Diarrheagenic *Escherichia coli* in Human Immunodeficiency Virus (HIV) Pediatric Patients in Lima, Perú

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Abstract. We conducted a prospective study in three hospitals in Lima in human immunodeficiency virus (HIV) children to determine the frequency of diarrheagenic *Escherichia coli*. Five *E. coli* colonies/patients were studied by a multiplex real-time polymerase chain reaction to identify the six currently recognized groups of diarrhea-associated *E. coli*. We have analyzed 70 HIV-associated diarrheal and 70 control samples from HIV-infected children without diarrhea. Among the diarrheal episodes 19% were persistent, 3% dysenteric, and 33% were associated with moderate or severe dehydration. The diarrheagenic *E. coli* were the most commonly isolated pathogens in diarrhea (19%) and control samples (26%) ($P = 0.42$), including enteroaggregative (6% versus 10%), enteropathogenic (6% versus 10%), and enterotoxigenic *E. coli* (4% versus 3%), respectively. The HIV-infected children with diarrhea had the worse age-related immunosuppression, higher viral loads, and were on highly active antiretroviral treatment (HAART) less often than HIV-infected children without diarrhea. Diarrheagenic *E. coli* were highly resistant to ampicillin (74%) and cotrimoxazole (70%).

INTRODUCTION

Diarrhea in patients infected with the human immunodeficiency virus (HIV) continues to be a common problem in developing countries,^{1,2} particularly in places where highly active antiretroviral treatment (HAART) is not widely available and where opportunistic infections continue to be a common problem. The lifetime incidence of diarrhea among HIV patients has been estimated to be 30% to 70%. However, the use of HAART has markedly improved the long-term outcome for patients with adequately treated HIV infection. Immune recovery related to HAART has resulted in a significant resolution of diarrhea caused by opportunistic pathogens previously thought to be untreatable, including microsporidia and cryptosporidia.^{3,4} Diarrhea in HIV-infected patients is typically difficult to treat because the specific etiology is infrequently determined. Better understanding of the region-specific etiologic agents could improve the outcomes of HIV-infected children with diarrhea. In particular, the long-term consequences of prolonged and repeated gastrointestinal infections with intestinal parasites, including permanent shortfalls in physical and cognitive development,^{5–7} might be averted. However, there is no evidence for cognitive deficits caused by asymptomatic gastrointestinal infections with bacterial enteropathogens at this time.

The diarrheagenic *Escherichia coli* as a group are responsible for 30% to 40% of acute diarrhea episodes in children who are not HIV infected in developing countries⁸ and now are also being recognized as important enteric pathogens in the developed world.^{9,10} However, stool samples are not routinely evaluated for these pathogens in clinical laboratories outside the research setting. The diarrheagenic *E. coli* are categorized into six groups, enterohemorrhagic or shiga toxin-producing (EHEC or STEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC

or EAaggEC), and diffusely adherent *E. coli* (DAEC).¹¹ Within these groups, EAEC has been more frequently associated as a causative agent of diarrhea in adults infected with HIV.^{12–15} However, there are few studies on the prevalence of diarrheagenic *E. coli* in pediatric HIV patients.^{16–20}

The aims of this study were 1) to determine the prevalence of diarrheagenic *E. coli* in Peruvian children infected with HIV with and without diarrhea, 2) to determine its association with viral load and immunosuppression, and 3) to determine the antimicrobial resistance patterns of diarrheagenic *E. coli* isolated from these patients.

PATIENTS AND METHODS

Study design. This was a prospective, cross-sectional, descriptive study of HIV-infected children from Lima to determine prevalence of diarrheagenic *E. coli*.

Patients. The HIV-infected children, 1 month to 18 years of age, were enrolled at three public hospitals in Lima, Peru (Instituto Nacional de Salud del Niño, Hospital Nacional Cayetano Heredia, and Hospital Nacional Hipolito Unanue). The patients were recruited at the outpatient clinics and inpatient wards in a consecutive manner over a period of 2 years (March 2007 and February 2009). Clinical data (type of diarrhea, duration, vomiting, presence of blood, dehydration), and information on the HIV status (viral load, CD4 counts, and use of HAART) were collected from the medical charts in collaboration with the attending physicians of each participating hospital. Anthropometric data (weight and height) were not available for all children during the diarrhea episodes, because most children were seen at the Emergency Room. Stool samples were collected during diarrhea episodes. Diarrhea was defined by the presence of three or more loose stools in 24 hours or one loose stool with blood. Control stool samples were collected from HIV-infected children who had no diarrhea or other gastrointestinal symptoms for 1 week before and 1 week after the stool sample collection.

Laboratory studies. Stool samples from patients with diarrhea were analyzed for common enteric bacteria (*Shigella*, *Salmonella*, *Campylobacter*, and *Vibrio*) by routine procedures

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at the microbiology laboratory of each hospital. Direct exam was used for eggs of parasites, and modified Ziehl-Nielsen stain for *Cryptosporidium*, after stool concentration. Stool samples from patients without diarrhea were cultured only on MacConkey agar plates for isolation of *E. coli* colonies. Detection of diarrheagenic *E. coli* was done for both diarrhea and control samples at the Tropical Medicine Institute "Alexander von Humboldt" in Lima. Five lactose-positive *E. coli* colonies per patient were isolated from MacConkey agar plates and analyzed by a multiplex real-time polymerase chain reaction (PCR) method using previously validated specific primers for each pathotype: EAEC (AggR), ETEC (*lt*, *st*), EPEC (*eaeA*), STEC (*stx1*, *stx2*), EIEC (*IpaH*), and DAEC (*daaD*).²¹ The PCR was performed using a PTC-200 thermal cycler and real-time fluorescence monitoring by a Chromo 4 optical detector (MJ Research/Biorad, Hercules, CA), using a five-colony pool analysis as previously described.²² Diarrheagenic *E. coli* colonies were analyzed for their antimicrobial susceptibility by disk diffusion according to the Clinical Laboratory Standards Institute (CLSI) guidelines.²³ Antibiotic susceptibilities were analyzed for ampicillin (AMP, 10 µg disk), amoxicillin-clavulanic acid (AMC, 30 µg disk), cefotaxime (CTX, 30 µg disk), ceftazidime (CAZ, 30 µg disk), gentamicin (GTM, 10 µg disk), nalidixic acid (NAL, 30 µg disk), ciprofloxacin (CIP, 5 µg disk), azithromycin (AZD, 15 µg disk), tetracycline (TET, 30 µg disk), chloramphenicol (CAF, 30 µg disk), cotrimoxazole (SXT, 23.75/1.25 µg disk), and nitrofurantoin (NIT, 300 µg disk). Multi-resistance was defined as resistance to three or more unrelated antimicrobial agents.

Ethical aspects. This study was reviewed and approved by the Institutional Review Board (IRB) of Universidad Peruana Cayetano Heredia and by the IRB of each participating hospital. Informed consent was obtained from the parents and assent from patients over the age of 7 years.

Statistical analysis. The results were analyzed using the statistical software EpiInfo 3.4.3 (CDC, Atlanta, GA). The χ^2 (Yates) or Fisher test were used to compare pathogens or clinical data between diarrhea and control samples. Student *t* test or non-parametric tests were used in the case of continuous variables. Differences were considered statistically significant if the *P* value was less than 0.05.

RESULTS

One hundred thirteen HIV patients were enrolled in the study (83 from Instituto Nacional de Salud del Niño, 27 from Hospital Nacional Cayetano Heredia, and 3 from Hospital Nacional Hipólito Unanue), representing 69% of the 164 HIV-infected patients regularly followed in the three participating hospitals. The median age of the children was 6 years ranging from 2 months to 17 years of age; 50% of children were female. Overall, the median viral load (RNA copies [log₁₀]/mL) was 4.5 (1.7–6.8); 70% had a viral load > 400 RNA copies/mL; 33% had severe age-related immunosuppression; and 85% were on HAART. The most commonly used treatment schemes were zidovudine, lamivudine, and nelfinavir (66%), and lamivudine, nelfinavir, and stavudine (23%).

Ninety-four patients provided one single stool sample (diarrhea or control) and 19 patients provided more than one sample at different times during the 2-year period. Overall, 70 diarrhea samples and 70 control samples without diarrhea

FIGURE 1. Age distribution of human immunodeficiency virus (HIV)-infected children with (*N* = 70) and without diarrhea (*N* = 70). **P* < 0.01 for the age group comparison between children with and without diarrhea.

were analyzed. The age of children with diarrhea was 5 ± 4 years while the controls averaged 7 ± 4 years. The majority of patients with diarrhea were within the age group of 1 to 5 years, whereas the majority of patients without diarrhea were found to be within the age group of 6 to 18 years (Figure 1). Children with diarrhea had significantly worse age-related immunosuppression²⁴ (based on CD4 cells by age), higher viral loads, and were less frequently on HAART (Table 1).

Diarrheagenic *E. coli* were isolated in 19% of diarrheal samples (13/70) and in 26% of control samples (18/70) (*P* = 0.416). The EAEC and EPEC were the most frequently isolated pathogens from both diarrhea and control samples (Table 2). There were no significant age-related differences in the frequency of each diarrheagenic *E. coli* group among children younger than 5 years of age and older. There were no *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio*, or parasites isolated in the diarrheal samples. Children without diarrhea, in addition to the diarrheagenic *E. coli*, were colonized with

TABLE 1
Characteristics of human immunodeficiency virus (HIV) infection in children with and without diarrhea

	With diarrhea (<i>N</i> = 70)	Without diarrhea (<i>N</i> = 70)
Viral load (VL) RNA copy (log ₁₀)/mL		
VL by age, mean $\pm$ SD		
< 12 months	5.6 $\pm$ 0.9	4.9 $\pm$ 1.3
1–5 years	4.8 $\pm$ 1.1	4.4 $\pm$ 1.3
6–18 years	4.2 $\pm$ 1.3	3.4 $\pm$ 1.1*
All age groups	4.7 $\pm$ 1.2	3.8 $\pm$ 1.3**
VL, median (range)	5.3 (1.6–6.8)	3.2 (2.6–6.0)
VL > 400 RNA copies/mL, n/N (%)	61/69 (88)	39/70 (56)
CD4, cells/mm ³		
CD4, mean $\pm$ SD	506 $\pm$ 440	710 $\pm$ 434*
CD4, median (range)	431 (37–1,854)	656 (37–2,489)
Severe immunosuppression,† n/N (%)		
< 12 months	9/10 (90)	4/6 (67)
1–5 years	21/40 (53)	7/21 (33)
6–18 years	7/20 (35)	3/43 (7)*
All age groups	37/70 (53)	14/70 (20)**
HAART, n/N (%)	50/70 (71)	69/70 (99)**

P* < 0.01 and *P* < 0.001 for the comparison between children with and without diarrhea.

† Based on CD4 cells by age (CDC, 1994).²⁴

TABLE 2

Diarrheagenic *Escherichia coli* isolated in diarrhea and control samples (without diarrhea) in human immunodeficiency virus (HIV) pediatric patients

Pathogen	Children < 5 years		Children ≥ 6 years		All children	
	Diarrhea (N = 50) n (%)	Control (N = 27) n (%)	Diarrhea (N = 20) n (%)	Control (N = 43) n (%)	Diarrhea (N = 70) n (%)	Control (N = 70) n (%)
All diarrheagenic <i>E. coli</i> *	8 (16)	9 (33)	5 (25)	9 (21)	13 (19)	18 (26)
EAEC	1 (2)	4 (15)†	3 (15)	3 (7)	4 (6)	7 (10)
EPEC	3 (6)	4 (15)	1 (5)	3 (7)	4 (6)	7 (10)
DAEC	0 (0)	1 (4)	1 (5)	1 (2)	1 (1)	2 (3)
ETEC	3 (6)	0 (0)	0 (0)	2 (5)	3 (4)	2 (3)
STEC	1 (2)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)

*There were no EIEC isolated.

† $P < 0.05$ for the comparison between diarrhea and control.

several parasites: *Blastocystis hominis* (13%), *Entamoeba coli* (11%), *Endolimax nana* (10%), *Chilomastix mesnili* (3%), *Cryptosporidium* sp. (3%), and *Giardia lamblia* (1%). Among the diarrheal episodes 19% were persistent, 3% dysenteric and 33% were associated with moderate or severe dehydration (Table 3). The clinical characteristics of the diarrheal episodes with an isolated diarrheagenic *E. coli* were similar to the characteristics of episodes without these pathogens (Table 3). The average number of positive colonies per samples was two. There were no differences among each *E. coli* pathotype or among the different symptoms; however, the number of positive samples was small overall for proper comparison among categories.

Twenty-three *E. coli* strains were available for antibiotic susceptibility testing (74%). Resistance to ampicillin and cotrimoxazol were common (Figure 2). Of interest, there were no strains with high level resistance to nitrofurantoin (furozolidone) and ciprofloxacin; however, 4 strains had intermediate resistance to ciprofloxacin (17%). Similarly, there were only 2 strains (9%) with high level resistance to amoxicillin-clavulanic, but 8 strains (35%) with intermediate resistance to this antibiotic. There were no differences in the resistance levels between diarrhea and control samples, except for nalidixic acid, which was significantly more resistant when isolated from diarrhea rather than control samples (63% versus 13%, $P < 0.05$). Multidrug resistance was present in 52% of strains. There are no break points approved for azythromycin resistance; the distribution of the growth inhibitory zones by disc diffusion for this antibiotic was 4% (1 strain) less than 10 mm, 57% (13 strains) 11–15 mm, and 39% (9 strains) more than 16 mm.

DISCUSSION

In Peru the number of reported HIV-infected patients from 1983 to March 2009 is 34,677, of which 23,143 have acquired immunodeficiency syndrome (AIDS).²⁵ The number of children reported by age group is 371 (0–4 years), 90 (5–9 years), 64 (10–14 years), and 528 (15–19 years). In 2007 there were 2,766 newly diagnosed HIV cases in all group ages; in 2008 there were 2,923. Overall, the male to female ratio was 2.8 (793 males/279 females) in 2008; this is a dramatic change from the previous decade; in 1990 this ratio was 11.8 (376 male/32 female). The mode of HIV transmission is 97% sexual and 3% vertical. Between 2004 and 2009 there were 506 children enrolled for management in the public hospitals of the entire country, most of them in Lima; 434 (86%) are on HAART; 30 (6%) have died during this period. In the current study we enrolled 113 HIV children, approximately 2/3 of the total number of HIV children from the three participating hospitals.

Diarrheagenic *E. coli* as a group were the most common isolated pathogens in HIV-infected children with or without diarrhea, including EAEC, EPEC, and ETEC. This finding is similar to a study we recently conducted in peri-urban districts of Lima, in which we found that diarrheagenic *E. coli* were the most common pathogens in infants with or without diarrhea (31% and 32%, respectively).²⁶ Thus, children in Lima are frequently exposed to these bacteria early in life. The interpretation of pathogen frequency in diarrhea versus control samples is complicated. Colonization rather than illness results from the interaction of several factors from the pathogen, the host, and the environment. The pathogens are sometimes heterogeneous, sharing specific virulence genes used for categorizing

TABLE 3

Clinical characteristics of the diarrheal episodes with or without a diarrheagenic *Escherichia coli* (DEC) isolated from human immunodeficiency virus (HIV)-infected children

	All children with diarrhea (N = 70) n/N (%)	Children with diarrhea and DEC (N = 13) n/N (%)	Children with diarrhea without DEC (N = 57) n/N (%)
Duration of diarrhea, days			
Mean ± SD	9 ± 14	12 ± 24	9 ± 11
Median (range)	5 (2–90)	5 (2–90)	5 (2–67)
Persistent diarrhea	13/70 (19)	1/13 (8)	12/57 (21)
Blood in the stools	2/70 (3)	0/13 (0)	2/57 (4)
Fever	28/70 (40)	7/13 (54)	21/57 (37)
Vomiting	15/70 (21)	4/13 (31)	11/57 (19)
Moderate and severe dehydration	23/70 (33)	6/13 (46)	17/57 (30)
Severe immunosuppression*	37/70 (53)	7/13 (54)	30/57 (53)
VL†, > 400 RNA copies/mL	61/69 (88)	9/12 (75)	52/57 (91)

* Based on CD4 cells by age (CDC, 1994).²⁴

† VL = viral load.

FIGURE 2. Antibiotic susceptibility (percent with high level resistance) of diarrheagenic *Escherichia coli* isolated from human immunodeficiency virus (HIV)-infected children ($N = 23$ strains). AMP = ampicillin; SXT = cotrimoxazole; TET = tetracycline; NAL = nalidixic acid; AMC = amoxicillin-clavulanic acid; CTX = cefotaxime; CAZ = ceftazidime; CAF = chloramphenicol; GTM = gentamicin. There were no strains resistant to ciprofloxacin or nitrofurantoin.

them in a group, but having accessory genes that affect virulence. Host susceptibility to infection is determined by the child's age, presence of protective maternal factors (i.e., transplacental antibodies), nutritional and immunological status, prior exposure and acquired immunity, and genetic susceptibility. Environmental factors such as poor hygiene and high fecal contamination result in early and frequent exposure with development of acquired immunity. We hypothesize that the high frequency of pathogens in samples from asymptomatic young children reflects these factors. Several epidemiological studies have compared the isolation rate of diarrheagenic *E. coli* among diarrhea and control samples, with different results. The factors mentioned previously, should be taken into consideration in interpretation and comparison of studies. This study is the first report of diarrheagenic *E. coli* in HIV-infected children in Peru.

EAEC is an emerging pathogen currently considered one of the main causes of diarrhea in adult HIV-infected patients,¹² accounting for 10% to 44% of acute diarrhea cases and 43–79% of persistent diarrhea cases in HIV patients from developing countries.²⁷ In a recent meta analysis, the presence of EAEC was found to be significantly associated with acute diarrheal illness among HIV-infected children (odds ratio [OR], 1.58; 95% confidence interval [CI], 1.36–1.83) and adults residing in developing regions (OR, 6.43; 95% CI, 2.91–14.16).¹² In this study, the frequency of EAEC in HIV-infected children with diarrhea was 6%, which is much lower than previously reported in children and adults from South Africa (29%),¹⁸ and infants from Zaire (41%).¹⁷

Enterotoxigenic and EPEC are important pathogens in the pediatric population because of their high morbidity and mortality rates.²⁸ The ETEC is usually associated with dehydrating diarrhea, caused by the presence of the heat-stable (ST) and/or heat-labile (LT) enterotoxin. The EPEC is relevant because it is not only associated with acute watery diarrhea, but also with persistent diarrhea, which accounts overall for 30% to 50% of diarrheal deaths in children from developing countries.²⁹ The ETEC and EPEC have been infrequently studied in HIV patients, and in those studies, these pathogens were also found infrequently. In a study in adult HIV patients

in Lima, ETEC accounted for 4% of all the diarrhea cases.¹ In a study conducted in Brazil of HIV-infected children less than 13 years of age, there was one EPEC strain isolated and no ETEC in diarrheal samples, however, the number of samples was very small.¹⁹ In a study in HIV-infected infants from Zaire, ETEC was isolated in 11% of diarrhea cases and EPEC in 15%.¹⁷

Although the studied groups (diarrhea and control) were not similar in age, an interesting finding was the important difference on the characteristics of the HIV infections among them. Children with diarrhea had worse immunosuppression (greater viral load and lower CD4 counts) than children without diarrhea, reflecting the lower percentage of patients in HAART in the group with diarrhea. This highlights the need to treat patients with HAART as soon as treatment criteria are present to improve their immunosuppression and decrease the co-morbidities. In this study the high frequency of persistent diarrhea (19%) stands out, with episodes lasting up to 3 months. This percentage is higher than reported in the general population, where only 5–10% of diarrhea episodes are persistent. However, it is known that persistent diarrhea is significantly more common among HIV-infected children.^{2,17,30} Persistent diarrhea is multi-factorial; it can result from multiple consecutive infections, a single pathogen related unresolved infection, secondary malabsorption, or as a post-infection syndrome.²⁹ In addition, chronic diarrhea in advanced AIDS, can be related to drug side effects, gastrointestinal malignancies, or HIV enteropathy.²⁹

The frequency of antibiotic resistance among diarrheagenic *E. coli* samples was high, especially for ampicillin and cotrimoxazole. However, these percentages were similar to those found in other studies of diarrheagenic *E. coli* isolated from non-HIV-infected infants in Lima (ampicillin 85% and cotrimoxazole 79% resistance).³¹ Of interest, half of the *E. coli* strains were multidrug resistant (resistant to more than three antimicrobial agents), as has been reported in EAEC samples isolated from adults and children with or without HIV in southern India; most of the multidrug resistant isolates were from children less than 5 years of age.³² One-third of *E. coli* isolated were resistant to nalidixic acid and none were highly resistant to ciprofloxacin (17% showed intermediate resistance to ciprofloxacin). However, resistance to nalidixic acid in a community is a marker of future resistance to quinolones; this is worrisome because these drugs are among the few available oral agents to treat bacterial gastroenteritis.

In this study, the recruitment of patients lasted 2 years. There were few children hospitalized with diarrhea ($N = 49$), because the majority were on HAART. This represents a significant improvement in the quality of care of HIV-infected children in comparison to the previous decade in Lima. Because the recruitment of children with diarrhea was spread over a 2-year period, we were not able to find matched-age and season controls without diarrhea. The age difference between the diarrhea and control groups represents one of the limitations of the study. However, the age distribution in the control samples reflects the age distribution of all HIV-infected children in our setting²⁵; the fact that diarrhea was more common in younger children shows that age is an important factor in pediatric HIV-associated diarrhea. The differences found in the characteristics of the HIV infection between children with and without diarrhea is an indicator of the success of the current HIV treatment regimens and HAART in Peru and elsewhere.

In summary, HIV children with diarrhea had worse immunosuppression and viral load, and less frequently were on HAART, than HIV children without diarrhea. Diarrheagenic *E. coli* were the most commonly isolated pathogens in HIV-infected children and were highly resistant to ampicillin and cotrimoxazole. Because diarrhea is sometimes treatable with antibiotics, molecular studies for diarrheagenic *E. coli* could be part of the workup of children with HIV and prolonged diarrhea, and for surveillance studies of antimicrobial resistance.

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