

# Quantitative Real-time Polymerase Chain Reaction for Enteropathogenic *Escherichia coli*: A Tool for Investigation of Asymptomatic Versus Symptomatic Infections

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**Background.** Enteropathogenic *Escherichia coli* (EPEC) strains are pediatric pathogens commonly isolated from both healthy and sick children with diarrhea in areas of endemicity. The aim of this study was to compare the bacterial load of EPEC isolated from stool samples from children with and without diarrhea to determine whether bacterial load might be a useful tool for further study of this phenomenon.

**Methods.** EPEC was detected by polymerase chain reaction (PCR) of colonies isolated on MacConkey plates from 53 diarrheal and 90 healthy children aged <2 years. DNA was isolated from stool samples by cetyltrimethylammonium bromide extraction. To standardize quantification by quantitative real-time PCR (qRT-PCR), the correlation between fluorescence threshold cycle and copy number of the intimin gene of EPEC E2348/69 was determined.

**Results.** The detection limit of qRT-PCR was 5 bacteria/mg stool. The geometric mean load in diarrhea was 299 bacteria/mg (95% confidence interval [CI], 77–1164 bacteria/mg), compared with 29 bacteria/mg (95% CI, 10–87 bacteria/mg) in control subjects ( $P = .016$ ). Bacterial load was significantly higher in children with diarrhea than in control subjects among children <12 months of age (178 vs 5 bacteria/mg;  $P = .006$ ) and among children with EPEC as the sole pathogen (463 vs 24 bacteria/mg;  $P = .006$ ).

**Conclusions.** EPEC load measured by qRT-PCR is higher in diarrheal than in healthy children. qRT-PCR may be useful to study the relationship between disease and colonization in settings of endemicity.

Enteropathogenic *Escherichia coli* (EPEC) strains are among the most important pathogens infecting children worldwide, because they are common and are often associated with prolonged illness with its attendant risk of

malnutrition [1]. These pathogens induce a distinctive histopathology known as attaching effacing (A/E) lesion, which is characterized by the intimate contact between bacteria and the epithelial surface of the enterocyte [2]. The protein intimin (*eaeA*), necessary for the A/E lesion, has been used for the molecular identification of EPEC. The current average prevalence of EPEC in pediatric diarrheal episodes in developing countries, using molecular methods, is 5%–10% [1]. There is a trend, although often not statistically significant, for more frequent isolation of this pathogen among children with diarrhea than among healthy control subjects [3–6]. Recently, in a passive surveillance diarrhea cohort study involving 1034 Peruvian children, we isolated EPEC with a similar frequency from children with diarrhea (7.6%) and from asymptomatic controls (9.9%) [7]. However, multiple

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studies have established the virulence of EPEC, and remarkable progress has been made to identify the virulence determinants required to mediate the pathogenesis of these infections [8]. Our hypothesis was that presence of symptoms in EPEC infection relates to the bacterial load. The aims of this study were to develop, standardize, and validate a quantitative real-time polymerase chain reaction (qRT-PCR) assay for EPEC and to determine whether bacterial load relates to symptom status.

## METHODS

### Patients and Samples

Patients and samples included in this study were selected from a passive surveillance diarrhea cohort study conducted with 1034 children <2 year of age from Lima, Perú [7]. A total of 143 stool specimens previously found to be positive for EPEC by PCR of colonies isolated on MacConkey plates of stool culture were analyzed in this study. All stool samples had been tested for specific genes of all 6 pathotypes of diarrheagenic *E. coli*, including *stx1* and *sxt2* of Shigatoxin-producing *E. coli* [9]. We excluded for this study *eaeA*-positive samples positive for Shigatoxin (*stx1* and *stx2*). Fifty-three stool specimens were from patients with diarrhea (26 aged <12 months and 27 aged ≥12 months), and 90 stool samples were collected from randomly selected healthy children without diarrhea 1 week before or after the stool sample collection (31 aged <12 months and 59 aged ≥12 months). Twenty-six samples were from children with a coinfection or cocolonization that included both an EPEC and another common enteric pathogen (*Campylobacter*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, or diffusely adherent *E. coli*). In 117 children, EPEC strains were the only pathogen isolated from the stool sample. Fresh stool samples were collected in capped containers and were stored at -20°C until evaluated by PCR. The prototypical strain EPEC E2348/69 was used as a positive control for standardization of the quantitative method.

Clinical information on the diarrheal episodes was obtained from the medical records filled by study personnel at the time of illness. We used a modified Vesikari score to determine the severity of an EPEC-associated diarrhea episode [7, 10]. The maximum possible score was 18. We defined illness as mild (score, 0–8 points), moderate (9–14 points), or severe (15–18 points).

### DNA Isolation From Culture and Stool Samples

Genomic DNA was isolated from a pure culture of EPEC strain E2348/69, grown for 12–18 hours at 37°C, and extracted with the phenol-chloroform method. Ten-fold serial dilutions (equivalent to 10<sup>1</sup> to 10<sup>6</sup> bacilli) were prepared to determine the detection limit. According to GenBank (NC\_011601), EPEC genome has 4 965 553 basepairs (bp), which is equivalent to 5.44 fg and represents 2 copies of *eaeA* gen. The DNA was isolated

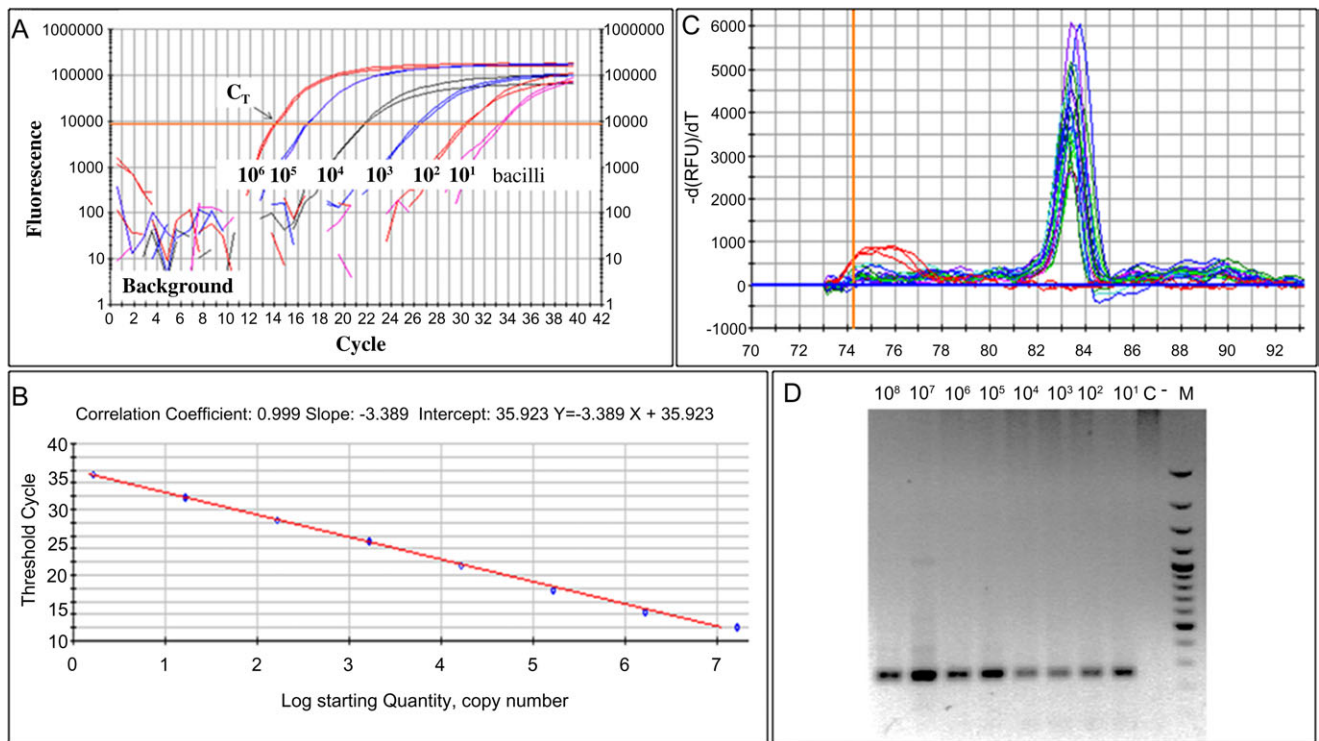
from stool specimens using a cetyltrimethylammoniumbromide extraction method modified from a method described elsewhere [11]. In brief, 50–300 mg of each stool specimen was dispersed in 250 µL of lysis buffer (0.25% sodium dodecyl sulfate in 0.1 M ethylenediaminetetraacetic acid [EDTA]; pH, 8.0), and 100 µg/mL of proteinase K was added. The lysate was incubated at 55°C for 2 hours. Then, 75 µL of 3.5 M sodium chloride (NaCl), followed by 42 µL of 10% CTAB/0.7 M NaCl (heated to 55°C), was added. After the components were mixed, the sample was incubated at 65°C for 30 minutes. This was followed by extractions with equal volumes of chloroform and then phenol-chloroform isoamyl alcohol (25:24:1), and the DNA was precipitated with ice-cold ethanol. The dried DNA pellet was dissolved in Tris-EDTA buffer and passed over a DNA clean-up spin column. The DNA was finally eluted from the spin column in 100 µL of Tris-EDTA 1× buffer; 2 µL of this DNA solution was used in the PCR.

### RT-PCR Assay for Quantification of EPEC E2348/69

PCR was performed using a iCycler IQ<sup>TM</sup> Multicolor Real-Time PCR Detection System (Bio-Rad, California) monitoring by an Optical System Software Version 3.1. Each PCR assay contained 0.5 U of Phusion polymerase (Finnzyme OY) in high-fidelity Phusion buffer, with a final concentration of 350 µM of each deoxyribonucleotide triphosphates and 4 mM MgCl<sub>2</sub>. The primers used for the detection of the *eaeA* gene were forward 5'-ATGCTTAGTGCTGGTTAGG-3' and reverse 5'-GCCTTCATCATTTTCGCTTTC-3' [9], and they were used at final concentrations of 0.5 µM. SYBR Green I (Cambrex Bio Science) was diluted as recommended by the manufacturer and mixed with 10 nM fluorescein. Hot-start of 98°C for 30 seconds was used to prevent nonspecific amplification. The amplification protocol consisted of incubation at 98°C for 20 seconds, 61°C for 20 seconds, 72°C for 30 seconds, and 75°C for 10 seconds. After 40 cycles, a melting curve using SYBR Green was determined with a ramp speed of 2.5°C per second at 73°C–95°C, with a reading every 0.2°C. Melting peaks were automatically calculated by the Opticon Monitor software 115, which plotted the negative derivative of fluorescence with respect to temperature (-d(F)/dT vs T). To make sure that we did not have inhibition of amplification because of the presence of contaminants in stool samples, we randomly selected some stool samples from children, mixed them with human DNA, and determined the presence of the *ERV3* (endogenous retrovirus) gene as an internal control [12]. We obtained positive *ERV3* results for all samples, including those that were negative for the *eaeA* gene.

### Amplification Efficiencies and Quantitation of Bacterial Load

We constructed standard curves by using known quantities of genomic DNA (1.04 × 10<sup>0</sup> to 1.04 × 10<sup>8</sup> fg) extracted from EPEC



**Figure 1.** Quantitative real-time polymerase chain reaction (RT-PCR) standardization for enteropathogenic *Escherichia coli* (EPEC). *A*, RT-PCR results from representative experiments using DNA from a pure culture of EPEC E2348/69. Fluorescence from the PCR products is plotted against the corresponding number of copies of intimin (*eaeA*) gene, corresponding to  $10^1$  to  $10^6$  bacilli, to obtain the threshold cycle ( $C_T$ ). *B*, Standard curve for the RT-PCR analysis was done from the same stock of DNA diluted 10-fold. We plotted  $C_T$  against the log of the number of *eaeA* copies; the reaction efficiency was  $>97.3\%$ . *C*, The melting temperature for the *eaeA* gene was  $83.8 \pm 0.23^\circ\text{C}$ ; curves are superimposed for the different DNA concentrations used in the analysis. *D*, Agarose gel (2%): (1–8) PCR products (248 pb) corresponding to the 10-fold dilutions ( $10^8$  to  $10^1$  bacilli); C, No template control; (M) 100-bp molecular weight ladder.

E2348/69 samples. For standard curve analyses, the threshold cycles ( $C_T$ ) were plotted against the corresponding log of input quantity DNA determining the detection limit of the assay. For a comparison of PCR amplification efficiencies and detection sensitivities among experiments, slopes of the standard curves were calculated by a linear regression analysis with iCycler 3.1 software (Bio-Rad). Amplification efficiency ( $E$ ) was estimated by using the slope of the standard curve and the formula  $E = (10^{-1/\text{slope}}) - 1$ . A reaction with 100% efficiency will generate a slope of  $-3.32$ . The amount of DNA was calculated as copies of *eaeA* gene per milligram of stool. From the quantification of the template DNA, an estimation of the relative bacterial load in the different samples was performed. To facilitate the interpretation, we have expressed the bacterial load as bacteria per milligram of stool instead of genome copies per milligram.

#### Statistical Analysis

To estimate bacterial load, an undetectable bacterial load in a previously positive isolated colony PCR was considered to be 0.01 bacteria/mg. To determine the differences in the bacterial load between diarrhea and control samples, the groups were compared using 2-sample Mann–Whitney  $U$  test. We fitted

logistic regression models with random intercept to assess whether bacterial load is associated with diarrhea for children with EPEC as part of a coinfection or as a single infection. We tested in the models for effects of potential confounders, such as age, sex, breastfeeding status, and complementary feeding. Only those variables that remained significant to the .05 level were retained in the final model. We used Stata software, version 10 (StataCorp), for analysis.

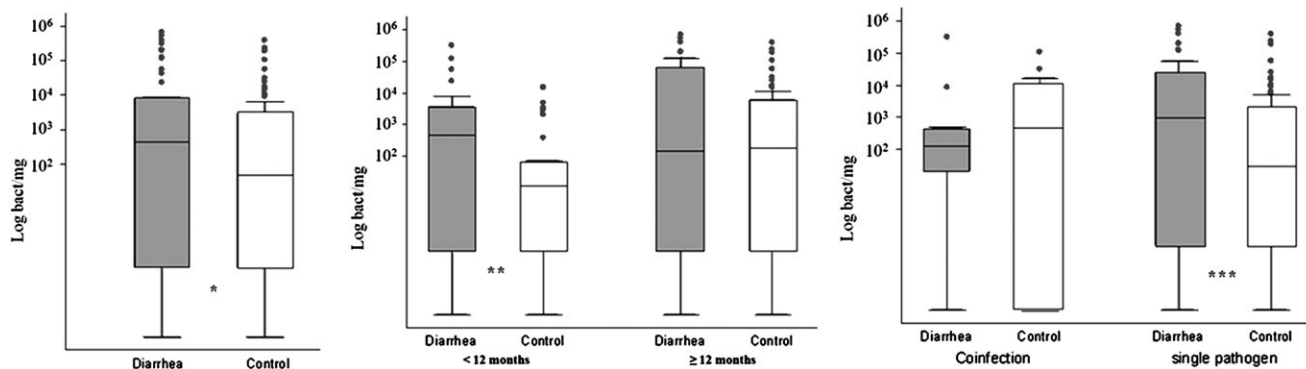
#### Ethical Aspects

The study was approved by the institutional review boards of Universidad Peruana Cayetano Heredia and Instituto de Investigación Nutricional, Lima, Perú.

## RESULTS

#### Standard Curves, Detection Limits, and Amplification Efficiencies

To demonstrate the detection range of the qRT-PCR, 10-fold serial dilutions containing  $1.04 \times 10^0$  to  $1.04 \times 10^8$  fg of genomic DNA  $\text{mL}^{-1}$  were assayed in triplicates for the *eaeA* gene of EPEC E2348/69 strain (Figure 1A). The results were reported as



**Figure 2.** Comparison of enteropathogenic *Escherichia coli* (EPEC) load among diarrhea and control samples. A, Diarrhea samples (gray bars) and samples from healthy control subjects (white bars). EPEC load in stool samples from children with (n = 53) and without (n = 90) diarrhea; \*P = .016. B, EPEC load in children <12 months of age (diarrhea, n = 26; control, n = 31) and children 12–24 months of age (diarrhea, n = 27; control, n = 59); \*\*P = .006. C, EPEC load in coinfections (diarrhea, n = 9; control, n = 13) and single-pathogen infection (diarrhea, n = 44; control, n = 77); \*\*\*P = .006.

threshold cycle numbers versus log starting quantities of DNA. The correlation coefficient was 0.999, and the PCR efficiency was 97.3% (Figure 1B). The detection limit of this PCR assay (standard curve method) was 10 copies of *eaeA* gene/mg stool (equivalent to 5 bacteria/mg stool). The melting curve analysis of the qRT-PCR *eaeA* product is shown in Figure 1C. The mean melting temperature (SD) for the *eaeA* gene was 84.3°C (0.3°C). The electrophoresis ran for the PCR products showed a unique specific band of 248 pb corresponding to the *eaeA* gene (Figure 1D).

#### Quantification of EPEC in Stool Samples

EPEC load in stool samples from infected or colonized children with a previously isolated colony positive by PCR ranged from ≤10 bacteria/mg to 4.03 × 10<sup>6</sup> bacteria/mg. The quantitative data showing EPEC load in diarrheal and healthy children are

shown in Figure 2 and Table 1. EPEC load was significantly higher (P = .016) in the diarrhea group than in the control group (Figure 2A). We found 46 samples with a fecal bacteria load ≤10 bacteria/mg, from which 71% were from healthy infants (33 samples). Because most reports on diarrheagenic *E. coli* show age-dependent differences in incidence rates, we divided the samples according to age groups (Figure 2B). Among children <12 months of age, the bacterial load in the diarrhea group was significantly higher than that in the control group (P = .006); there were no statistically significant differences among older children. Coinfections were common, accounting for 13% of diarrheal samples and 6% of control samples; there was not a statistically significant difference in the EPEC load between diarrhea and control groups when present as part of a mixed infection (Figure 2C). There were no differences in the

**Table 1. Enteropathogenic *Escherichia coli* Load in Stool Samples From Children With Diarrhea and Without Diarrhea (Control), Measured by Quantitative Real-time Polymerase Chain Reaction**

	Diarrhea geometric mean (95% CI) bacteria/mg	Control geometric mean (95% CI) bacteria/mg	P value
All stool samples	299 (77–1164) (n = 53)	29 (9–87) (n = 90)	.016
By age group			
<12 months	178 (27–1147) (n = 26)	5 (1–26) (n = 31)	.006
≥12 months	220 (18–2685) (n = 27)	29 (5–156) (n = 59)	.188
By type of EPEC infection			
Sole pathogen isolated	463 (97–2221) (n = 44)	24 (7–81) (n = 77)	.006
Coinfection	44 (1–1289) (n = 9)	35 (1–1657) (n = 13)	.732

Abbreviations: CI, confidence interval; EPEC, enteropathogenic *Escherichia coli*.

age distribution among sole pathogen and coinfection groups. The most common coinfections were EPEC with *Campylobacter* (n = 3) and EPEC with enterotoxigenic *E. coli* (n = 3). However, among stool samples with EPEC as a single pathogen, the bacterial load was significantly higher in diarrhea than in control samples (463 vs 24 bacteria/mg;  $P = .006$ ; Figure 2C). Of interest, we have not found differences in the mean number of positive EPEC colonies per patient (5 colonies were collected per patient from the MacConkey plate) from the initial PCR between diarrhea and control samples. Similarly, there was no correlation between the number of positive colonies per patient from the initial PCR from the MacConkey plate and the bacterial load determined directly from the stool samples.

### EPEC Load and Clinical Data

Among diarrhea cases, stool samples were collected on a variable number of days into illness. Overall, there was a tendency for a higher bacterial load during the first days of illness (Table 2). Complete clinical information was available in 39 episodes; 29 (74%) corresponded to a mild episode, 9 (23%) to a moderate episode, and 1 (3%) to a severe episode. The bacterial load was similar between mild and moderate cases (144 vs 95 bacteria/mg;  $P = .722$ ). The duration of the diarrhea was known in 45 episodes; the mean duration (SD) was 6.8 (5.9) days (median, 5 days; range, 1–24 days). Twenty-eight episodes lasted <7 days, 10 lasted 7–14 days, and 7 lasted >14 days. We did not find any difference in the bacterial load as related to the duration of the episode (444 vs 184 vs 146 bacteria/mg for <7, 7–14, and >14 days, respectively). We also explored bacterial load as related to breastfeeding of the child at the time of the sample collection. Ninety-six children were breastfeeding (37 diarrheal and 59 controls), whereas 46 were not (16 diarrheal and 30 controls). Among children breastfeeding, there were no statistically significant differences in the bacterial load of diarrheal and control samples (299 vs 54 bacteria/mg;  $P = .133$ ). Among children who were not breastfeeding at the time of sampling,

a significantly higher bacterial load was found in diarrhea samples than in healthy control samples (300 vs 6 bacteria/mg, respectively;  $P = .038$ ).

### Association Between Bacterial Load and Diarrhea

The coefficients for the logistic regression with random effects for children with EPEC as part of a coinfection or as a single infection were determined after adjusting for age, sex, and an interaction of both. For a given child, the odds of diarrhea increased by 29% (odds ratio, 1.29; 95% CI, 1.08–1.53) for each  $\log_{10}$  unit increase in bacterial load in the stool sample.

## DISCUSSION

The interpretation of the isolation rate of a pathogen in stool samples from children with diarrhea, compared with asymptomatic colonization, is complex. Factors that should be considered in interpretation include bacterial factors (bacteria with more virulent genes), host factors (age, genetic susceptibility, previous exposure, development of immunity, and protective factors from breastfeeding), and environmental factors (poor hygiene and high and frequent fecal contamination). With the development of new diagnostic methods, such as RT-PCR, the detection limit is now very low; therefore, detection of pathogens can occur in samples from healthy control children. Our results show that EPEC load was significantly higher in children with diarrhea than in asymptomatic control subjects, especially in single pathogen. Therefore, we propose that, in addition to the aforementioned factors, the bacterial load should be considered in the interpretation of illness and colonization of pathogens in the gut.

Several epidemiological studies from developing countries have been conducted in children to determine age-related differences in diarrheagenic *E. coli* infections [7, 13–19]. Similar to these studies, we found a relationship between age and symptoms. Our analysis showed that, in children <12 months of age with diarrhea, the bacterial load was higher than that in healthy control subjects infected with the same pathogen, consistent with the generally accepted view that EPEC is a true pathogen in young children. There appears to be a reduced threshold of bacterial load required for the initiation of EPEC infection in younger infants; however, the bacterial load in patients with diarrhea appears to remain unchanged in younger and older infants, probably related to presence or absence of protective factors.

In developing countries, mixed infections are common, especially in diarrheal samples [7], making it difficult to determine which pathogen is responsible for symptoms. In this study, among mixed infections, we found a similar EPEC load in children with and without diarrhea, suggesting that EPEC may not have had a role in these episodes of diarrhea. However, among stool samples with EPEC as a single-pathogen infection,

**Table 2. Enteropathogenic *Escherichia coli* Load in Relation to the Day of Illness**

Day of illness	No. (%) of samples <sup>a</sup>	Geometric mean (95% CI) bacteria/mg
1	4 (9)	855 (.2–4 028 568)
2	10 (22)	154 (.7–34 157)
3	9 (20)	384 (4–34 476)
4	6 (13)	283 (11–7482)
5	7 (15)	149 (2–12 685)
6	1 (2)	3520
≥7	9 (20)	188 (15–2318)

Abbreviation: CI, confidence interval.

<sup>a</sup> Forty-six samples were included; 7 samples were excluded because there was no recorded date of the beginning of the episode.

we found a strong association between EPEC and diarrhea, shown by the higher bacterial load in children with diarrhea than in healthy controls. This suggests that EPEC is a true pathogen when present as the sole pathogen isolated; however, in mixed infection, we hypothesize that higher EPEC loads would be required to overcome a mixed infection. Similarly, in children breastfeeding at the time of the sample collection, there was no difference in the bacterial load in diarrhea and control samples. However, in children not breastfeeding, we found a higher bacterial load in children with diarrhea. This finding supports the notion that breast milk protects these children from symptomatic infection [20–23]. Conversely, breast milk may have decreased the load but not totally prevented EPEC-related symptoms in these children. Further studies will be needed to confirm each of these important conclusions.

With the implementation of molecular tools, the detection limit of pathogens has improved, and quantification is now possible [24]. In this study, we developed, standardized, and validated a qRT-PCR for EPEC in stool samples. The approach reported here is not entirely new. RT-PCR assays have been developed in the past for the detection and quantification of enteropathogens in food, animal, and clinical samples [25], including assays for *Campylobacter*, *Cryptosporidium*, *Salmonella*, *E. coli* O157:H7, and other Shiga toxin-producing *E. coli* [26–32]. Overall, the most common DNA isolation method has been the use of commercial kits, which are expensive and are not easily available in developing countries. The current study proposes the application of a bromide-extraction method as an alternative DNA isolation method, which is cheaper than commercial kits. It should be noted that this study is, to our knowledge, the first study that applies a qRT-PCR analysis for EPEC in human samples and compares stool samples from children with and without diarrhea.

This study has some limitations. First, we did not search for all possible enteric pathogens (norovirus, astrovirus, enteric adenovirus, *Yersinia*, and parasites) to define the single-pathogen infection with EPEC. This could account for some overlap in groups of patients with only EPEC infection found, because in some cases, unrecognized mixed infections might have been present. However, we evaluated stool samples for the most common pathogens in children (rotavirus, all diarrheagenic *E. coli*, *Shigella*, *Salmonella*, and *Campylobacter*). Second, we did not evaluate factors that may explain why EPEC was excreted in asymptomatic individuals (eg, protective antibody levels, lower ingestion dose, transplacental maternal immunity, and zinc levels). Third, the number of diarrheal samples was not sufficient to optimally determine the relationship between bacterial load and duration of illness and diarrhea severity or the effect of breastfeeding and age on bacterial load. Future studies are needed with larger numbers of EPEC diarrheal samples to clarify these important issues.

In summary, we developed, standardized, and validated a qRT-PCR for EPEC in stool samples with use of an inexpensive bromide DNA extraction method that can be used as a diagnostic tool to clarify the differences between colonization and illness. We found that the bacterial load of EPEC, measured by qRT-PCR of stool samples, is higher in children with diarrhea than in healthy control subjects. We have identified areas, outlined above, for further investigation using this approach.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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