

## Diarrheagenic *Escherichia coli*: Prevalence and Pathotype Distribution in Children from Peruvian Rural Communities

Gonzalo J. Acosta,<sup>1†</sup> Natalia I. Vigo,<sup>1†</sup> David Durand,<sup>1</sup> Maribel Riveros,<sup>1</sup> Sara Arango,<sup>1</sup> Mara Zambruni,<sup>2</sup> and Theresa J. Ochoa<sup>1,3\*</sup>

<sup>1</sup>Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; <sup>2</sup>University of Texas Medical Branch, Galveston, Texas; <sup>3</sup>University of Texas Health Science Center at Houston, School of Public Health, Houston, Texas

**Abstract.** Diarrheagenic *Escherichia coli* (DEC) are common pathogens of childhood gastrointestinal infections worldwide. To date, research tracking DEC has mainly been completed in urban areas. This study aims to determine the prevalence and pathotype distribution of DEC strains in children from rural Peruvian communities and to establish their association with malnutrition. In this prospective cohort, 93 children aged 6–13 months from rural communities of Urubamba (Andes) and Moyobamba (jungle) were followed for 6 months. Diarrheal and control stool samples were analyzed using multiplex real-time polymerase chain reaction to identify the presence of virulence genes of DEC strains. The overall isolation rate of DEC was 43.0% (352/820). Enteroaggregative *E. coli* (EAEC, 20.4%), enteropathogenic *E. coli* (EPEC, 14.2%), and diffusely aggregative *E. coli* (DAEC, 11.0%) were the most prevalent pathotypes. EAEC was more frequently found in Moyobamba samples ( $P < 0.01$ ). EPEC was the only strain significantly more frequent in diarrheal than asymptomatic control samples ( $P < 0.01$ ). DEC strains were more prevalent among younger children (aged 6–12 months,  $P < 0.05$ ). A decline in height-for-age Z-score (HAZ) was observed in 75.7% of children overall. EAEC was more frequently isolated among children who had a greater HAZ decline ( $P < 0.05$ ). In conclusion, DEC strains were frequently found in stool samples from children in rural communities of the highlands and jungle of Peru. In addition, children with a greater decline in their growth rate had higher EAEC isolation rates, highlighting the importance of this pathogen in child malnutrition.

### INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) account for 30–40% of cases of childhood diarrhea,<sup>1</sup> one of the main causes of morbidity and mortality among children in developing countries.<sup>2</sup> DEC may also colonize the infant gastrointestinal tract and result in asymptomatic infection, which confounds diagnosis and treatment.<sup>3</sup>

DEC are classified into six groups or pathotypes based on their pathogenic mechanisms: enteroaggregative (EAEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), diffusely aggregative (DAEC), and shiga toxin-producing *E. coli* (STEC).<sup>3</sup> Although several analytic methods distinguish pathotypes, polymerase chain reaction (PCR) has the highest sensitivity and specificity.<sup>4</sup> Nonetheless, this methodology is usually available only in research facilities.

Although the World Health Organization (WHO) has named specific DEC strains as the highest priority for vaccine development after rotavirus,<sup>5</sup> detection has not been routinely conducted in clinical laboratories, particularly in rural areas. Others have shown that prevalence and distribution of DEC pathotypes vary significantly between and within countries.<sup>6–8</sup> However, studies have been mostly conducted in periurban and urban areas and less frequently in rural communities, especially those in the geographical areas of the highlands and jungle.<sup>9–12</sup>

Malnutrition and growth retardation have remained a significant population health challenge in low-income regions. Previous studies suggest that the presence of EAEC may contribute to growth impairment in children, even when diarrhea is

not present.<sup>13</sup> Further exploring this association in rural populations may help public health initiatives prioritize therapy.

The present study aims to determine the prevalence and pathotype distribution of DEC strains isolated from children in rural communities of Peruvian highlands and jungle, as well as to establish whether an association between DEC pathotypes and malnutrition exists.

### MATERIALS AND METHODS

**Study design and population.** This research was conducted as a subgroup analysis of an environmental enteropathy project. A cohort of 93 children aged between 6 and 13 months were prospectively enrolled from the Peruvian rural communities of Urubamba (Yucay, Huayllabamba, and Ccotohuincho) and Moyobamba (Yantalo and Calzada), representing the highlands and jungle, respectively. Subjects were followed up for 6 months (December 2014 to June 2015). We excluded children with history of congenital disease, severe neonatal complications, prematurity, or previously diagnosed acute or chronic malnutrition. Length and weight were measured by trained field workers monthly and analyzed according to WHO standards (Anthro version 3.2.2 [Department of Nutrition, WHO, Geneva, Switzerland]).

**Specimen collection and processing.** Stool samples were collected every 2 weeks and with each diarrheal episode (within 48 hours). Nondiarrheal (asymptomatic control) samples were collected and included when diarrhea was absent for 1 week before or after stool sample collection to evaluate colonization. Diarrhea was defined as having three or more liquid or semiliquid stools in a 24-hour period or at least one loose stool with visible blood.<sup>14</sup> All specimens were placed in Cary–Blair transport medium and shipped in iced boxes at 4°C to the Instituto de Medicina Tropical Alexander von Humboldt in Lima (Moyobamba samples) and Cuzco (Urubamba samples) for processing. Average time to analysis postcollection was 96 hours for Moyobamba and 32 hours for Urubamba.

\*Address correspondence to Theresa J. Ochoa, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Avenida Honorio Delgado 430, San Martín de Porres, Lima 31, Peru. E-mail: theresa.j.ochoa@uth.tmc.edu

†These authors contributed equally to this work.

TABLE 1  
Sociodemographic characteristics of study population at enrollment

Characteristic	Moyobamba (jungle)	Urubamba* (Andes)	Total (N = 93)
	(N = 52)	(N = 41)	
Male sex	30 (57.7)	25 (61.0)	55 (53.4)
Age (months)			
6–9	28 (53.8)	31 (75.6)†	59 (57.3)
10–13	24 (46.2)	10 (24.4)	34 (33.0)
Mean age (months) ± SD	9.4 ± 2.4	8.7 ± 1.7	9.1 ± 2.1
Breastfeeding of any duration	52 (100.0)	34 (100.0)	86 (100.0)
Exclusive breastfeeding at 6 months	30 (57.7)	17 (63.0)	47 (59.5)
Mean age at start of solid food (months) ± SD	5.8 ± 1.2	5.8 ± 0.8	5.8 ± 1.0
Maternal age, mean ± SD	27.2 ± 6.7	26.8 ± 6.6	27.0 ± 6.6
Maternal education			
None	8 (15.4)	1 (2.9)	9 (10.4)
Grammar school	25 (48.0)	11 (32.4)	36 (41.9)
High school	11 (21.2)	16 (47.1)†	27 (31.4)
Technical school or university	8 (15.4)	6 (17.6)	14 (16.3)
Access to piped water inside house	52 (100.0)	34 (100.0)	86 (100.0)
Domestic sewage	26 (54.2)	29 (85.3)†	55 (67.1)
Boiling of water before consumption	46 (93.9)	27 (100.0)	73 (96.1)
Having animals at house	22 (45.8)	2 (5.9)†	24 (29.3)

SD = standard deviation. Data are no. (%) of children, unless otherwise indicated.

\*Data not available for all children.

†*P* < 0.05.

Samples were inoculated onto MacConkey agars and incubated for 24 hours at 37°C. Five lactose-fermenting colonies with typical *E. coli* morphology and two non-lactose fermenting colonies (when found) were selected and maintained in nutrient agar slants at room temperature (seven per stool sample). No biochemical tests for identification were carried out.

To determine specific DEC pathotypes, pooling of the seven isolated colonies was done after thermal shock DNA extraction for posterior multiplex real-time PCR. This method simultaneously detects the presence of the following genes:

*aggR* (EAEC), *st1*, *st2*, *eltA*, *eltB* (ETEC), *eae* (EPEC), *eae*, *stx1*, *stx2* (STEC), *ipaH* (EIEC), and *daaD* (DAEC).<sup>4</sup> This methodology has been validated and used since 2005 in the Instituto de Medicina Tropical Alexander von Humboldt (Lima, Peru).

**Statistical analysis.** The statistical analyses were performed using STATA, version 14 (StataCorp LP, College Station, TX). Categorical data were compared by  $\chi^2$  test. Comparisons between means were made using two-sample *t* test or non-parametric test when applicable. A *P* value < 0.05 was considered statistically significant.

**Ethical considerations.** The study was approved by the Ethical Review Board of the University of Texas Health Science Center at Houston and Universidad Peruana Cayetano Heredia. Written informed consent was obtained from the parent or guardian of each child.

## RESULTS

Of the 93 children enrolled, 41 were from Urubamba and 52 from Moyobamba. Urubamba children were younger, had a higher percentage of high-school graduate mothers, and lived in houses with better sanitation (i.e., sewage system present and fewer animals inside the house) (Table 1).

Nineteen children (20.4%) were lost during follow-up period; still, their specimens were processed and analyzed. A total of 820 stool samples were collected, of which 46 (5.6%) were diarrheal, whereas 774 (94.4%) were nondiarrheal or asymptomatic control samples. A median of 10 stool samples per child were collected.

Overall, diarrheagenic *E. coli* were isolated in 43.0% (353/820) of stool samples. Of the 93 enrolled children, 75 (80.6%) had at least one stool positive for *E. coli* (median of 3, range 1–10 positive samples for 1–5 different pathotypes per child) during follow-up (Table 2). DEC were identified in 50% (*N* = 23) of diarrheal samples and 42.6% (*N* = 330) of control samples. EAEC (20.8%), EPEC (13.3%), and DAEC (11.1%) were the most frequent pathotypes—as single or mixed infections—in control samples, whereas EPEC (28.3%), EAEC

TABLE 2  
Distribution of DEC strains found in stool samples

DEC pathotype	Moyobamba (jungle)		Urubamba (Andes)		All children	
	Diarrhea (N = 23)	Control (N = 415)	Diarrhea (N = 23)	Control (N = 359)	Diarrhea (N = 46)	Control (N = 774)
Single pathotype						
EAEC ( <i>aggR</i> )	3 (13.0)	81 (19.5)*	0 (0.0)	46 (12.8)*	3 (6.5)	127 (16.4)
EPEC ( <i>eaeA</i> )	4 (17.4)	39 (9.4)	5 (21.7)	32 (8.9)	9 (19.6)†	71 (9.2)†
DAEC ( <i>daaD</i> )	0 (0.0)	18 (4.3)	2 (8.7)	27 (7.5)	2 (4.3)	45 (5.8)
ETEC ( <i>st</i> and/or <i>lt</i> )	1 (4.3)	12 (2.9)	3 (13.0)	18 (5.0)	4 (8.7)	30 (3.9)
STEC ( <i>stx1</i> and/or <i>stx2</i> , <i>eae</i> )	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.1)
EIEC ( <i>ipaH</i> )	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.1)
DEC coinfections						
EAEC + DAEC	1 (4.3)	10 (2.4)	0 (0.0)	6 (1.7)	1 (2.2)	16 (2.1)
EPEC + DAEC	0 (0.0)	6 (1.4)	1 (4.3)	9 (2.5)	1 (2.2)	15 (1.9)
EAEC + EPEC	1 (4.3)	8 (1.9)	1 (4.3)	5 (1.4)	2 (4.3)	13 (1.7)
DAEC + ETEC	0 (0.0)	3 (0.7)	0 (0.0)	2 (0.6)	0 (0.0)	5 (0.6)
EAEC + ETEC + DAEC	0 (0.0)	1 (0.2)	0 (0.0)	1 (0.3)	0 (0.0)	2 (0.3)
EAEC + DAEC + EPEC	0 (0.0)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.3)
EPEC + ETEC	1 (4.3)	0 (0.0)	0 (0.0)	1 (0.3)	1 (2.2)	1 (0.1)
EAEC + EPEC + ETEC	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)	0 (0.0)	1 (0.1)
All DEC	11 (47.8)	180 (43.4)	12 (52.2)	150 (41.8)	23 (50.0)	330 (42.6)

DEC = diarrheagenic *E. coli*; DAEC = diffusely aggregative *E. coli*; EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; STEC = shiga toxin-producing *E. coli*. Data are no. (%) of children, unless otherwise indicated.

\**P* = 0.005, for the comparison of EAEC in control samples between Moyobamba and Urubamba.

†*P* = 0.004, for the comparison of EPEC between diarrhea and control samples.

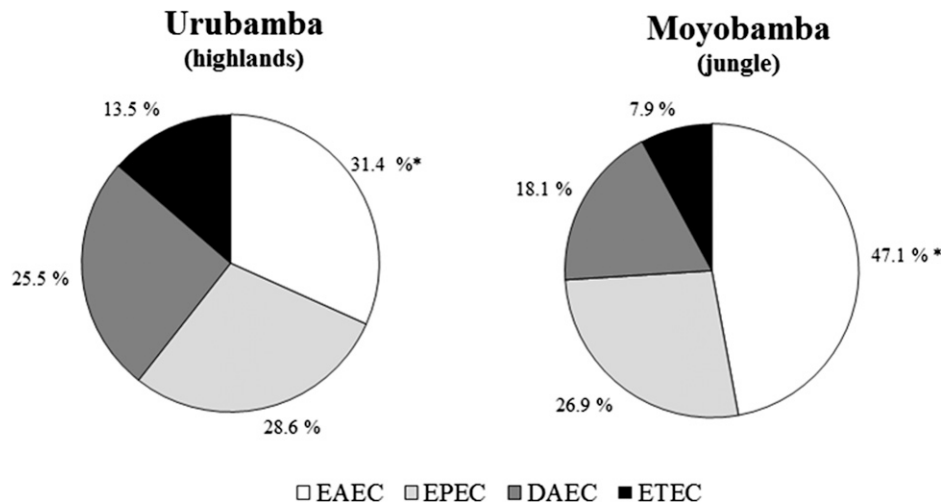


FIGURE 1. Distribution of diarrheagenic *Escherichia coli* (DEC) pathotypes in rural communities of the highlands and jungle. Urubamba  $N = 194$  positive DEC samples; Moyobamba  $N = 227$  positive DEC samples. \*  $P < 0.05$ .

(13.0%), and ETEC (10.9%) were the most common in diarrheal samples. EPEC was the only pathotype significantly more common in diarrheal than in control samples ( $P < 0.01$ ).

The prevalence of EAEC, as single or coinfection, was higher in control samples collected from the jungle site (Moyobamba) than in those from Urubamba, the highland site (102/415 [24.6%] versus 59/359 [16.4%],  $P < 0.01$ ). Although pathotypes were similarly arranged by frequency, the distribution varied between both geographical regions (Figure 1).

DEC colonization and behavior over time was frequent and heterogeneous in subjects from both communities (Figure 2). Children were found to be colonized with at least one DEC pathotype for about 50% of the time of follow-up (median number of months with positive stool was 3/6, interquartile range = 2–5). Reinfection with EAEC was common, being detected in 39 (54.9%) of 71 children with an initial infection (median of two infections per child, interquartile range = 2–3). This proportion was lower for EPEC (43.9% [29/66]), DAEC (30.8% [16/52]), and ETEC (21.9% [7/32]). On the other hand, shedding after a new infection, defined as the time elapsed from the first to the last positive consecutive sample, ranged from 2 to 16 weeks for EAEC, 2 to 5 weeks for EPEC, 2 to 7 weeks for DAEC, and 2 weeks for ETEC.

To determine age-related susceptibility to infection with DEC, stool samples were divided into two groups according to the age at which they were obtained. DEC strains were more frequently isolated in samples from children aged 6–12 months than in the 13–19 months group (222/400 [55.5%] versus 197/420 [46.9%],  $P < 0.05$ ), EAEC contributing most to this difference ( $P < 0.05$ ).

With regard to the evolution of nutritional status of those who completed follow-up (74/93), a decline in height-for-age Z-score (HAZ) was observed in 70.3% of children from Moyobamba (mean HAZ change of  $-0.49 \pm 0.94$ ) and 81.1% of children from Urubamba (mean HAZ change of  $-0.47 \pm 0.73$ ). Of these, six children from Moyobamba (6/37, 16.2%) and 11 from Urubamba (11/37, 29.7%) were clinically stunted (HAZ  $< -2$ ) by the end of follow-up. EAEC was more frequently isolated among the children who had a decline greater

than one unit in their HAZ score as opposed to those with a smaller change ( $P < 0.05$ ) (Figure 3).

## DISCUSSION

This prospective cohort study was performed to determine the importance of DEC infections in children from rural communities of the highlands and jungle, which has not been thoroughly described previously. We found that the overall isolation rate of DEC strains in the present study (43.0%) was higher than previously reported in coastal periurban areas of Peru (31.5%)<sup>9</sup> and some rural communities in other geographical areas of the world.<sup>15,16</sup> Similar to other reports, no significant difference between the frequency of DEC infections in diarrheal (50%) and nondiarrheal samples (42.6%) was observed. This finding underscores the high prevalence of the carrier state in these areas.

Previous studies in Peru have predominantly been conducted in periurban Lima, located in the coast. In 2011, Ochoa and others studied the frequency and pathotypes of DEC in a cohort of 1,034 children aged 2–12 months with and without diarrhea followed for 1 year, and found DEC in 31.0% of 936 diarrheal samples and 31.8% of 424 asymptomatic control samples.<sup>9</sup> In accordance to our findings, EAEC, EPEC, and DAEC were the most prevalent strains in asymptomatic infections. Similar results were obtained in another large study including 8,000 samples from children younger than 5 years, although ETEC was the third most common pathotype identified in that study.<sup>10</sup> This data suggests that pathotype distribution in control samples is similar throughout the three major regions of Peru: coast, mountains, and jungle.

In congruence with our study, EAEC has been previously described as the DEC strain with the highest isolation rate overall.<sup>9,10,15,17</sup> Nonetheless, its association with diarrhea requires further analysis: studies conducted in Brazil<sup>18,19</sup> and Vietnam<sup>20</sup> found EAEC to be significantly more prevalent in diarrheal than control samples, whereas others did not (including the present study).<sup>9,10,15,16</sup> Notably, EAEC was more frequently identified in nondiarrheal samples from Moyobamba than Urubamba. This could be explained by the less favorable

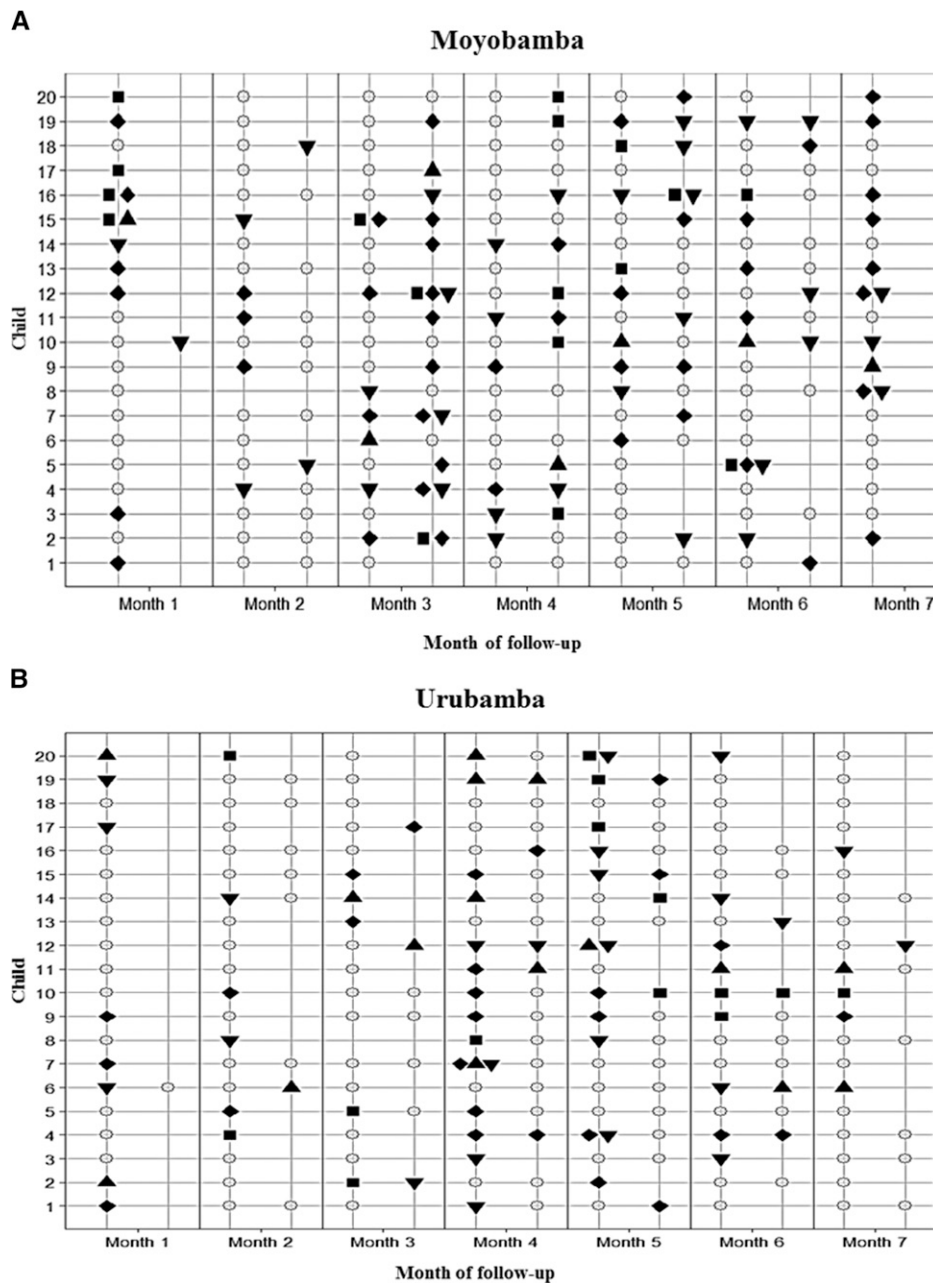


FIGURE 2. Diarrheagenic *Escherichia coli* infections in children from both communities over time. Sample of 20 children randomly selected in each study site, among children with complete follow-up and at least one stool sample collected per month. ○ = negative sample, ◆ = positive for enteroaggregative *E. coli*, ▼ = positive for enteropathogenic *E. coli*, ■ = positive for diffusely aggregative *E. coli*, ▲ = positive for enterotoxigenic *E. coli*.

sociodemographic conditions (Table 1) or the distinct environmental and climatic characteristics in the former study site.

In diarrheal samples, EPEC was the most frequently isolated pathotype, followed by EAEC and ETEC. Furthermore, EPEC was significantly associated with diarrheal episodes, in concordance with previous reports by Estrada-Garcia and others<sup>21</sup> in children < 2 years of age in Mexico and Moreno and others<sup>19</sup> in infants from northeast Brazil. However, mostly atypical EPEC has been shown to contribute to the aforementioned association, information lacking in our study given that the *bfpA* gene was not sought for.

The role of DAEC in diarrhea is still undefined. This association has been described in several reports, which describe

an age-related susceptibility in favor of older children.<sup>9,16,22</sup> Although we also found DAEC to be an important cause of infection, these cases were mostly asymptomatic even after age stratification. Interestingly, DAEC was the most prevalent pathotype among DEC coinfections (Table 2).

The analysis of colonization over time demonstrates that children living in rural areas of Peru are frequently and repeatedly colonized with DEC in their first years of life; prolonged shedding, reinfections, and coinfections are common. The clinical implications of these findings need further investigation.

As mentioned before, we analyzed samples according to the age at which they were obtained to explore the age-related



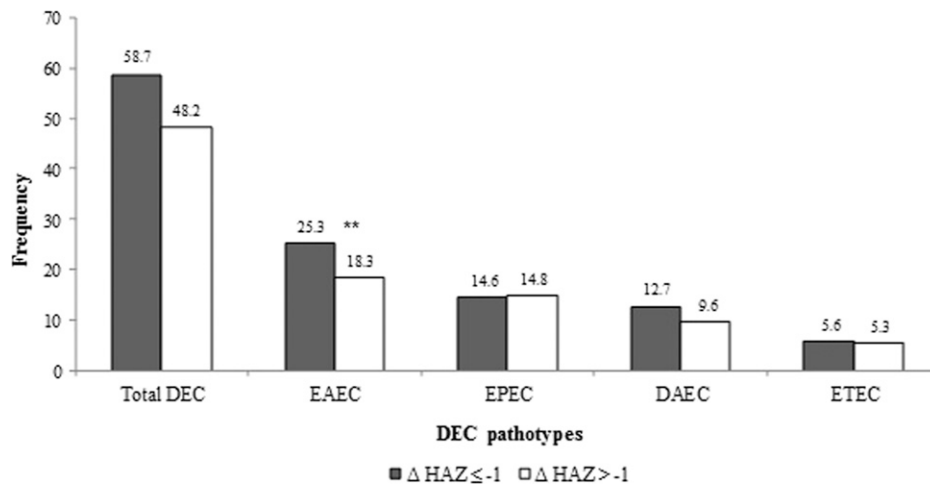


FIGURE 3. Frequency of diarrheagenic *Escherichia coli* pathotypes according to variation of height-for-age Z-score (HAZ) during follow up. Sample size:  $N = 22$  children in  $\Delta\text{HAZ} \leq -1$  group,  $N = 52$  children in  $\Delta\text{HAZ} > -1$ . Significant difference was found among the enteroaggregative *Escherichia coli* (EAEC) groups, \*\*  $P < 0.05$ .

susceptibility to infection with DEC strains. We found that all pathotypes were more commonly identified in the younger group (6–12 months), especially EAEC, which was the only one with a significant difference between the two groups. This correlates with the results of Scaletsky and others in children  $< 2$  years of age in Brazil for EAEC, EPEC, and DAEC.<sup>22</sup>

Several studies have suggested that EAEC plays a role in growth impairment mainly through intestinal inflammation and diminished nutrient absorption.<sup>11,23</sup> To evaluate this effect, we compared the prevalence of EAEC infections in children with an HAZ decline greater than one standard deviation (SD) during follow-up ( $\Delta\text{HAZ} \leq -1$ ) versus those with a decline smaller than one SD ( $\Delta\text{HAZ} > -1$ ). We observed that EAEC infection was more frequently detected in children with a greater decline in their HAZ (Figure 3). This association may reflect a microbiota preferred in the starvation state and is not necessarily causal, but an important observation nonetheless.

It is important to take the limitations of this study into account. The lack of complete demographic information may have altered our perspective of baseline characteristics in both study sites. The reduced number of diarrheal episodes may have contributed to the underestimation of the real importance of DEC strains as causal agents of symptomatic events. Delay in sample transportation from the Moyobamba study site to Lima, as well as the cold temperatures at which samples were kept (4°C), may have led to a decreased detection rate of DEC pathotypes. With regard to the diagnostic methods used, *bfpA* evaluation for further typification of isolated EPEC strains as typical or atypical would have allowed a more detailed insight on its association with diarrhea. Finally, the time interval between consecutive samples was very variable among participants, making it difficult to derive consistent information regarding shedding of pathogens after diarrheal events.

In summary, DEC strains were found to be an important cause of symptomatic and asymptomatic infection in rural communities of the Peruvian highlands and jungle. This information is valuable as it complements previous knowledge from coastal and periurban areas, provides further

insight for countries with distinct geographical regions and completes the epidemiological panorama of DEC in Peru.

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Authors' addresses: Gonzalo J. Acosta, Natalia I. Vigo, David Durand, Maribel Riveros, and Sara Arango, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru, E-mails: gjacosta89@gmail.com, natalia.vigo@outlook.com, david.durand@upch.pe, maribel.riveros@upch.pe, and sara.arango29@gmail.com. Mara Zambruni, Department of Pediatric Infectious Diseases, University of Texas Medical Branch, Galveston, TX, E-mail: mazambru@utmb.edu. Theresa J. Ochoa, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru, and University of Texas Health Science Center at Houston, School of Public Health, Houston, TX, E-mails: theresa.j.ochoa@uth.tmc.edu or theresa.ochoa@upch.pe.

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