Trans R Soc Trop Med Hyg 2015; **109**: 493–502
doi:10.1093/trstmh/trv054

ORIGINAL ARTICLE

Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies in Lima, Peru

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Received 30 March 2015; revised 29 May 2015; accepted 11 June 2015

Background: Antibiotic resistance is increasing worldwide, being of special concern in low- and middle-income countries. The aim of this study was to determine the antimicrobial susceptibility and mechanisms of resistance in 205 enterotoxigenic *Escherichia coli* (ETEC) isolates from two cohort studies in children <24 months in Lima, Peru.

Methods: ETEC were identified by an in-house multiplex real-time PCR. Susceptibility to 13 antimicrobial agents was tested by disk diffusion; mechanisms of resistance were evaluated by PCR.

Results: ETEC isolates were resistant to ampicillin (64%), cotrimoxazole (52%), tetracycline (37%); 39% of the isolates were multidrug-resistant. Heat-stable toxin producing (ETEC-*st*) (48%) and heat-labile toxin producing ETEC (ETEC-*lt*) (40%) had higher rates of multidrug resistance than isolates producing both toxins (ETEC-*lt-st*) (21%), $p<0.05$. Only 10% of isolates were resistant to nalidixic acid and none to ciprofloxacin or cefotaxime. Ampicillin and sulfamethoxazole resistance were most often associated with bla_{TEM} (69%) and *sul2* genes (68%), respectively. Tetracycline resistance was associated with *tet*(A) (49%) and *tet*(B) (39%) genes. Azithromycin inhibitory diameters were ≤15 mm in 36% of isolates, with 5% of those presenting the *mph*(A) gene.

Conclusions: ETEC from Peruvian children are often resistant to older, inexpensive antibiotics, while remaining susceptible to ciprofloxacin, cephalosporins and furazolidone. Fluoroquinolones and azithromycin remain the drugs of choice for ETEC infections in Peru. However, further development of resistance should be closely monitored.

Keywords: Antibiotic resistance, Children, Diarrhoea, Enterotoxigenic *Escherichia coli*, Peru

Introduction

Among children under 5 years of age, there are approximately 2.5 billion cases of diarrhoea per year reported worldwide,[1] resulting in 700 000–800 000 deaths per year globally, principally in low- and middle-income countries.[2,3] In Peru, childhood diarrhoea is the third leading cause of death in children under 5,[4] with its highest incidence in periurban and rural areas.

Rotavirus and diarrhoeagenic *Escherichia coli* (DEC) account for approximately 200 000 and 120 000 deaths per year, respectively, accounting collectively for 40% of all deaths due to childhood diarrhoea.[2] Among DEC pathotypes, enterotoxigenic *E. coli* (ETEC) accounts for more than 40 000 deaths each year in children from developing countries, in addition to causing significant morbidity in adult travellers from industrialised countries to the developing world.[5–7]

Diarrhoea due to ETEC occurs between 8 and 72 hours after initial infection, usually following the ingestion of contaminated food or water.[6] The severity of disease varies from a mild illness to severe disease and potentially to death. Typical symptoms

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include nausea, vomiting and resulting dehydration. Leukocytes and blood are usually not found in faeces.[8] Oral rehydration therapy or, in severe cases, intravenous fluid replacement is the cornerstone of treatment. Antibiotic treatment plays a role in specific cases, such as persistent diarrhoea, nutritional deficiencies, or the presence of other pathology.[9,10] Patients with persistent diarrhoea and abdominal pain due to ETEC may benefit from antibiotic therapy, with a more rapid resolution of symptoms.[9] Patients with nutritional deficiencies or medical comorbidities may similarly have more severe presentations and require the use of antimicrobial agents.[10] Unfortunately, the rapid emergence of antibiotic-resistant strains limits their usefulness.[7,11,12] The dissemination of antimicrobial resistance genes among bacteria is an increasingly serious problem throughout the world.[13–15]

Peru is a developing country with an expanding tourism sector. Diarrhoea caused by contaminated food or water is very common in Peru and is a potentially serious threat for travellers visiting historical sites, including Lima. Thus, circulating strains in a particular country that primarily affect children and contaminate local water and food sources (as well as the hands of the food handlers) may determine the type of ETEC infecting travellers.[6]

Given the widespread geographical reach of ETEC, studies in different regions are mandatory to evaluate local variations in its virulence profile.[16,17] Until a licensed ETEC vaccine is available, the profile of ETEC antimicrobial susceptibility must also be known in order to avoid inadequate therapy.[6] Unfortunately, resistance to older, inexpensive antimicrobial agents, such as ampicillin or cotrimoxazole, is widespread among ETEC isolates in most low-income countries.[17,18] In upper-middle-income countries such as Peru, the scenario is more complex, due to the relatively easy access to most modern antimicrobial agents, the availability of antibiotics without a prescription, and both the overprescription and misuse of antimicrobial agents.[19]

The aims of this study were to determine the antibiotic susceptibility of ETEC isolates from two cohort studies in Peruvian children, establishing the molecular mechanisms of resistance in these strains.

Materials and methods

Study population

The specimens analysed in this study were obtained as part of two previous studies.[21,22] The first study (Cohort 1) was a prospective, passive surveillance cohort diarrhoea study of children 2 to 24 months of age, conducted in low socioeconomic communities in the southern districts of Lima, Peru, between September 2006 and December 2007 (1034 children younger than 1 year of age) and from January to July 2008 (529 children, 1–2 years of age, from the initial cohort were followed during this period).[20] The second study (Cohort 2) was a community-based randomised double-blind placebo controlled trial comparing supplementation with bovine lactoferrin versus placebo conducted in low socioeconomic communities in the northern districts of Lima, Peru, between January 2008 and May 2011, in which 555 weaned children were enrolled at 12–18 months of age and followed for 6 months with daily home visits for data and sample collection and supplement administration.[21] In this study, there were no differences in the frequency of ETEC isolation in both groups. Therefore, all strains were included in the current study. Both cohort studies were approved by the Institutional Review Board of the Universidad Peruana Cayetano Heredia, in Lima, Peru, and other participating institutions.

Strains

A total of 120 ETEC strains from children with diarrhoea (defined as three or more liquid or semiliquid stools in 24 hours or a single bloody semiliquid stool in 24 hours) and 85 ETEC strains from control children without diarrhoea 1 week before and 1 week after the stool collection sample were obtained from both cohorts. Briefly, stool samples were evaluated for common enteric pathogens (*Shigella, Salmonella, Vibrio, Campylobacter*, *Giardia lamblia*, *Cryptosporidium* and rotavirus) by conventional methods.[23] Five lactose-positive colonies were isolated from MacConkey plates and tested by a multiplex PCR with specific DNA primers to detect virulence factors associated with enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), Shiga toxin-producing *E. coli* (STEC) and ETEC, as described previously, using a validated five-colony pool analysis method.[24,25] Subsequently, individual colonies from positive ETEC samples were analysed using separate PCR assays for the LT (heat-labile toxin) and ST (heat-stabile toxin) genes.[24] Only one ST gene-positive and/or LT gene-positive colony per sample was then selected for further analysis. The ETEC strains were stored at -70°C in skim milk broth until use. In all cases, the isolates were re-identified prior to use amplifying the *uidA* gene as previously described.[26]

Antimicrobial susceptibility testing

Antimicrobial susceptibility was established by disk diffusion, according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[27] The antibiotics analysed were: ampicillin (Amp; 10-μg), cotrimoxazole (Sxt; 23.75/1.25-μg), tetracycline (Tet; 30-μg), nitrofurantoin (Nit; 300-μg), nalidixic acid (Nal; 30-μg), chloramphenicol (Chl; 30-μg), ciprofloxacin (Cip; 5-μg), gentamicin (Gm; 10-μg), cefotaxime (Ctx; 30-μg), amoxicillin-clavulanic acid (Amc; 30-μg), ceftazidime (Caz; 30-μg), and azithromycin (Azm; 15-μg). As no specific furazolidone resistant breakpoints are available, those of Nit were examined. The antibiotics tested were selected considering both their usefulness in the treatment of diarrhoea or their relevance at epidemiological level. The *E. coli* strain ATCC 25922 was used as quality control. Multidrug resistance (MDR) was defined as resistance to three or more classes of antimicrobial agents. Additionally, the presence of extended-spectrum β-lactamases (ESBLs) was established by the double disk synergy test as described elsewhere.[28]

Molecular mechanisms of antimicrobial resistance

Genes encoding common resistance mechanisms to β-lactams ($bla_{TEM\text{-}like}$, $bla_{CARB\text{-}like}$, $bla_{SHV\text{-}like}$, $bla_{OXA1\text{-}like}$, $bla_{OXA2\text{-}like}$ and $bla_{OXA5\text{-}like}$), to Tet (*tet*(A) and *tet*(B) genes), Chl (*cmlA*, *floR* and *cat* genes), Sxt (*dfrA1, dfrA5, dfrA6, dfrA7, dfrA8, dfrA12, dfrA13, dfrA14, dfrA15, dfrA15b, dfrA16, dfrA16b*, and *dfrA17* genes for trimethoprim and *sul1*, *sul2*, and *sul3* genes for sulfamethoxazole), and Azm (*mph*(A), *erm*(A) and *erm*(B) genes) were studied by conventional PCR using previously described primers and conditions (Table 1) in those isolates presenting resistance to the

Table 1. Primers and conditions for identification of molecular mechanisms of antibiotic resistance on enterotoxigenic *Escherichia coli* (ETEC) strains

Antibiotic	Gene	Primer sequence		Size of product (bp)	Annealing temperature (°C)	Reference
		Forward (5′–3′)	Reverse (5′–3′)			
β-Lactams	$bla_{TEM\text{-}like}$	ATTCTTGAAGACGAAAGGGC	ACGCTCAGTGGAACGAAAAC	1150	60	35
	$bla_{SHV\text{-}like}$	ATGCGTTATATTCGCCTGTG	TTAGCGTTGCCAGTGCTCG	841	55	61
	$bla_{CARB\text{-}like}$	AATGGCAATCAGCGCTTC	GGGGCTTGATGCTCACT	586	56	58
	$bla_{OXA1\text{-}like}$	ACCAGATTCAACTTTCAA	TCTTGGCTTTTATGCTTG	598	55	59
	$bla_{OXA2\text{-}like}$	CGATAGTTGTGGCAGACGAA	CCACTCAACCCATCCTACCC	550	55	64
	$bla_{OXA5\text{-}like}$	TATATTCCAGCATCAACATT	ATGATGCCCTCACTTGCCAT	605	55	61
Chloramphenicol	*cmlA*	TGTCATTTACGGCATACTCG	ATCAGGCATCCCATTCCCAT	435	55	35
	floR	CACGTTGAGCCTCTATAT	ATGCAGAAGTAGAACGCG	868	55	35
	cat	GGTGAGCTGGTGATATGG	GGGATTGGCTGAGACGA	209	48	62
Tetracycline	*tet*(A)	GTAATTCTGAGCACTGTCGC	CTGCCTGGACAACATTGCTT	950	62	35
	tet(B)	CTCAGTATTCCAAGCCTTTG	CTAAGCACTTGTCTCCTGTT	435	57	35
Trimethoprim	*dfr1A, dfrA5, dfrA15, dfrA15b, dfrA16, dfrA16b*	GTGAAACTATCACTAATGG	TTAACCCTTTTGCCAGATTT	474	55	29
	dfrA6, dfrA14	GAGCAGCTICTITTIAAAGC	TTAGCCCTTTIICCAATTTT	393	60	29
	dfrA7, dfrA17	TTGAAAATTTCATTGATT	TTAGCCTTTTTTCCAAATCT	474	55	29
	dfrA8	GAGCTTCCGGGTGTTCGTGAC	CTTCCATGCCATTCTGCTCGTAGT	247	43	63
	dfrA12, dfrA13	GGTGGCGCAGAAGATTTTTCGC	TGGGAAGAAGGCGTCACCCTC	319	60	29
Sulfamethoxazole	*sul1*	TGGTGACGGTGTTCGGCATTC	GCGAGGGTTTCCGAGAAGGTG	789	63	35
	sul2	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722	50	35
	sul3	CATTCTAGAAAACAGTCGTAGTTCG	CATCTGCAGCTAACCTAGGGCTTTGGA	990	51	35
Azithromycin	*mph*(A)	GTGAGGAGGAGCTTCGCGAG	TGCCGCAGGACTCGGAGGTC	403	60	60
	erm(A)	TCTAAAAAGCATGTAAAAGAAA	CGATACTTTTTGTAGTCCTTC	533	52	60
	erm(B)	GAAAAAGTACTCAACCAAATA	AGTAACGGTACTTAAATT	639	45	60

corresponding antibiotic. As no clinical breakpoints have been established for Azm, the genes encoding resistance to this antimicrobial agent were sought in those isolates with an Azm inhibitory diameter zone of ≤15 mm.[11] In all cases, the amplified products were visualized in 1.5% agarose gels containing 5% of SYBR® Safe Gel Stain (Invitrogen, Eugene, OR, USA). To determine the specific *dfr* genes, the amplified products were analysed by restriction fragment length polymorphism (RFLP) analysis as previously described.[29]

In all cases, *E. coli* positive controls from bacterial collection of the Centre de Recerca en Salut Internacional de Barcelona (Barcelona, Spain) were used.

Statistical analysis

Results were analysed using EpiInfo version 3.4.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA). The χ^2 test or Fisher's exact test was used for comparisons between groups, as appropriate. Differences were considered statistically significant when $p<0.05$.

Results

A high proportion of ETEC isolates were resistant to Amp (64%), Sxt (52%), and Tet (37%). Only 10% of all ETEC isolates were resistant to Nal, while resistance rates for the other antibiotics evaluated were ≤10%. No isolate was resistant to Cip or Ctx. Thirty-nine percent (79/205) of the isolates were multidrug-resistant. ETEC-*st* isolates were more resistant than ETEC-*lt-st* to Amp (74% vs 47%, $p<0.01$), Sxt (62% vs 33%, $p<0.01$) and Tet (34% vs 16%, $p<0.001$), whereas ETEC-*lt-st* isolates presented higher resistance to Nal than ETEC-*lt* isolates (21% vs 7%, $p<0.05$) (Table 2). ETEC-*st* and ETEC-*lt* isolates (48% and 40%, respectively) presented with high rates of multidrug resistance than ETEC-*lt-st* (21%), $p<0.05$.

The rates of antibiotic resistance of Cohort 1 (n=83) were significantly more resistant to Sxt (61% vs 46%, $p<0.05$) and to Amc (18% vs 2%, $p<0.001$) when compared with those of Cohort 2 (n=122). Cohort 1 also had higher resistance rates than Cohort 2 for most commonly-used antibiotics such as Amp (71% vs 59%), Tet (41% vs 34%) and Nit (13% vs 5%) (Table 2). ETEC isolates from Cohort 1 tended to be more multiresistant than Cohort 2 (41% vs 37%), however this difference was not statistically significant. Although no evidence of ESBLs was observed, 1% of strains (three isolates) were resistant to Caz. Resistance to three or more antibiotics was more commonly detected in ETEC strains from Cohort 1 than those of Cohort 2 (47% vs. 32%, respectively, $p<0.05$). The most common multi-drug resistance phenotype was Amp^R Sxt^R Tet^R present, with or without resistance to other antibiotics (57 out 205 strains, 27.8%) (Table 3).

The distribution of the growth inhibitory zones to Azm varied between Cohort 1 and Cohort 2, with inhibitory zone diameters less than 10 mm in 5% and 2%; 11–15 mm in 46% and 24%; 16–20 mm in 18% and 68%; and more than 21 mm in 31% and 6%, respectively.

Among all ETEC isolates, antibiotic resistance was related mainly to the presence of $bla_{TEM\text{-}like}$ β-lactamases (91/131, 69.5%) for Amp; *dfrA15* gene (14/107, 13.1%) for trimethoprim, *sul2* gene (73/107, 68.2%) for sulfamethoxazole, *tet*(A) gene (37/76, 49%) for Tet, and *cat* gene (10/14, 71%) for Chl resistance (Table 4). Seventy-three out of 205 isolates (36%) had Azm inhibitory diameter zones of less than 15 mm, and 5% (4/73) of those were related to the presence of *mph*(A).

Table 2. Antibiotic resistance rates of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from Peruvian children

Antibiotic	No. (%) of ETEC strains									
	Cohort 1			Cohort 2			Toxin type			All strains
	Diarrhoea (n=57)	Control (n=26)	Total (n=83)	Diarrhoea (n=63)	Control (n=59)	Total (n=122)	LT (n=99)	ST (n=63)	LT-ST (n=43)	(n=205)
Amp	42 (74)	17 (65)	59 (71)[a]	36 (57)	36 (61)	72 (59)[a]	73 (74)	38 (60)[c]	20 (47)[c]	131 (64)
Sxt	35 (61)	16 (62)	51 (61)	29 (46)	27 (46)	56 (46)	61 (62)	32 (51)[c]	14 (33)[c]	107 (52)
Tet	22 (39)	12 (46)	34 (41)	20 (32)	22 (37)	42 (34)	34 (34)	35 (56)[c]	7 (16)[c]	76 (37)
Amc	11 (19)	4 (15)	15 (18)[b]	1 (2)	2 (3)	3 (2)[b]	12 (12)	3 (5)	3 (7)	18 (9)
Nit	8 (14)	3 (12)	11 (13)	4 (6)	2 (3)	6 (5)	5 (5)	12 (19)	0	17 (8)
Nal	4 (7)	3 (12)	7 (8)	7 (11)	7 (12)	14 (11)	7 (7)[a]	5 (8)	9 (21)[a]	21 (10)
Chl	2 (4)	3 (12)	5 (6)	5 (8)	4 (7)	9 (7)	11 (11)	2 (3)	1 (2)	14 (7)
Caz	2 (4)	0	2 (2)	0	1 (2)	1 (1)	2 (2)	0	1 (2)	3 (1)
Gm	0	0	0	0	1 (2)	1 (1)	1 (1)	0	0	1 (0)
Multiresistant	22 (39)	12 (46)	34 (41)	24 (38)	21 (36)	45 (37)	40 (40)[a]	30 (48)[a]	9 (21)[a]	79 (39)

Amc: amoxicillin-clavulanic acid; Amp: ampicillin; Caz: ceftazidime; Chl: chloramphenicol; Gm: gentamicin; LT: heat-labile toxin; Nal: nalidixic acid; Nit: nitrofurantoin; ST: heat-stabile toxin; Sxt: cotrimoxazole; Tet: tetracycline. No isolate was resistant to ciprofloxacin or cefotaxime.
[a] $p<0.05$.
[b] $p<0.001$.
[c] $p<0.01$.

Table 3. Antibiotic resistance patterns for the enterotoxigenic *Escherichia coli* (ETEC) strains isolated from Peruvian children

Pattern	MDR resistance patterns	Resistant ETEC isolates, n (%)				
		Cohort 1 (n=83)	Cohort 2 (n=122)	Diarrhoea (n=120)	Control (n=85)	All strains (n=205)
I	Amp^R Caz^R Amc^R Sxt^R Tet^R Nit^R	0	1 (1)	0	1 (1)	1 (0.5)
II	Amp^R Amc^R Sxt^R Tet^R Nit^R	3 (4)	0	3 (3)	0	3 (1.5)
III	Amp^R Nal^R Gm^R Sxt^R Tet^R	0	1 (1)	0	1 (1)	1 (0.5)
IV	Amp^R Sxt^R Tet^R Nit^R	5 (6)	2 (2)	6 (5)	1 (1)	7 (3.4)
V	Amp^R Nal^R Sxt^R Tet^R	1 (1)	4 (3)	1 (1)	4 (5)	5 (2.4)
VI	Amp^R Amc^R Sxt^R Tet^R	3 (4)	1 (1)	1 (1)	3 (4)	4 (2.0)
VII	Amp^R Sxt^R Tet^R Chl^R	0	3 (2)	2 (2)	1 (1)	3 (1.5)
VIII	Nal^R Sxt^R Tet^R Chl^R	2 (2)	0	0	2 (2)	2 (1.0)
IX	Amp^R Caz^R Amc^R Sxt^R	1 (1)	0	1 (1)	0	1 (0.5)
X	Amp^R Amc^R Tet^R Chl^R	1 (1)	0	0	1 (1)	1 (0.5)
XI	Amp^R Sxt^R Tet^R	14 (17)	19 (16)	18 (15)	15 (18)	33 (16.1)
XII	Amp^R Nal^R Sxt^R	2 (2)	4 (3)	5 (4)	1 (1)	6 (2.9)
XIII	Amp^R Amc^R Sxt^R	5 (6)	0	4 (3)	1 (1)	5 (2.4)
XIV	Amc^R Tet^R Chl^R	1 (1)	2 (2)	3 (3)	0	3 (1.5)
XV	Amp^R Sxt^R Nit^R	0	2 (2)	2 (2)	0	2 (1.0)
XVI	Amp^R Amc^R Chl^R	1 (1)	0	1 (1)	0	1 (0.5)
NA	Resistance to three or more antibiotics (MDR)	39 (47)[a]	39 (32)[a]	47 (39)	31 (36)	78 (38)
NA	Resistance to two antibiotics	21 (25)	25 (20)	28 (23)	18 (21)	46 (22.4)
NA	Resistance to only one antibiotic	6 (7)	22 (18)	14 (12)	14 (16)	28 (13.7)
NA	Pan-susceptible	17 (20)	36 (30)	31 (26)	22 (26)	53 (25.9)

Amc: amoxicillin-clavulanic acid; Amp: ampicillin; Caz: ceftazidime; Chl: chloramphenicol; Gm: gentamicin; MDR: multidrug resistance; NA: not applicable; Nal: nalidixic acid; Nit: nitrofurantoin; Sxt: cotrimoxazole; Tet: tetracycline.
[a] $p<0.05$

The presence of antibiotic resistance mechanisms was also analysed by source (Cohort 1 vs Cohort 2). All studied molecular mechanisms of resistance present in ETEC from Cohort 2 were also present in the strains from Cohort 1, except for *dfrA5*, *dfrA14* and *dfrA17* genes conferring trimethoprim resistance and $bla_{CARB\text{-}like}$ β-lactamase conferring Amp resistance (Table 4). The $bla_{TEM\text{-}like}$ genes (83% vs 53%, $p<0.001$) and *tet*(B) gene (52% vs 24%, $p<0.05$) were more frequently detected in Cohort 2-strains than in Cohort 1-strains, respectively ($p<0.05$). Additionally, in other cases, as account for *sul2*, a clear trend toward higher presence in Cohort 2 was also observed, but under significance breakpoint. The concomitant presence of multiple resistance mechanisms of resistance affecting the same antibacterial family was detected in a subset of cases, most often from Cohort 2 (Table 4).

No $bla_{SHV\text{-}like}$, $bla_{OXA2\text{-}like}$ and $bla_{OXA5\text{-}like}$ genes were detected. Similarly, we did not detect any isolates with *cmlA, dfrA6, dfrA8, dfrA12, dfrA13, dfrA15b, dfrA16,* or *dfrA16b* genes.

Significant proportions of antibiotic-resistant strains did not exhibit any of the resistance genes tested. These isolates predominated in Cohort 1, in which a significant number of isolates did not have identifiable mechanisms of resistance to trimethoprim ($p<0.05$), tetracycline, or β-lactam agents ($p=0.0001$ in both cases). In Cohort 1, only 58% of Amp-resistant strains had at least one of the β-lactamase genes identified, while 68% of Tet-resistant strains had at least one specific mechanism of resistance found (Table 4). A small proportion of Sxt-resistant isolates possessed at least one associated resistance mechanisms (29%) (Figure 1). In Cohort 2, the majority of the Amp-resistant isolates had at least one β-lactamase gene detected (92%), while all Tet-resistant isolates had the *tet*(A) and/or *tet*(B) genes. Only 39% of Sxt-resistant isolates in Cohort 2 possessed any of the Sxt-resistance genes under evaluation.

Discussion

Antibiotics are often used in patients with severe enteritis, dysentery and persistent diarrhoea.[30] However, in paediatric diarrhoea, the risks and benefits of antibiotic use are not fully defined because of the multiple bacterial and viral agents implicated in childhood infections and the inability to distinguish between pathogens on clinical grounds. In addition, conventional laboratory diagnostics are limited in their ability to detect many common enteropathogens, particularly ETEC and other DEC. As such, it has been difficult to study the effect of antimicrobials in children with specific pathogens such as ETEC. This often results in widespread empiric use of antimicrobial agents which drive towards antibiotic resistance. Antimicrobial resistance in Peru is currently a great challenge, related in part to the high rates of antibiotic

Table 4. Mechanisms of resistance of enterotoxigenic *Escherichia coli* (ETEC) isolates from Peruvian children

Antimicrobial	Mechanism of resistance	No. of isolates with indicated mechanism of resistance/total no. of resistant isolates (%)				
		Cohort 1	Cohort 2	Diarrhoea	Control	All strains
Ampicillin[a]	$bla_{TEM\text{-}like}$	31/59 (53)[b]	60/72 (83)[b]	53/78 (68)	38/53 (72)	91/131 (69)
	$bla_{OXA1\text{-}like}$	3/59 (5)	8/72 (11)	7/78 (9)	4/53 (8)	11/131 (8)
	$bla_{CARB\text{-}like}$	0/59 (0)	2/72 (3)	1/78 (1)	1/53 (2)	2/131 (2)
	Non determined	25/59 (42)[c]	6/72 (8)[c]	20/78 (26)	11/53 (21)	31/131 (24)
Chloramphenicol	*cat*	3/5 (60)	7/9 (78)	6/7 (86)	4/7 (57)	10/14 (71)
	floR	1/5 (20)	1/9 (11)	1/7 (14)	1/7 (14)	2/14 (14)
	Non determined	1/5 (20)	1/9 (11)	0/7 (0)	2/7 (29)	2/14 (14)
Tetracycline[d]	*tet(A)*	15/34 (44)	22/42 (52)	21/42 (50)	16/34 (47)	37/76 (49)
	tetB	8/34 (24)[e]	22/42 (52)[e]	17/42 (40)	13/34 (38)	30/76 (39)
	Non determined	11/34 (32)[c]	0/52 (0)[c]	5/42 (12)	6/34 (18)	11/76 (14)
Sulfamethoxazole[f]	*sul2*	30/51 (59)[g]	43/56 (77)[g]	44/64 (69)	29/43 (67)	73/107 (68)
	sul1	13/51 (25)	13/56 (23)	14/64 (22)	12/43 (28)	26/107 (24)
	sul3	1/51 (2)	0/56 (0)	1/64 (2)	0/43 (0)	1/107 (1)
	Non determined	9/51 (18)	5/56 (9)	7/64 (11)	7/43 (16)	14/107 (13)
Trimethoprim[h]	*dfrA1*	4/51 (8)	7/56 (13)	8/64 (13)	3/43 (7)	11/107 (10)
	dfrA15	8/51 (16)	6/56 (11)	8/64 (13)	6/43 (14)	14/107 (13)
	dfrA7	2/51 (4)	5/56 (9)	4/64 (6)	3/43 (7)	7/107 (7)
	dfrA5	0/51 (0)	2/56 (4)	2/64 (3)	2/43 (5)	4/107 (4)
	dfrA14	0/51 (0)	3/56 (5)	2/64 (3)	1/43 (2)	3/107 (3)
	dfrA17	0/51 (0)	2/56 (4)	0/64 (0)	2/43 (5)	2/107 (2)
	Non determined	38/51 (75)[c]	31/56 (55)[c]	41/64 (64)	27/43 (63)	68/107 (64)
Azithromycin	*mph*(A)	1/42 (2)	3/31 (10)	2/29 (7)	2/44 (5)	4/73 (5)
	Non determined	41/42 (98)	28/31 (90)	27/29 (93)	42/44 (95)	69/73 (95)

[a] Two isolates from Cohort 2 presented $bla_{TEM\text{-}like}$ and $bla_{OXA1\text{-}like}$ concomitantly, also both $bla_{CARB\text{-}like}$ were found concomitantly with $bla_{TEM\text{-}like}$.
[b] $p<0.001$.
[c] $p=0.0001$.
[d] Two isolates from Cohort 2 possessed both the *tet(A)* and *tet(B)* genes.
[e] $p<0.05$.
[f] Seven isolates (2 from Cohort 1, and 5 from Cohort 2) presented the *sul1* and *sul2* genes concomitantly.
[g] $p=0.061$.
[h] the *dfrA7* gene was found concomitantly with *dfrA1* gene (1 case) and *dfrA15 gene* (1 case).

use as well as inadequate dosing and inappropriate antibiotic selection for a given infection.[31] This situation may be extrapolated to most other low and middle income countries, each one with its particularities.

Antibiotic resistance was widespread among analysed ETEC strains, mainly to common antibiotics such as Amp (64%) and Sxt (52%). This finding is similar to descriptions of children in different countries, such as Egypt (63% and 52%), Mexico (73% and 65%) and Mozambique (46% and 61%) or to that described in international travellers (63% in both cases).[7,18,32,33] These antimicrobial agents had been largely used for treatment of diarrhoea in children for years,[34] and despite the aforementioned general high resistance rates, they remained in common use in many countries due to the lack of available alternatives. EUCAST criteria (http://www.eucast.org/clinical_breakpoints/), which are based on clinical parameters and epidemiological cut-off values, generally use lower breakpoints to classify Enterobacteriaceae as resistant, especially for β-lactam antibiotics and fluoroquinolones. However, the application of EUCAST criteria does not alter the quantified level of antibiotic resistance described in terms of zone inhibition. Qualitatively, only a few isolates in our study would be re-classified as resistant to a given antimicrobial agent, mainly due to the fact that relatively few microorganisms classified as susceptible in our study had borderline-to-intermediate levels of susceptibility.

This is a worrisome finding because ETEC isolates, and especially ETEC-*st*, are among the diarrhoeagenic pathogens most associated with child mortality worldwide.[2,5] In the present study and in accordance with that described by different authors,[35,36] the most commonly detected β-lactamases encoding genes were the $bla_{TEM\text{-}like}$ genes, sometimes co-existing with other β-lactamases such as $bla_{OXA1\text{-}like}$ or $bla_{CARB\text{-}like}$. Meanwhile different *dfr*A and *sul* genes were identified.

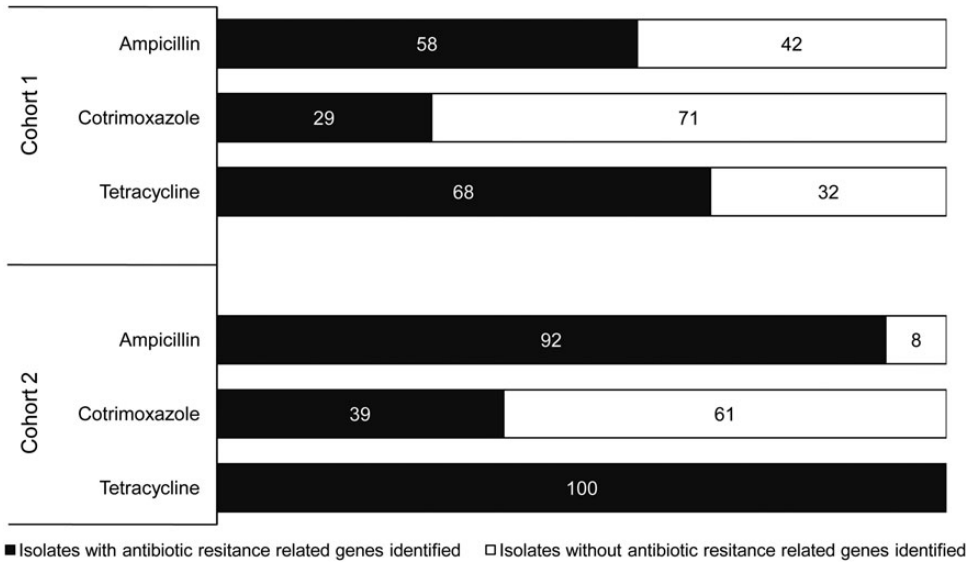

Figure 1. Presence of antimicrobial resistance-related genes. Percentages of resistant ETEC isolates presenting at least one of the analysed mechanisms of antibiotic resistance for each included antibiotic family. For Sxt it is considered the concomitant presence of at least one gene related to sulfamethoxazole resistance and at least another for trimethoprim resistance. In black, presence of antibiotic resistance genes. In white, absence of antibiotic resistance genes.

Based on our findings, these high levels of resistance should preclude the empiric use of Amp or Sxt if ETEC is suspected or confirmed. Different alternatives may be considered, including fluoroquinolones, cephalosporins, macrolides or furazolidone.

Cip remains appropriate as empiric therapy in adults, and its safety and utility in children with enteric infections has been previously shown.[37,38] However, the 10% of strains resistant to Nal observed in this study, and the 68% and 28% of resistance to Nal and Cip, respectively, detected in commensal *E. coli* strains isolated from Peruvian children, highlight the importance of ongoing surveillance.[39] Given the additive effect of fluoroquinolone resistance mechanisms, the presence of Nal-resistance is a risk factor for the development of complete fluoroquinolone resistance and subsequent treatment failures.[40,41] Of additional concern is the spread of different transferable mechanisms of quinolone resistance,[13,42] which may facilitate the development of fluoroquinolone resistance in absence of previous detected Nal resistance.[41]

Our results show that cephalosporins remain highly active against ETEC isolates. Only three isolates showed resistance to Caz and none to Ctx. Although no ESBL production was detected by double disk diffusion tests in our study, high rates of ESBLs (in excess of 75%) have been described among *Enterobacteriaceae*, including pathogenic *E. coli*, in other clinical syndromes in Peru.[43] In the present study, in the absence of ESBLs, Caz resistance may be secondary to other mechanisms such as overexpression of efflux pumps, or, more probably, to the presence of plasmid-encoded AmpC. The ability of AmpC to hydrolyse cephalosporins is not uniform across the class and may result with resistance to some specific cephalosporins, with others retaining activity. An analysis of the effect of plasmid-encoded AmpC showed that 33% and 50% of *Enterobacteriaceae* isolates carrying AmpC were Ctx-susceptible and -intermediate respectively, while only 16% of them were susceptible or intermediate to Caz.[44]

The situation of Azm is also worrisome, with a high number of isolates with a halo diameter lower than 15 mm. However, our analysis of the most prevalent transferable mechanisms of resistance showed the presence of only four isolates carrying the *mph*(A) gene, the most extensively described worldwide among *Enterobacteriaceae*.[45,46] This may be partially explained by the problems related to the use of Azm disks, as previously described.[47] The presence of a number of isolates in which no halo was observed confirms the presence of Azm-resistance in the ETEC isolates circulating in the study area, which may be related to target alterations, other transferable mechanisms of resistance (http://faculty.washington.edu/marilynr/), or the overexpression of efflux pumps.[48]

Low resistance levels to Nit were observed. Despite concerns about the carcinogenic potential of nitrofurans, furazolidone is considered acceptable for the treatment of paediatric diarrhoea in Peru and other Latin American countries.[49,50] These data, together with the low frequency of selection of furazolidone-resistant mutants,[51] show its utility in clinical usage.

In most low and middle income countries, enteric co-infections may be the rule rather than the exception. Moreover, asymptomatic carriage of diarrhoeagenic pathogens, as described in the present study, is common.[52] With the high sensitivity of current molecular diagnostic techniques,[53] co-infections (or co-colonization) may be more frequently identified. In certain cases, quantitative measurement of pathogen DNA in stools may clarify the specific aetiology of a particular case of diarrhoea.[54] The high rate of asymptomatic carriage in developing countries highlights the need to include samples from apparent healthy people in epidemiological studies,[55] as well as the need for a greater understanding of the role of the general microbiome of symptomatic and asymptomatic hosts alike.

Overall, the study showed a wide variety of transferable antibiotic-resistance mechanisms among isolates. The ease with which ETEC may acquire antibiotic resistance mechanisms

highlights the need for stricter control of antimicrobial agents in order to maximize the benefits of antibiotic therapies when needed.

Both cohorts showed differences in the rates of antimicrobial resistance. Interestingly, we were unable to detect specific antibiotic resistance mechanisms in some of the strains analysed. In the case of Sxt, the overall high number of isolates from both cohorts in which no detectable *dfrA* gene was found, suggests that the most frequent mechanisms for Sxt resistance in the region of Lima remain undetected. In the case of Tet and Amp, the resistant isolates in which no mechanisms of resistance were detected were limited to Cohort 1. This may reflect differences in the nature of the antibiotic pressure which is exerted over microorganisms on both studied areas. Interestingly, high levels of resistance to rifaximin mediated by the overexpression of efflux pumps have been described in the same area as Cohort 1.[56] Overexpression of efflux pumps may also be involved in the resistance to Amp and other antibiotics and may be induced by different bacterial stressors, including the presence of specific environmental toxics.[57]

A limitation of the study is that molecular mechanisms of antibiotic resistance were only studied in antibiotic-resistant or intermediate isolates but not in susceptible isolates; these could hypothetically act as a hidden antibiotic resistance reservoir while remaining unexpressed.

In conclusion, ETEC isolates in periurban Lima showed high rates of resistance to the most available and inexpensive antibiotics while remaining broadly susceptible to cephalosporins, furazolidone and fluoroquinolones. An effort to control the use of antimicrobial agents is needed in order to preserve their utility when necessary. Ongoing surveillance of antimicrobial resistance is also needed to detect the presence of isolates exhibiting resistance to first-line therapies.

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Authors' contributions: AMM and FPR contributed equally to this work. RCM, CFL, JR and TJO conceived the study; AMM, FPR, MJP, MR, CG, MB, RM, LH, EC-W, AIG develop all the laboratory procedures. AMM, FPR, TJO and JR analyse the data. AMM, FPR, TJO and HR drafted the manuscript. MJP, CG, TJO and JR critically revised the manuscript for intellectual content. All authors read and approve the final manuscript. TJO and JR are guarantors of the paper.

Funding: This work was partially supported by: Agencia Española de Cooperación Internacional para el Desarrollo (AECID), Spain, Programa de Cooperación Interuniversitaria e Investigación Científica con Iberoamérica [D/019499/08, D/024648/09, D/030509/10, and A1/035720/11] (JR and TJO); National Institute of Child Health and Human Development, USA [Public Health Service award R01-HD051716] (EC-W. and TJO); by the Spanish Network for the Research in Infectious Diseases [REIPI RD12/0015] (JR); and by Generalitat de Catalunya, Departament d'Universitats, Recerca i Societat de la Informació [2014 SGR 26] (JR); AMM attended a 3-month training through the CRESIB-UPCH agreement, training was partially funded by Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica (CONCYTEC, FONDECYT) in Peru. JR has a fellowship from the program I3, of the ISCIII [grant number CES11/012]. MJP has a postdoctoral fellowship from CONCYTEC/FONDECYT.

Competing interests: None declared.

Ethical approval: Not required.

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