A Comparison of Stool Enteropathogen Detection by Semiquantitative PCR in Adults With Acute Travelers' Diarrhea Before and 3 Weeks After Successful Antibiotic Treatment

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We evaluated stool enteropathogen detection by semiquantitative polymerase chain reaction (PCR) in 108 subjects with travelers' diarrhea before and 3 weeks after treatment. Stool samples from 21 subjects were positive for the same pathogen species at both visits. We discuss factors that should be considered when interpreting stool PCR data after treatment.

Keywords. travelers' diarrhea; PCR; military; infectious disease.

Acute infectious diarrhea remains a common illness in deployed military personnel and travelers. Polymerase chain reaction (PCR)–based detection is being increasingly used for high-throughput rapid detection of enteropathogens from fecal specimens in travelers' diarrhea (TD) field studies. The increased sensitivity of PCR compared with culture-based methods has led to interpretative challenges in differentiating true pathogens from noncontributing bystanders. Several studies have compared the distribution of fecal pathogens among TD cases and asymptomatic travelers to determine the

odds of TD associated with detected pathogens [1–3]. In addition, the growing availability of culture-independent diagnostics for use in the ill-returning traveler with persistent abdominal symptoms has created a diagnostic conundrum, as multiple organisms are often identified due to the potential accumulation of bystanders in travel exposures [4]. There are few data comparing pathogen detection by PCR during a TD episode and after successful treatment [5]. The objectives of this study were to compare pathogen distribution by semiquantitative PCR in stool samples obtained before (day 0) and 3 weeks (day 21) after TD treatment and to describe the PCR data of subjects who were positive for the same pathogen species in paired samples.

We used a prospective, well-characterized cohort of deployed military personnel enrolled in a TD treatment trial to conduct this post hoc analysis [6]. The trial evaluated the effectiveness of 3 single-dose antibiotic regimens (azithromycin, rifaximin, or levofloxacin) plus loperamide for treatment of acute watery diarrhea (defined as ≥3 unformed stools in 24 hours or >2 loose stools in 24 hours associated with nausea, vomiting, abdominal cramps, or tenesmus) and the use of azithromycin with and without loperamide for treatment of febrile diarrhea or dysentery. Subjects provided a stool sample before treatment and, optionally, 21 days (± 7 days) after treatment. Subjects were assessed for TD-associated symptoms at 24 hours, 72 hours, and 7 days post-treatment but were not assessed for TD symptoms at the day 21 visit. We included subjects who provided stool samples at day 0 and day 21 and achieved clinical cure. Clinical cure was defined as (1) no reported diarrheal stools beyond 24 hours after initiation of therapy, (2) all diarrhea-associated symptoms present at 24 hours post-therapy being no greater than mild in severity, and (3) no impact on activity and no evidence of treatment failure.

Fresh stool specimens were processed by on-site laboratories, and an aliquot (180–220 mg or 220 μL if liquid stool) was placed in a cryovial, stored at −80°F, and batch-shipped to the Naval Medical Center at Portsmouth, Virginia, for PCR testing. Nucleic acid was extracted with the QIAamp Fast DNA Stool mini kit (Qiagen, Valencia, CA) using a modified protocol that included spiking with external controls (MS2 bacteriophage and phocine herpes virus) and bead beating [7]. Extraction blanks were included to monitor contamination. A customized semiquantitative PCR assay, TaqMan Array Card, was used for detection of 20 enteropathogens and associated virulence genes (Supplementary Table 1) [8–10]. A quantification cycle (Cq) value of 35 was used as the cutoff for positivity [8, 9].

Three hundred eighty-seven subjects were enrolled in the study, and of these 108 (28%) had paired day 0 and day 21 stool samples that met inclusion criteria. Participants who did not
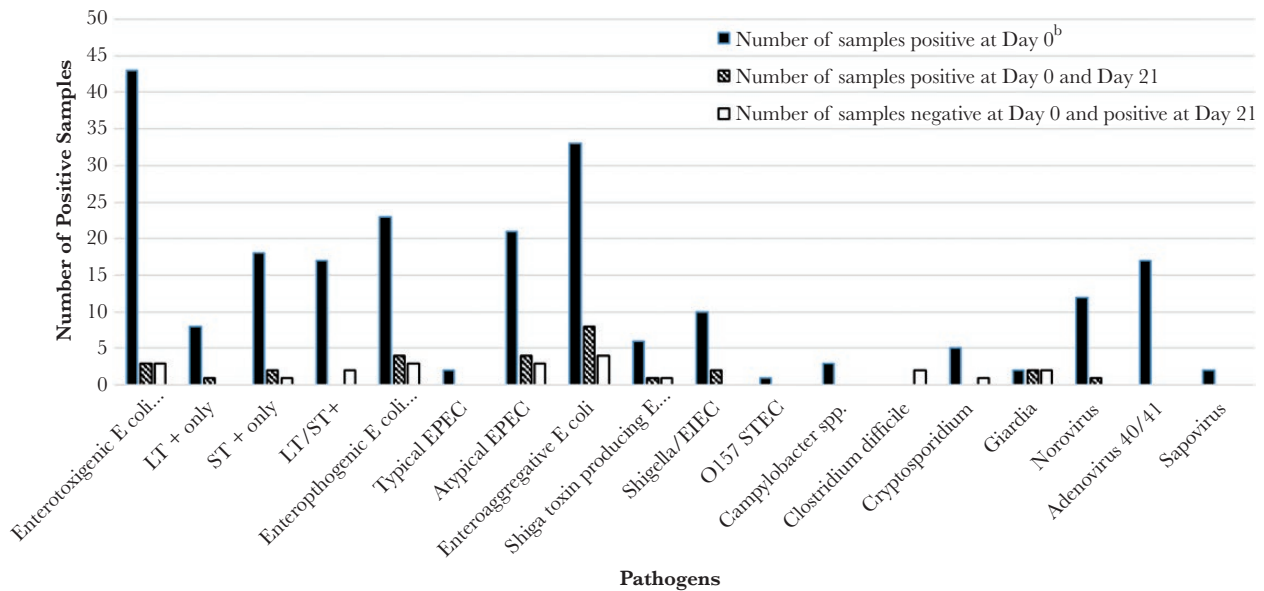
Figure 1. Distribution of pathogens in the 108 paired stool samples from subjects with travelers' diarrhea collected at day 0 before antibiotic therapy and day 21 after treatment.[a] [a]No samples were positive for *Aeromonas, Astrovirus, Campylobacter coli, Cyclospora, Entameoba histolytica, Rotavirus, Salmonella, Vibrio parahemolyticus.* [b]Represents all samples positive at day 0 (regardless of positivity at day 21). Abbreviations: EIEC, enteroinvasive *Escherichia coli*; EPEC, enteropathogenic *E. coli*; LT, heat-labile; ST, shiga toxin; STEC, shiga toxin–producing *E. coli.*

provide a paired sample (n = 168), had an insufficient stool volume for testing (n = 93) due to a freezer breakdown at a study site, or did not achieve clinical cure (n = 1) were excluded. Based on the external control data, PCR results were deemed indeterminate for 17 paired specimens. Paired samples that met inclusion criteria were from subjects enrolled in Honduras (n = 35, 32.4%), Kenya (n = 53, 49.1%), and Djibouti (n = 20, 18.5%). The median duration from day 0 and day 21 sample collection to testing was 15.8 and 20.8 months, respectively, as samples were batch-shipped from the study sites at the end of the trial. At least 1 pathogen was identified in 76.9% (83/108) of day 0 samples, with 55.0% (46/83) positive for multiple pathogens (average number of pathogens ± SD, 1.9 ± 1.0) (Figure 1). The overall detection rate in day 21 samples was 25.0% (27/108), with 9 (33.3%) samples positive for >1 pathogen (average number of pathogens ± SD, 1.4 ± 0.6). Twenty-one pathogens detected at day 0 were also detected in the corresponding sample at day 21 (Table 1). Diarrheagenic *Escherichia coli* (ie, enterotoxigenic *E. coli*, enteroaggregative *E. coli* [EAEC], and enteropathogenic *E. coli*) accounted for the majority of pathogens detected in paired samples (n = 15). Other pathogens detected in paired samples were *Shigella*/enteroinvasive *E. coli* (n = 2), shiga toxin–producing *E. coli* (n = 1), *Giardia* (n = 2), and norovirus (n = 1). A difference in the toxin gene profile between day 0 and day 21 samples was noted for pathogens with multiple gene targets (ie, enterotoxigenic *E. coli* [2 of 3 paired samples showed a difference in toxin gene profile] and enteroaggregative *E. coli* [6 of 8 paired samples showed a difference in toxin gene profile]), possibly due to acquisition of new strains after treatment. Paired positive samples with the same toxin gene profile

Table 1. Paired Samples Positive for the Same Pathogen Species at Day 0 and Day 21 – Average Quantitative Cycle (Cq) Value[a] of Day 0 and Day 21 Samples

Pathogen (No. of Positive Paired Samples)	Day 0	Day 21
Enterotoxigenic *E. coli* (n = 1)[b]	27.6	33.9
Enteroaggregative *E. coli* (n = 2)[c]	25.2 ± 0.4	31.7 ± 4.1
Atypical enteropathogenic *E. coli* (n = 4)	26.3 ± 3.2	28.4 ± 1.7
Shigella/enteroinvasive *E. coli* (n = 2)	20.7 ± 0.4	28.7 ± 2.8
Shiga toxin–producing *E. coli* (n = 1)	31.5	34.8
Giardia (n = 2)	27.1 ± 4.8	23.7 ± 6.4
Norovirus (n = 1)	29.9	33.9

[a]Average Cq value across all positive gene targets for a pathogen. ETEC and EAEC had multiple gene targets, and the remaining pathogens had 1 gene target.

[b]One of 3 paired samples positive for ETEC retained the gene profile between days 0 and 21 (STp+, CS6+ at day 0 and 21) and was included in table above; 2/3 paired samples demonstrated a difference in ETEC toxin profile between paired samples and were not included in the table above (Sample 1: LT+, STp+, STh+, and CS6+ on day 0, and only STp+ on day 21; Sample 2: LT+, STp+, CS1, CS2, CS21, CS3, and CS6+ on day 0, and only LT+ only on day 21).

[c]Two of 8 paired samples positive for EAEC retained the gene profile between day 0 and day 21 (Sample 1: aaiC+, aatA+, aggR+ on days 0 and 21; Sample 2: aaiC+ on days 0 and 21) and were included in table above. Six of 8 paired samples positive for EAEC demonstrated a change in toxin profile between days 0 and 21 and were not included in the table. Two samples were aaiC+, aatA+, aggR+ on day 0 and aaiC+ and aggR+ on day 21, and 1 sample was positive for each of the following: day 0: aaiC+, aatA+, and aggR+; day 21: aatA+ and aggR+; day 0: aaiC+; day 21: aatA+ and aggR+; day 0: aaiC+ and aggR+; day 21: aaiC+; day 0: aatA+ and aggR+; day 21: aaiC+.

demonstrated an increase in the mean Cq value between days 0 and 21, indicating a decrease in the target gene copy numbers after treatment. Samples positive at day 0 that remained positive at day 21 had a similar mean Cq value compared with samples positive at day 0 but negative at day 21. The proportion of subjects with paired positive samples was similar among the 3 treatment arms: rifaximin 22% (6/27), azithromycin 28% (10/36), and levofloxacin 16% (5/31).

Certain pathogens detected by the TaqMan Array Card were not covered by the prescribed antibiotic regimens. A decrease in the number of samples positive for *Cryptosporidium* spp. and norovirus was noted between day 0 and day 21, suggesting that these were potentially mild infections or short-lived carriage in immunocompetent hosts (Figure 1) [11, 12]. Two subjects had paired samples positive for *Giardia* but achieved clinical cure, indicating that they may have had self-limited infection. Lastly, the *Clostridium difficile* tcdB gene was detected in 2 subjects at day 21, but the PCR data are difficult to interpret in the absence of symptom data and testing with assays for toxin production (eg, enzyme immunoassay for glutamate dehydrogenase or toxin B).

Our findings indicate that most subjects with TD (74%) do not have detectable bacterial DNA 3 weeks after successful antibiotic treatment. In subjects with detectable pathogen by quantitative or qualitative PCR after treatment, several factors must be considered when interpreting PCR data, including antibiotic coverage, PCR efficiency, changes in toxin gene profile, presence of symptoms, and timing of sample collection. In addition, this was a cohort of military personnel on long-term deployment to high–TD risk regions; these individuals were at continued risk of exposure after successful TD treatment. These findings may not apply to short-term travelers who return to a developed country setting after TD treatment and are no longer exposed to TD pathogens. Among subjects with persistent detection despite appropriate treatment and resolution of symptoms, higher target gene Cq values were observed after treatment, possibly due to detection of residual DNA from nonviable organisms or acquisition of a new infection. A change in gene profile was detected between day 0 and day 21 in two-thirds of paired samples positive for ETEC or EAEC, suggesting that some instances of "persistent detection" may be due to acquisition of new strains of the same bacterial species after appropriate treatment. Analyzing stool in its entirety limits the ability to determine if enterotoxin detection is from the same or different strains of *E. coli*. Lastly, persistent detection was also noted with asymptomatic carriage and self-limited or mild infection caused by pathogens that were not covered by antibiotic therapy (ie, *Giardia* and norovirus).

There are important limitations of this post hoc analysis, including the lack of TD symptom data between achievement of clinical cure and day 21. This makes it difficult to assess whether pathogen detection at day 21 was due to additional TD episodes and if collection of day 21 samples was biased (ie, subjects who were symptomatic at day 21 were more likely to provide a stool sample). The challenge of obtaining a day 21 sample in a deployment setting limited the sample size of available paired samples and our ability to evaluate the impact of PCR efficiency and the prolonged duration of storage on Cq values. Additional studies that perform PCR testing at predefined intervals and include symptom surveillance are needed to understand the duration of pathogen detection after TD treatment and the value of semiquantitative PCR assays to identify attribution of pathogenicity of identified organisms using these platforms, which are finding their way into vaccine and drug development and clinical management.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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