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High Prevalence of *bla*$_{CTX-M}$ in Fecal Commensal *Escherichia coli* from Healthy Children

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ABSTRACT

Background: Antibiotic-resistant *Escherichia coli* can colonize the intestinal tract of healthy children, causing concern when antibiotic resistance is related to the presence of transferable mechanisms, such as extended-spectrum β-lactamases (ESBLs).
Materials and Methods: Fecal samples from 41 healthy children from two villages of rural Peru were cultured on ceftriaxone-disks. ESBL production was confirmed with double disk synergy. In all ESBL-produced isolates, antibiotic susceptibility to 12 antibacterial agents was established by disk diffusion, while clonal relationships were determined by repetitive extragenic palindromic-polymerase chain reaction (REP-PCR). Presence of ST131 was determined using PCR.
Results: Ceftriaxone-resistant microorganisms were recovered from 39 samples belonging to 22 out of 41 children (53.7%). Of these, 80 ceftriaxone-resistant and two ceftriaxone-intermediate *E. coli* from inside ceftriaxone-halos were confirmed as ESBL-producers. All isolates were multidrug-resistant. In 79/80 (98.8%) ceftriaxone-resistant isolates, the presence of *bla*$_{CTX-M}$ was detected alone (58 isolates, or together with other β-lactamase (*bla*$_{TEM}$, 17 isolates; *bla*$_{OXA-1-like}$, 3 isolates; *bla*$_{TEM}$ + *bla*$_{OXA-1-like}$, 1 isolate), while in one isolate no such ESBL was identified. The two ceftriaxone-intermediate isolates recovered from the same sample, carried a *bla*$_{TEM}$ and *bla*$_{SHV}$ respectively. Thirty-four different clones were identified, with 4 clones being recovered from different samples from the same child. Twelve clones were disseminated among different children, including 5 clones disseminated between both villages. Two clones, accounting for 3 isolates and both recovered from the same children, belonged to *E. coli* ST131.
Conclusion: This study demonstrates high prevalence of ESBL-carriers among healthy children living in a rural area of Peru, stressing the need for continuous surveillance and search for public health control measures.

Keywords: Antibiotic-resistance; Commensal; Extended-spectrum β-lactamases; *Escherichia coli*; Healthy carriers

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Conflict of Interest
No conflict of interest.

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INTRODUCTION

The increasing levels of antibacterial resistance are one of the most relevant human health challenges of the present century. This phenomenon affects pathogenic microorganisms leading to human and/or animal infections, and also is extended to commensal inhabitants of human/animal microbiotas and environmental microorganisms [1-5]. This finding has been related to a series of factors such as the widespread use of antibacterial agents which are often misused and abused, as well as to the fragile sanitary conditions present in different geographical areas [6-8].

The gastrointestinal microbiota plays a crucial role as reservoir of antibiotic-resistant genes. *Escherichia coli* is a relevant member of this microbiota, often exhibiting resistance to antibiotics, mostly related to the induction of chromosomal mutations leading to antibiotic resistance or the acquisition of transferable antibiotic-resistant genetic determinants [4, 9-11], this later may be transferred to pathogenic bacteria in the course of infections or transient asymptomatic colonization [12, 13]. Among transferable antibiotic-resistant determinants, the current widespread of extended-spectrum β-lactamases (ESBLs) is involved in the acquisition of resistance to 3th and 4th generation cephalosporins, such as ceftazidime and cefepime. These enzymes are often encoded in genetic structures carrying other antibiotic-resistant determinants producing multidrug-resistant (MDR) or extended-resistant phenotypes (XDR) [14, 15]. Currently, a great variety of ESBL encoding genes have been described, but only a few ESBL families account for the majority of the descriptions, with *bla*$_{CTX-M}$ being the most prevalent in recent years and *bla*$_{CTX-M-15}$ being the most disseminated worldwide [16-18].

In Peru, a high frequency of ESBL-carrying pathogens have been reported in patients admitted to hospitals [2, 3] but data about the ESBL prevalence among healthy people is scarce, underreported and mostly outdated [19, 20].

Therefore, the aim of the present study was to determine the presence of ESBL-carriers among healthy children from a rural area of Peru.

MATERIALS AND METHODS

1. Samples

Fecal samples from 41 healthy children 10 - 20 months of age were collected in Calzada (19 children) and Yantalo (22 children), 2 rural districts from Moyobamba (Jungle area of Peru), between April and June 2015. The sampling was performed every 15 days.

2. Ethics statement

The study was approved by the Ethical Review Board of the University of Texas Health Science Center at Houston (HSC-SPH-14-0261) and Universidad Peruana Cayetano Heredia (UPCH) (63407). Written informed consent was obtained from the parent or guardian of each child.

3. Microorganisms

The feces were directly cultured in McConkey agar No 3 (Oxoid, Thermo Scientific, Basingstone, United Kingdom) in the presence of a ceftriaxone disk (CRO, 30 μg), in accordance with Bartoloni *et al* [21]. A maximum of 3 colonies presumptive for *E. coli* were collected from

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inside ceftriaxone halos. Thereafter, selected microorganisms were preliminary identified by biochemical tools [22], and confirmed by polymerase chain reaction (PCR) amplification of the *uidA* gene [23]. For PCR controls, the *E. coli* ATCC 25922 was used as the positive, an *Enterobacter* spp. from the internal UPCH collection was used as the negative, and distilled water was used as the blank control.

4. Antibiotic susceptibility

In all selected microorganisms, the antibiotic susceptibility to CRO was confirmed by disk diffusion test. In those isolates confirmed as CRO-intermediate/resistant, the susceptibility to ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), aztreonam (ATM, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX, 30 µg), meropenem (MEM, 10 µg), chloramphenicol (CHL, 30 µg), trimethoprim-sulfamethoxazole (TMP/SMX, 25 µg), nalidixic acid (NAL, 30 µg) and ciprofloxacin (CIP, 5 µg) was also assessed following the same methodology and in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines [24].

5. Phenotypic detection of production of ESBL

In all isolates, when resistance to ceftriaxone was confirmed, the presence of ESBL was determined by the double disk methodology. A subset of 21 ESBL-positive isolates was confirmed by measuring the differences in the halos of CAZ and CAZ/CAZ-AMC, CTX and CTX/CTX-CLA pairs [25].

6. Detection of β-lactamases

The presence of *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA-1}$-like was determined by PCR using primers and conditions previously described [26]. The *E. coli* JP27 (*bla*$_{CTX-M}$) and *Klebsiella pneumoniae* Sb108 (*bla*$_{OXA-1}$, *bla*$_{TEM}$ and *bla*$_{SHV}$) from internal UPCH bacterial collection were used as positive controls, while *E. coli* ATCC25922 was used as negative control.

7. REP-PCR (Repetitive Extragenic Palindromic - PCR)

Clonal relationships of ESBL-producing *E. coli* were determined by REP-PCR using the primer (5'-GCG CCG ICA TGC GGC ATT- 3') and conditions described by Riveros *et al* [27]. The PCR products were resolved in 1.5% agarose gel and stained with 2% ethidium bromide.

The band patterns were analyzed through the GelJv2.0 software (https://sourceforge.net/projects/gelj/) [28]. Clonal relationships were established using the DICE coefficient, while clustering was based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with a 1.2% tolerance in band position differences. The isolates were considered to belong to the same epidemiological group when the profiles showed ≥80% of identity [15]. The clones were arbitrarily named using Roman numbers.

8. Detection of ST131 clone

The presence of *E. coli* ST131 was sought in one isolate representative for each one of the different ESBL genotypes belonging to each REP-PCR pattern. Thus, a PCR using the primers (5'-AGCAACGATATTTGCCCATT-3' and 5'-AGCAACGATATTTGCCCATT -3'), and following the methodology previously described by Matsumura *et al*, was performed [29]. The ST131 uropathogenic *E. coli* U255, isolated in a previous study was used as positive control [30], while *E. coli* ATCC 25922 was used as negative control.

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RESULTS

A total of 167 samples of feces were collected, of these 17 were discharged because lack of grown. The presence of microorganisms presumptive for *E. coli* being detected inside CRO halos in 26.0% cases (39/150) belonging to 53.66% (22/41) children. Overall, 112 bacteria isolates were collected. Of these, 82.1% (92/112) were confirmed to be *E. coli* by both phenotypic and molecular methods. These *E. coli* were recovered from 38 of the selected samples, Meanwhile 16% (18/112) were identified as *K. pneumoniae*, and 1 non-fermenter bacteria remained unidentified. The remaining microorganism was unable to grown after pick-up.

The 86.9% (80/92) of the *E. coli* were confirmed as CRO-resistant and 2.2% (2/92) were CRO-intermediate. The remaining 10.9% (10/92) *E. coli*, despite being isolated from inside CRO disk halos, were susceptible to CRO. These CRO-resistant/intermediate isolates were detected in 92.3% (36/39) of the selected samples. Thus, in 17 samples were recovered, 51 CRO-resistant *E. coli* isolates (3 *E. coli*/sample), while 22 CRO-resistant *E. coli* were recovered from 11 samples (2 *E. coli*/sample) and for each of the remaining seven samples were recovered one CRO-resistant *E. coli*. Furthermore, two CRO-intermediate isolates were recovered from the same sample. At least one CRO-resistant/intermediate isolate was recovered from the feces of 95.4% (21/22) children included in the study.

Phenotypically, all CRO-resistant and intermediate isolates were ESBL-producers. When the ESBLs were determined by molecular tools, all CRO-resistant isolates carried a *bla*$_{CTX-M}$ alone (59 isolates) or together other β-lactamase (*bla*$_{TEM}$, 17 isolates; *bla*$_{OXA-1}$*-like*, 3 isolates; *bla*$_{TEM}$ + *bla*$_{OXA-1}$-like, 1 isolate). Regarding the two intermediate isolates, one of them carried a *bla*$_{TEM}$, and another carried a *bla*$_{SHV}$.

Regarding antibiotic resistance, the ESBL-producing isolates showed high levels of resistance to AMP (82 isolates, 100%), CTX (80 isolates, 97.6%), ATM (54 isolates, 65.9%), FEP (36 isolates, 43.9%), and CAZ (33 isolates, 40.2%). In addition, a high number of cephalosporin and AMC intermediate isolates was also observed. In addition to β-lactam related antibiotics, the isolates showed high levels of resistance to NAL (67 isolates, 81.7%), TMP/SMX (55 isolates, 67.1%) and CIP (41 isolates, 50%). The isolates only were susceptible to MEM (**Table 1**). Of note, all ESBL isolates showed MDR.

The clonal analysis of ESBL-producing *E. coli* showed a total of 34 different REP-PCR patterns. In 13 out of 21 children (61.9%) with ESBL-producing *E. coli* isolates, two or more different clones were observed, either in the same or in different samples. On the other side, four out of ten (40.0%) patients from which two or more samples were available, had the same *E. coli* clone recovered at different sampling times. While several clones were only recovered from the feces of one child, 12 clones were recovered from at feces of at least two children, with clones IX and III being recovered from the feces of five and four of the children respectively.

Of note, while most of the clones seems to be specific for each village, five of them, the clones II, III, IX, XII and XVIII were recovered from feces of children of both villages (**Tables 2**, **3**). Finally, the results showed the presence of isolates belonging to the same REP-PCR pattern with different β-lactamase patterns.

The presence of *E. coli* ST131 was established, with the isolates representative for the clones XIV and XXI showing a positive result, both recovered from the same children at different times.

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Table 1. Antibiotic resistance patterns in 82 ESBL-producing *Escherichia coli* from healthy children living in a rural area of Peru

ATB	ESBL-producing *E. coli* (N = 82)		
	R	I	S
	N (%)	N (%)	N (%)
AMP	82 (100.0)	0 (0.0)	0 (0.0)
CRO	80 (97.6)	2 (2.4)	0 (0.0)
CTX	80 (97.6)	2 (2.4)	0 (0.0)
NAL	67 (81.7)	0 (0.0)	15 (18.3)
TMP/SMX	55 (67.1)	2 (2.4)	25 (30.5)
ATM	54 (65.9)	14 (17.1)	14 (17.1)
CIP	41 (50.0)	3 (3.7)	38 (46.3)
FEP	36 (43.9)	43 (52.4)	3 (3.7)
CAZ	33 (40.2)	23 (28.0)	26 (31.7)
CHL	20 (24.4)	0 (0.0)	62 (75.6)
AMC	4 (4.9)	22 (26.8)	56 (68.0)
FOX	1 (1.2)	5 (6.1)	76 (92.7)
MEM	0 (0.0)	0 (0.0)	82 (100.0)

ESBL, extended-spectrum β-lactamase; ATB, antibiotic; R, resistant; I, intermediate; S, susceptible; AMP, ampicillin; CRO, ceftriaxone; CTX, cefotaxime; NAL, nalidixic acid; TMP/SMX, trimethoprim-sulfamethoxazole; ATM, aztreonam; CIP, ciprofloxacin; FEP, cefepime; CAZ, ceftazidime; CHL, chloramphenicol; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; MEM, meropenem.

DISCUSSION

In hospital admitted patients, the asymptomatic carriage of ESBL-producing microorganisms has been related to increased number of acquired antibiotic-resistant infections, longer hospitalizations, and worse outcomes [31-33]. Further, the presence of ESBL-carriers may become the hospital entry backdoor of ESBL microorganism, or ESBL-carrying genetic structure, which may be disseminate throughout the hospital on medical and personal devices, eventually colonizing different hospital wards [32]. These introduced ESBL-producing microorganisms may cause nosocomial infections or transfer the ESBL-carrying genetic structure to local nosocomial pathogens, which therefore will increase their antibiotic resistance armamentarium, exacerbating treatment difficulties. Most of these microorganisms belong to *Enterobacteriales* such as *E. coli* or *K. pneumoniae*, which are usual inhabitants of gut tract [16]. This scenario highlights the importance to establish community rates of gut colonization by ESBL-producing microorganisms. In this scenario we have analyzed the carriage of ESBL in healthy children living in a rural area of the Peruvian jungle.

Two previous studies designed to evaluate the fecal carriage of antibiotic-resistant *E. coli* in children were conducted in the early 2000's in the same geographical area of Moyobamba, both using the same methodology followed in the present analysis [18, 19]. Thus, a 2002 sampling showed a very low prevalence (1 isolate, 0.1%) of ESBL in the area [1, 19], and a posterior study of samples collected in 2005 showed an increase in the fecal carriage of ESBL-producing *E. coli*, determined to be 1.5% [20]. In contrast, present results have shown extremely worrisome high prevalence of ESBL in this rural area of the Peruvian jungle. Despite the young age of the enrolled children (10 - 20 months of age), ESBL-producing *E. coli* were isolated from feces of >50% children included in the study. Most concerning was how all ESBL-producing isolates presented MDR, showing levels of non-susceptibility (resistance plus intermediate isolates) higher than 20% for all tested antibacterial agents excepting FOX and MEM. These findings, which highlights the exponential increase in the prevalence of ESBL in the area, are in agreement with the overall current high rates of antibiotic resistance, including cephalosporins, which has been described in recent studies performed in Peru

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Table 2. Clonal relationships among ESBL-producing *Escherichia coli* from healthy children living in a rural area of Peru

Children	Area[a]	Date[b]	Colony 1[c]		Colony 2		Colony 3	
			ESBL	REP[d]	ESBL	REP	ESBL	REP
1	C	April 10, 2015	CTX-M + TEM	VI				
1	C	May 22, 2015	CTX-M	XIX	CTX-M	III	CTX-M	XX
2	C	April 10, 2015	CTX-M	XXI	CTX-M	XVIII		
2	C	April 24, 2015	CTX-M+OXA-1	XIV	CTX-M+OXA-1	XIV	CTX-M+OXA-1	XII
3	Y	April 17, 2015	CTX-M + TEM	XII	CTX-M	XII	CTX-M	XII
4	C	April 17,2015	CTX-M	XIII	CTX-M	XIII	CTX-M	IX
4	C	June 09, 2015	CTX-M	II	CTX-M	XXII		
5	Y	April 24, 2015	CTX-M	XVIII	CTX-M	XVIII	CTX-M	XVIII
5	Y	May 08, 2015	CTX-M	XVIII	CTX-M	XVIII	CTX-M	XVIII
5	Y	May 22, 2015	CTX-M	XV	CTX-M	XV	CTX-M	XVII
5	Y	June 05, 2015	CTX-M	XVII	CTX-M	XVII	CTX-M	XVI
6	Y	April 24, 2015	CTX-M+TEM	XVI	CTX-M+TEM	XVII	CTX-M+TEM	XVII
6	Y	June 05, 2015	CTX-M+TEM	XVII	CTX-M+TEM	V	CTX-M+TEM	V
7	Y	April 24, 2015	CTX-M+TEM	V	CTX-M+TEM	XXIII		
7	Y	May 08, 2015	CTX-M+TEM	IV	CTX-M	IV		
7	Y	May 22, 2015	CTX-M	IV	CTX-M	IV	CTX-M	IV
7	Y	June 05, 2015	CTX-M	IV	CTX-M	III		
8	Y	April 24, 2015	CTX-M+TEM	VII	CTX-M	IX	CTX-M	XXIV
8	Y	May 22, 2015	CTX-M+TEM+OXA-1	XXV	CTX-M+TEM	XXVI	CTX-M	XXVII
8	Y	June 05, 2015	CTX-M	XXXIV				
9	Y	April 24, 2015	CTX-M	III	CTX-M	XVIII	CTX-M	VII
9	Y	May 08, 2015	CTX-M	VII	CTX-M	VII		
10	Y	May 08, 2015	CTX-M	I				
10	Y	May 22, 2015	CTX-M+ TEM	IX	CTX-M+TEM	II	CTX-M	VIII
11	Y	May 22, 2015	CTX-M+TEM	XXVIII	CTX-M	I		
11	Y	June 05, 2015	CTX-M	XXIX	CTX-M	XXX		
12	Y	June 05, 2015	CTX-M	III	CTX-M+TEM	III		
13	Y	June 05, 2015	CTX-M	IX	CTX-M	XXXI	CTX-M	IX
14	Y	June 09, 2015	CTX-M	XI	CTX-M	XI	CTX-M	VIII
15	Y	June 09, 2015	CTX-M	XXXIII	CTX-M	IX		
16	C	June 09, 2015	CTX-M	X	CTX-M	X		
17	C	June 13, 2015	TEM	VI	SHV	VI		
18	Y	April 24, 2015	CTX-M	XXXII				
19	Y	May 08, 2015	CTX-M	ND				
20	Y	May 08, 2015	CTX-M	ND				
21	C	June 09, 2015	CTX-M	ND				

[a]Town in which was collected the sample (C: Calzada; Y: Yantalo).
[b]Day in which the sample was taken.
[c]Samples with at least one colony displaying the presence of extended-spectrum β-lactamases.
[d]The REP-PCR patterns were arbitrarily named following Roman numbers.
ESBL, extended-spectrum β-lactamases; REP, repetitive extragenic palindromic; CTX-M, cefotaxime-hydrolyzing; OXA, oxacillin-hydrolyzing; SHV, sulfhydryl reagent variable; TEM, temoneira; ND, no determined; PCR, polymerase chain reaction.

focused on *E. coli* or other microorganisms [26, 34, 35]. In this regard, in Peru it has been observed rates of 73.3% of ESBL among *K. pneumoniae* causing neonatal sepsis and of 52.3% among *E. coli* causing bacteremia in children <5 years of age [3, 34]. Further, ESBL-producing *E. coli* dissemination though marketed food has also been shown. Thus, 59.4% of *E. coli* isolates recovered from marketed chicken meat was positive for ESBL in Peru [36].

From its first description in 1990 [37], *bla*$_{CTX-M}$ has become the most widely disseminated ESBL encoding genes, with *bla*$_{CTX-M-15}$ being the most frequently described worldwide, superseding other genes such as *bla*$_{TEM}$ or *bla*$_{SHV}$ [16-18]. In Peru, including the Moyobamba province, a high diversity of *bla*$_{CTX-M}$ has been reported with the presence of pathogenic, commensal, or environmental *E. coli* carrying *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-24}$, or *bla*$_{CTX-M-65}$ among others [20, 26, 38]. While in the present study no specific *bla*$_{CTX-M}$ were

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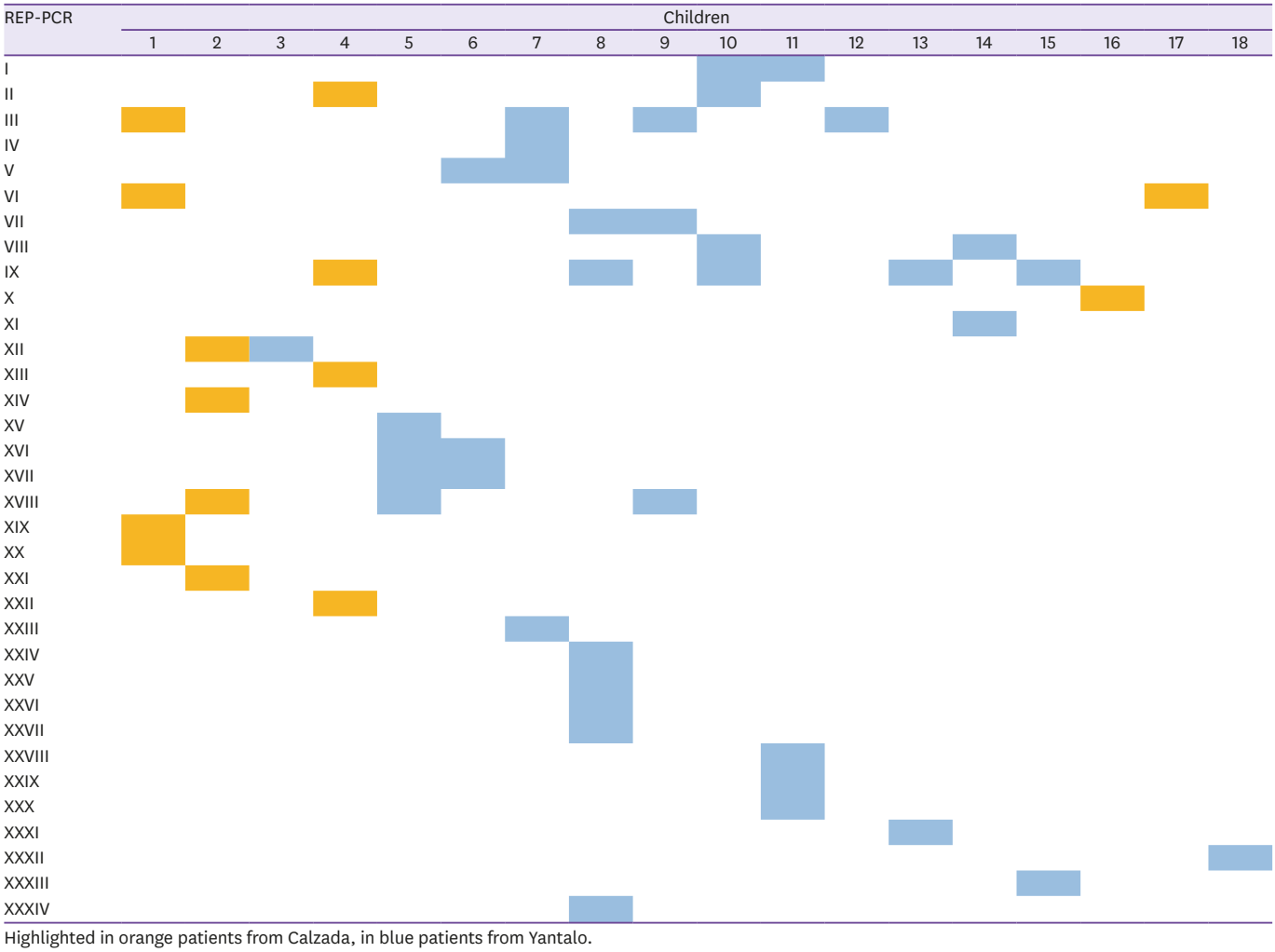
Table 3. Distribution of ESBL-clones



Highlighted in orange patients from Calzada, in blue patients from Yantalo.
ESBL, extended-spectrum β-lactamases; REP, repetitive extragenic palindromic; PCR, polymerase chain reaction.

determined, in agreement with above-mentioned wide dissemination, all CRO-resistant *E. coli* isolated in the present study carried *bla*$_{CTX-M}$.

Retrospective analysis has shown the presence of *E. coli* ST131 in Peru as early as 1968, and it has been associated with fluoroquinolone resistance and presence of CTX-M [39, 40]. Nevertheless, while the presence of *E. coli* ST131 is confirmed in the present series, only two clones accounting for three out of 82 isolates belong to this ST pattern.

In addition, two other β-lactamases were detected (*bla*$_{TEM}$ and *bla*$_{SHV}$ respectively) from CRO-intermediate isolates, which might underly this phenotype. While in the present study the most relevant ESBL families were sought, the number of unrelated β-lactamase encoding genes either ESBL or not is enormous [16]. Thereby, the presence of few unidentified ESBL should not be ruled out. Meanwhile, the presence of ESBL-producing *Enterobacteriaceae* susceptible or intermediate to cephalosporins has been observed [31].

While 40% of the 22 children from which ESBL-producing *E. coli* were recove red at different times become stably colonized by specific ESBL-producing *E. coli* clones, as shown by the

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isolation of the same clone at different sampling times, a high number of different ESBL-producing *E. coli* clones have been identified, demonstrating a high heterogeneity of ESBL-producing *E. coli* populations. This finding, together with the continuous increase and current high prevalence of ESBL-producing *E. coli* clones among healthy young children from the Moyobamba province, reflects a heavy external ESBL-selective pressure. This may be explained by several factors such as direct pressure exerted by an high use of cephalosporins in pediatric populations, although unlikely, or it could be related to the use of other antibiotics in pediatric populations, which might select specific genetic structures concomitantly carrying a mechanism of resistance for the antibiotic used but also a *bla*$_{CTX-M}$ gene or other ESBL encoding gene [14, 15]. Nonetheless, the most probable scenario is an acquisition of the ESBL in the family environment as previously suggested [20]. This possibility is supported by the fact that a few clones were recovered from the feces of different children, including children from both villages. It is possible these common clones were disseminated by adults traveling between both villages.

The main limitation of the study is the sample size. Despite this fact, the obtained results clearly highlight the magnitude of the problem, with ESBL-producing *E. coli* being detected in the feces of >50% of healthy children. Further, in those isolates carrying an identified ESBL, more than one ESBL may be present either co-amplifying with used primers (*e.g.* 2 different *bla*$_{CTX-M}$ genes) or remaining hidden (*i.e.* presence of an ESBL unable to be amplified with used primers). In this sense it is worth mentioning the presence of different isolates co-carrying *bla*$_{CTX-M}$ with other β-lactamases such as *bla*$_{TEM}$, *bla*$_{OXA-1}$-like or both concomitantly.

In summary, present results showed a worrisome prevalence of ESBL in feces of healthy children living in a rural area of Peru. These commensal *E. coli*, while non-pathogenic, are a severe health-risk because they may act as a hidden reservoirs of antibiotic resistant genes, such as ESBL. Further, if children need to be admitted to a health facility, these apparent innocent gut dwellers would contribute to the ESBL background of nosocomial pathogens by transferring the resistance determinants to hospital-resident microorganisms or cause nosocomial infections.

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