diarrhoea.

Mayatepek *et al.*, 1993; Ochoa *et al.*, 2009; Pallecchi *et al.*, 2004, 2007). Finally, the antibiotic resistance level in *E. coli* from children with/without diarrhoea has not yet reached the high levels of resistance to the most common antibiotics used for diarrhoea treatment as in other countries. However, CTX-M-5 or CTX-M-15 production was detected in some multi-antibiotic-resistant DEC and non-DEC clones, which suggests the emergence of ESBLs in the Nicaraguan community, and this may indicate future treatment complications.

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