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# Real-Time PCR Threshold Cycle Cutoffs Help To Identify Agents Causing Acute Childhood Diarrhea in Zanzibar

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**Molecular assays might improve the identification of causes of acute diarrheal disease but might lead to more frequent detection of asymptomatic infections. In the present study, real-time PCR targeting 14 pathogens was applied to rectal swabs from 330 children aged 2 to 59 months in Zanzibar, including 165 patients with acute diarrhea and 165 asymptomatic control subjects. At least one pathogen was detected for 94% of the patients and 84% of the controls, with higher rates among patients for norovirus genogroup II (20% versus 2.4%; $P < 0.0001$), rotavirus (10% versus 1.8%; $P = 0.003$), and *Cryptosporidium* (30% versus 11%; $P < 0.0001$). Detection rates did not differ significantly for enterotoxigenic *Escherichia coli* (ETEC)-*estA* (33% versus 24%), ETEC-*eltB* (44% versus 46%), *Shigella* (35% versus 33%), and *Campylobacter* (35% versus 33%), but for these agents threshold cycle ($C_T$) values were lower (pathogen loads were higher) in sick children than in controls. In a multivariate analysis, $C_T$ values for norovirus genogroup II, rotavirus, *Cryptosporidium*, ETEC-*estA*, and *Shigella* were independently associated with diarrhea. We conclude that this real-time PCR allows convenient detection of essentially all diarrheagenic agents and provides $C_T$ values that may be critical for the interpretation of results for pathogens with similar detection rates in patients and controls. The results indicate that the assessment of pathogen loads may improve the identification of agents causing gastroenteritis in children.**

Acute diarrheal disease is the second most common cause of death worldwide in children younger than 5 years (1). Most of these deaths occur in low-income countries, where the etiologies of diarrheal infections have been incompletely understood because there are few comprehensive studies (2, 3). Such studies often used traditional diagnostic methods, such as culture, microscopy, or antigen detection, or focused on only one or a few diarrheal pathogens.

New multitargeting molecular PCR methods allow detection of diarrheal pathogens with high specificity and sensitivity (4–7), and their application may lead to improved understanding of diarrheal disease epidemiology. These methods provide better identification of viruses that cannot be cultured (e.g., *Caliciviridae*) or that previously have been diagnosed with methods with relatively low sensitivity (e.g., antigen testing for rotavirus) (8–10). They also have been shown to improve the detection of bacteria because of their higher sensitivity than culture (11–15). However, the mere presence of a pathogen in a fecal sample does not necessarily imply that it is the cause of disease, since high detection rates have been reported also for asymptomatic individuals with both conventional (2, 16) and molecular (12) methods. This is of particular importance in low-income countries, where children may be exposed to multiple enteric pathogens due to poor sanitary conditions. Thus, understanding the causes of diarrheal disease and how test results should be interpreted requires knowledge of the presence of pathogens in feces from both ill and healthy individuals.

In the present study, we used a broad real-time PCR assay to analyze pathogens in children, with or without diarrhea, in Zanzibar. In addition to comparing detection rates, we aimed at evaluating the potential utility of pathogen loads, in terms of real-time PCR threshold cycle ($C_T$) values, to separate symptomatic from asymptomatic infections, as suggested by studies on norovirus, rotavirus, and *Shigella* infections (17–19).

## MATERIALS AND METHODS

**Study participants. (i) Patients.** Children 2 to 59 months of age who presented to the Kivunge Primary Health Care Centre (PHCC) in rural Zanzibar (North A district) with fever (measured axillary temperature of ≥37.5°C or a history of fever during the preceding 24 h, according to the accompanying guardian) and diarrhea (history of loose stools during the preceding 24 h) were eligible for study inclusion. Children with signs of severe disease according to Integrated Management of Childhood Illness (IMCI) guidelines (http://www.who.int/child_adolescent_health/documents/IMCI_chartbooklet/en/index.html) were excluded. Recruitment was performed in April to July 2011, corresponding to the end of the rainy season and the beginning of the dry season.

**(ii) Asymptomatic control subjects.** Control subjects matched for living area and sampling time period, i.e., asymptomatic children 2 to 59 months of age, were recruited once a week during the entire study period, together with local representatives from 8 villages in the study area. No more than 2 children per household were recruited. An asymptomatic child was defined as having no history of diarrhea, cough, running nose, or fever in the preceding 10 days.

The study was approved in Zanzibar by the Zanzibar Medical Research

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Ethics Committee and in Sweden by the regional ethical review boards in Stockholm and Gothenburg. Written informed proxy consent was obtained from a guardian of all enrolled patients and asymptomatic control subjects. No financial incentives were given.

**Samples.** Rectal swab samples were collected in a standardized manner with flocked swabs (Copan regular flocked swab 502CS01; Copan Italia Spa, Brescia, Italy) introduced 2 to 3 cm into the rectum and rotated. Directly after sampling, the swabs were placed in sterile vials containing 1 ml of 0.9% NaCl. Directly after rectal swab collection from asymptomatic community controls, the vials were placed in a vaccine carrier with a controlled temperature of 2 to 8°C. All swabs from controls and patients were transferred to microtubes, using disposable transfer pipettes, within 2 h after collection and were stored at a controlled temperature of −70°C. After completion of the field trial, all samples were transported to Sweden, on dry ice, for molecular analyses.

**Extraction of nucleic acids and real-time PCR.** Following defrosting and brief vortex-mixing, 250 μl of the suspension was mixed with 2 ml of lysis buffer. Nucleic acids were then extracted into 110 μl of elution buffer with a NucliSENS easyMAG robot (bioMérieux, Marcy l'Etoile, France). By diluting samples and extracting nucleic acids with an easyMAG instrument, inhibition of PCR was effectively prevented (20).

Amplification was carried out in an ABI 7900 instrument (Applied Biosystems, Foster City, CA). After a reverse transcription step, 45 cycles of two-step PCR (95°C for 15 s and 56°C for 60 s) were performed in 10 parallel reactions, targeting a broad range of diarrheagenic agents as described in Table 1. The result for each agent was recorded as the $C_T$ value, which is inversely related to the pathogen load in each specimen. The potential utility of this quantitative information was evaluated by comparing $C_T$ values for patients and controls, as discussed below.

**Microbial agents and target sequences.** The targets for real-time PCR are presented in Table 1. The amplified regions of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus were located in conserved genomic regions (21–25), and these assays have been used in our diagnostic laboratory for several years. Bacterial PCRs were developed with guidance from available publications with respect to suitable target regions (26–35), usually by adapting a traditional PCR method to real-time PCR (when this study was planned, suitable real-time PCR assays were lacking for most nonviral targets). Thus, established target regions were used, and primers and probes were designed with the aim of obtaining similar melting temperatures (~58 to 60°C for primers and ~68 to 70°C for probes). For enterotoxigenic *Escherichia coli* (ETEC), both heat-labile toxin (*eltB*) and heat-stable toxin (*estA*) coding regions were targeted. *Shigella* was identified by amplification of the invasion plasmid antigen H (*ipaH*) gene (which also may be present in enteroinvasive *E. coli* [EIEC]), *Campylobacter jejuni* by the fibronectin-binding protein (*cadF*) gene, and *Cryptosporidium parvum/hominis* by the oocyst wall protein (OWP) gene.

Sufficient amplification efficiencies were documented for each real-time PCR by analyzing serial dilutions of pUC57 plasmids carrying synthetic target inserts. By comparing $C_T$ values for each target amplified alone or in duplex or triplex reactions, it was confirmed that performance was not compromised by multiplexing. In addition to optimization, which focused on analytical sensitivity, diagnostic accuracy was evaluated by analyzing well-characterized bacterial strains from the Culture Collection, University of Gothenburg (*Campylobacter jejuni*, *Salmonella enteritidis*, *Shigella flexneri*, *Vibrio cholerae*, and *Yersinia enterocolitica*), and the Department of Bacteriology at Sahlgrenska University Hospital (ETEC producing heat-labile [*eltB*] or heat-stable [*estA*] toxin). *Cryptosporidium* PCR was tested by analyzing *Cryptosporidium parvum* DNA purchased from the American Type Culture Collection (ATCC PRA-67D). The specificity of PCR for adenovirus types 40 and 41 was confirmed by analyzing DNA extracted from cultures of adenoviruses representing different genogroups.

**Identification of $C_T$ cutoff values.** For pathogens with significant differences in $C_T$ values between patients and controls (Mann-Whitney *U* test), a cutoff value was identified as the $C_T$ value that gave the highest balanced accuracy, defined as 0.5 × (sensitivity + specificity). This cutoff value also produced the lowest *P* values with the $\chi^2$ test. Agents classified as more likely causes of diarrhea were those with marked differences (odds ratio [OR] of >4) in either crude detection rates or proportions with $C_T$ values below the defined cutoff value.

**Statistics.** Statistical analysis was performed using Statview (SAS Institute), JMP (SAS), and Stata (StataCorp LP) software. Fisher's exact test was used for group comparisons of proportions of samples that were positive or negative by PCR assays or that were below or above the $C_T$ cutoff values that were set for some agents. $C_T$ values were compared with the Mann-Whitney *U* test. Multivariate analysis was performed as logistic regression with patient/control as the dependent variable and age (continuous), gender, and $C_T$ values (continuous) as independent variables. This analysis first included $C_T$ values for all agents (with negative results given a $C_T$ value of 45) and then omitted, in a stepwise manner, factors not independently associated with symptoms.

## RESULTS

**Patients and samples.** In total, fecal samples from 165 children with acute diarrhea and 165 asymptomatic controls were included in the analysis. The gender distributions were similar for the two groups, with 50% female subjects. The mean age was lower for patients than for controls (13.7 versus 26.3 months; $P < 0.0001$). For patients, the reported median duration of diarrhea was 3 days, and the reported median frequency of stools was 3 per day. At least one pathogen was detected for 94% of patients and 84% of controls.

**Detection rates and median $C_T$ values.** Crude detection rates for each agent by real-time PCR are presented in Table 2. *Cryptosporidium*, rotavirus, and norovirus genogroup II (GII) were significantly more common in patients, whereas ETEC carrying an *eltB* gene (ETEC-*eltB*) (overall the most frequent finding), *Campylobacter*, *Shigella*, and adenovirus were detected at similar rates in the two groups. Adenovirus was detected in around 30% of samples in both patients and controls, but only a relatively small proportion of these (~5% of all samples) were adenovirus 40 or 41, which are considered the diarrheagenic adenovirus types.

The $C_T$ values were significantly lower in patients than controls for *Shigella* and ETEC-*eltB*, which had similar crude detection rates (Table 2 and Fig. 1). $C_T$ values were also lower for *Cryptosporidium* and ETEC-*estA*, which both were more common among patients.

$C_T$ values were further compared by multivariate logistic regression analysis that also included age and gender. For agents that were independently associated with disease in this analysis, a $C_T$ cutoff value was established. Comparing patients and controls with $C_T$ values below or above this cutoff value resulted in better distinction in terms of odds ratios, compared with crude detection rates, for *Cryptosporidium*, ETEC-*estA*, and *Shigella*, as shown in Table 3. The most important diarrheagenic agents, when $C_T$ cutoff values were considered (as described in Materials and Methods), were *Cryptosporidium* (25% with $C_T$ values of <35), *Shigella* (20% with $C_T$ values of <30), norovirus GII (19% with any $C_T$ value), ETEC-*estA* (16% with $C_T$ values of <31), and rotavirus (9.1% with any $C_T$ value).

**Infections with multiple pathogens.** Infection with more than one pathogen was a common finding for both patients and asymptomatic controls, as shown in Table 4. Thus, 2 or 3 pathogens were detected for approximately 50% of both patients and controls, and a single pathogen was found in only 22% of patient samples.

**Age and pathogen detection.** Both patients and controls who

**TABLE 1** Primers and probes targeting RNA or DNA of diarrheagenic agents

| Pathogen | Mixture | Forward primer | Reverse primer | Probe | Fluorophore[a] | Target gene/region | Reference or source |
|---|---|---|---|---|---|---|---|
| Norovirus GII | 1 | TGGAYTTTTAYGTGCCCAG | CGACGCCATCTTCATTCAC | AGCCAGATTGCGATCGCCC | VIC-TAMRA | Pol-capsid junction | 22 |
| Rotavirus | 1 | AACCATCTACACATGACCCTCTATGA | GGTCACATAACGCCCCTATAGC | CAATAGTTAAAAGCTAACACTGTCAAA | FAM-MGB | Nonstructural protein 3 | 23 |
| | 1 | AACCATCTTCACGTAACCCTCTATGA | | | | | |
| Astrovirus | 2 | GACTGCWAAGCAGCTTCGTGA | GCTAGCCATCACACTTCTTTGGTCCT | TCACAGAAGAGCAACTCCATCGCATTTG | FAM-BQ1 | Pol-capsid junction | 24 |
| Sapovirus | 2 | TTGGCCCTCGCCACCTAC | CCCTCCATYTCAAACACTA | CCRCCTATRAACCA | VIC-MGB | Pol-capsid junction | 25 |
| | 2 | GAYCASGCTCTCGCYACCTAC | | | | | |
| Norovirus GI | 3 | TGGCAGGCCATGTTCCGCT | TTTGKTGGGGCGTCCTTAGAC | ATTGCGATCTCCTGTCCA | VIC-MGB | Pol-capsid junction | 22 |
| | 3 | | CGCTTGATGTAGCGTCCTTAGAC | | | | |
| *Campylobacter* | 4 | ATGCAAACCATAATTGGGTTTCAAC | CGAGTATCAGCAACTTCTTCTACAGCT | TTGCCACCAAAACCAAAACT | NED-MGB | Fibronectin-binding protein | 26[b] |
| *Yersinia* | 5 | GCTKGATTGTCAGGAGTTGGTC | ATCCCCCGCAGTTGGCAT | ACCCGCTAATGAAGCA | VIC-MGB | Enterotoxin Yst precursor | 33[b] |
| *Vibrio cholerae* | 6 | CCACTTAGTGGGTCAAACTATATTGTC | ATGCCCCTAATACATCATTAACGTT | AGCCACTGCACCCAA | FAM-MGB | Cholera toxin A subunit | 34[b] |
| *Salmonella* | 7 | CGGGTTGCGTTATAGGTCTGA | TGAAATACGATGCGAACAACATC | AATACTGCGCTGCCAGAT | VIC-MGB | Outer membrane protein C | 35[b] |
| ETEC-*estA* | 7 | AAGCATGAATAGTAGCAATTACTGCT | TTAATAGCACCCGGTACAAGCA | AACAACACAATTCAC | NED-MGB | Heat-stable enterotoxin | 27[b] |
| ETEC-*eltB* | 8 | TCCGGCAGAGGATGGTTACA | CCAGGGTTCTTCTCTCCAAGC | AGCAGGTTTCCCACCGGATCACC | FAM-BQ1 | Heat-labile enterotoxin | 28[b] |
| *Shigella-ipaH* | 8 | ACCGGCGCTCTGCTCTC | GCAATGTCCTCCAGAATTTCG | CTGGGCAGGGAAATGTTCCGCC | JOE-BQ1 | Invasion plasmid antigen H | 31[b] |
| *Cryptosporidium* | 9 | CAAATTGATACCGTTTGTCCTTCTG | TGGTGCCATACATTGTTGTCCT | TGTCCTCCTGGATTCA | NED-MGB | Oocyst wall protein | 32[b] |
| Adenovirus | 10 | GCCACGGTGGGGTTTCTAAACTT | GCCCCAGTGGTCTTACATGCACATC | TGCACCAGACCCGGGCTCAGGTACTCCGA | FAM-BQ1 | Hexon | 21 |
| Adenovirus 40/41 | 11[c] | TGCCCGCGCCACCGAT | GAGCCACAGTGGGGTTTCTG | CCAGGCTGAAGTACG | FAM-BQ1 | Hexon | This study |

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGB, minor groove binding; BQ1, black hole quencher 1; JOE, 4,5-dichloro-dimethoxy-fluorescein; VIC, 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein. The chemical structure for NED fluorescent dye (ABI) is not publicly available.

[b] Adaptation of traditional PCR to real-time PCR.

[c] Run separately as a complementary analysis.

**TABLE 2** Comparison between patients and controls with respect to crude PCR detection rates and $C_T$ values

| Pathogen | No. (%) of patients | No. (%) of controls | OR | $P^a$ | Median $C_T$ for patients | Median $C_T$ for controls | $P^b$ |
|---|---|---|---|---|---|---|---|
| Viruses | | | | | | | |
| Adenovirus (any) | 45 (27) | 53 (32) | 0.79 | 0.40 | 38.2 | 39.3 | 0.05 |
| Adenovirus 40/41 | 10 (6.1) | 6 (3.6) | 1.71 | 0.44 | 36.6 | 35.0 | 0.66 |
| Astrovirus | 4 (2.4) | 1 (0.6) | 4.07 | 0.37 | 19.9 | 31.5 | |
| Norovirus GI | 1 (0.6) | 1 (0.6) | 1.00 | | | | |
| Norovirus GII | 33 (20) | 4 (2.4) | 10.1 | <0.0001 | 25.1 | 26.9 | 0.28 |
| Rotavirus | 16 (10) | 3 (1.8) | 5.80 | 0.003 | 24.4 | 26.0 | 0.50 |
| Sapovirus | 14 (8.5) | 7 (4.2) | 2.09 | 0.18 | 25.6 | 28.3 | 0.50 |
| Bacteria | | | | | | | |
| *Campylobacter* | 58 (35) | 54 (33) | 1.11 | 0.73 | 31.8 | 33.3 | 0.12 |
| *Vibrio cholerae* | 1 (0.6) | 0 (0) | | | | | |
| ETEC-*eltB* | 72 (44) | 76 (46) | 0.91 | 0.74 | 31.3 | 34.6 | 0.002 |
| ETEC-*estA* | 55 (33) | 39 (24) | 1.62 | 0.07 | 32.6 | 37.3 | 0.0001 |
| *Salmonella* | 9 (5.5) | 4 (2.5) | 2.32 | 0.26 | 42.2 | 40.6 | 0.22 |
| *Shigella* | 57 (35) | 54 (33) | 1.08 | 0.82 | 29.2 | 34.5 | <0.0001 |
| *Yersinia* | 0 (0) | 0 (0) | | | | | |
| Protozoa | | | | | | | |
| *Cryptosporidium* | 49 (30) | 18 (11) | 3.45 | <0.0001 | 32.1 | 36.8 | 0.0009 |
| Negative | 10 (6) | 27 (16) | | | | | |

[a] Fisher's exact test.
[b] Mann-Whitney *U* test for agents with a total of >10 positive samples.

were PCR positive for *Shigella* or adenovirus were older than those who were PCR negative for these agents, whereas detection of norovirus GII was associated with younger age among patients and older age among controls (Table 5). Accordingly, detection rates were related to age for some agents. As shown in Fig. 2, norovirus GII was found mainly in children younger than 1.5 years, who all had symptomatic infections. Detection rates for rotavirus, *Cryptosporidium*, and *Shigella* were (or tended to be) higher for patients than controls, for children with ages below ($P = 0.07$, $P = 0.03$, and $P = 0.01$, respectively) or above ($P = 0.08$, $P = 0.02$, and $P = 0.07$, respectively) 18 months.

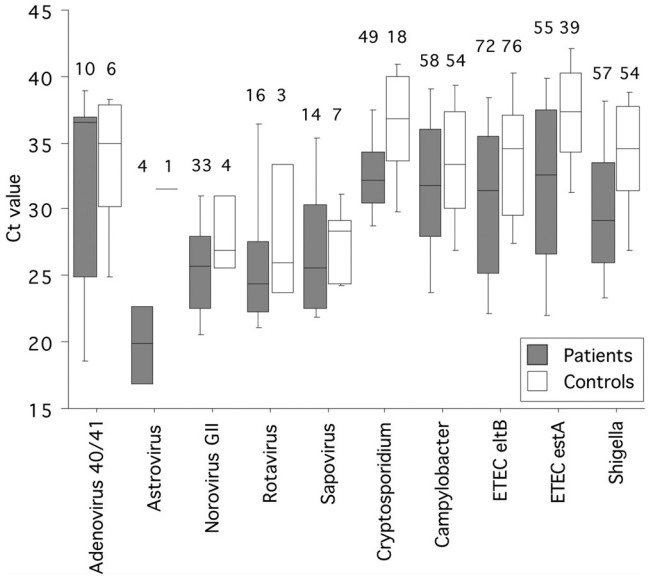


**FIG 1** Box plot showing $C_T$ values for agents targeted by real-time PCR among patients and controls. Boxes show the median (midline) and the 25th and 75th percentiles, and bars show the 10th and 90th percentiles. Numbers indicate the number of positive subjects for the respective agent.

## DISCUSSION

This study demonstrates the utility of a real-time PCR assay that targets essentially all pathogens that may cause acute infectious diarrhea in children. Previous studies have shown that molecular methods have higher analytical sensitivities than conventional techniques (12–15), but data are lacking from clinical studies applying PCR assays targeting the whole spectrum of causative agents.

In this study of children younger than 5 years, detection rates were high, identifying at least one agent for 94% of patients. Two or more agents were observed for 72% of patients and for 52% of

**TABLE 3** Multivariate logistic regression analysis and discrimination by $C_T$ cutoff values

| Pathogen | Logistic regression analysis | | $C_T$ cutoff value analysis[a] | | |
|---|---|---|---|---|---|
| | Coefficient (SE) | $P$ | $C_T$ value | OR (CI) | $P^b$ |
| Norovirus GII | 0.18 (0.04) | <0.0001 | 45 | 10.1 (3.5–29.1) | <0.0001 |
| Rotavirus | 0.12 (0.04) | 0.002 | 45 | 5.8 (1.7–20.3) | 0.003 |
| *Cryptosporidium* | 0.16 (0.04) | <0.0001 | 35 | 8.5 (3.5–20.6) | <0.0001 |
| ETEC-*estA* | 0.08 (0.03) | 0.008 | 31 | 10.1 (3.0–34.1) | <0.0001 |
| *Shigella* | 0.16 (0.03) | <0.0001 | 30 | 4.3 (2.0–9.4) | <0.0001 |

[a] Patient/control was the dependent variable, and $C_T$ values, gender, and age were independent variables. Only agents with $P$ values of <0.05 were included in the final analysis and are shown here. CI, confidence interval.
[b] Fisher's exact test.

**TABLE 4** Number of pathogens detected by PCR in patients and controls

| No. of pathogens detected | No. (%) of healthy controls[a] | No. (%) of patients with[a]: | |
|---|---|---|---|
| | | All agents | Selected agents[b] |
| None | 27 (16) | 10 (6.1) | 11 (6.7) |
| 1 | 46 (28) | 37 (22) | 91 (55) |
| 2 | 55 (33) | 50 (30) | 26 (16) |
| 3 | 31 (18) | 37 (22) | 1 (0.6) |
| 4 | 5 (3.0) | 23 (14) | 0 (0) |
| 5 | 1 (0.6) | 6 (3.6) | 0 (0) |
| 6 | 0 (0) | 2 (1.2) | 0 (0) |

[a] Adenoviruses other than 40/41 were excluded from this analysis.
[b] *Cryptosporidium* with $C_T$ values of <35, ETEC-*estA* with $C_T$ values of <31, *Shigella* with $C_T$ values of <30, norovirus GII, and rotavirus.

**TABLE 5** Median ages of patients and controls who tested positive or negative by real-time PCR

| Pathogen | Patients | | | Controls | | |
|---|---|---|---|---|---|---|
| | Median age (mo [*n*]) | | | Median age (mo [*n*]) | | |
| | Positive | Negative | *P*[a] | Positive | Negative | *P*[a] |
| Adenovirus (all) | 14 (45) | 11 (120) | 0.003 | 30 (53) | 21.5 (112) | 0.01 |
| Norovirus GII | 9 (33) | 12 (132) | 0.0002 | 40 (4) | 24 (161) | 0.03 |
| Rotavirus | 12 (16) | 12 (149) | 0.74 | 24 (3) | 24 (162) | 0.64 |
| *Cryptosporidium* | 12 (49) | 12 (116) | 0.36 | 21.5 (18) | 24 (147) | 0.23 |
| ETEC-*estA* | 12 (55) | 12 (110) | 0.49 | 24 (39) | 24 (126) | 0.73 |
| *Salmonella* | 14 (9) | 12 (156) | 0.24 | 48 (4) | 24 (161) | 0.01 |
| *Shigella ipaH* | 13 (57) | 11 (108) | 0.0007 | 36 (54) | 18 (111) | <0.0001 |

[a] Mann-Whitney *U* test. Data for agents showing associations with age are presented. The median age was 12 months for all 165 patients, in comparison with 24 months for all 165 controls.

children without diarrhea. Whereas some agents (norovirus GII and rotavirus) were found mainly among patients, others were found at high rates also among asymptomatic controls. The latter group included ETEC, *Shigella*, and *Campylobacter*, which previously have been found at significant rates among children without diarrhea (3, 10, 16, 36–38). These findings suggest substantial exposure to enteric pathogens in the study population, probably due to poor sanitation. This underlines the importance of including asymptomatic controls in diarrheal studies conducted in low-income countries. Even if a control group is included, however, it may be difficult to assess the pathogenic importance of some agents if they appear at similar rates among patients and controls, as observed in this study. Therefore, we explored the potential utility of $C_T$ values as markers for pathogen loads in the interpretation of results. This was done first by including $C_T$ values when analyzing the associations between PCR results and disease and then by setting and applying $C_T$ cutoff values for some agents, in an effort to improve the distinction of causative agents.

Thus, when $C_T$ values were analyzed, ETEC, *Shigella*, *Campylobacter*, and sapovirus (all with similar detection frequencies among patients and controls) were found to be associated with symptomatic infections. Multivariate analysis confirmed independent associations with diarrhea for norovirus GII, rotavirus, *Cryptosporidium*, ETEC-*estA*, and *Shigella*. For the latter three agents, $C_T$ cutoff values improved the distinctions between symptomatic and asymptomatic children. When $C_T$ cutoff values were considered, the 5 most important causative agents among patients were *Cryptosporidium* (25%), *Shigella* (20%), ETEC-*estA* (16%), norovirus GII (19%), and rotavirus (9.1%). This is in agreement with the findings of the Global Enteric Multicenter Study (GEMS), with the exception that norovirus GII was more common than rotavirus, which was classified as the causative agent for only 9% of our patients. An explanation for this low rate may be that the study was conducted during the rainy season, when rotavirus might be less common (39).

ETEC carrying *estA* or *eltB* genes was found at high rates among both patients and asymptomatic children. Whereas ETEC-*estA* tended to be more common among patients (33% versus 24%), ETEC-*eltB* was detected at equal rates for patients and controls (44% and 46%, respectively). When $C_T$ values were included in the comparison, both ETEC-*estA* and ETEC-*eltB* were associated with diarrhea, but this association remained significant only for ETEC-*estA* in the multivariate analysis. A stronger association with diarrhea for ETEC-*estA* than ETEC-*eltB* has been observed by others (40). The crude detection rates for ETEC among our patients were higher than those observed in most previous studies, which typically reported rates of 10 to 20% (40–43).

*Shigella* infections were identified by PCR targeting the *ipaH* gene, a multicopy gene that is present in all *Shigella* species but also may be found in enteroinvasive *E. coli* (EIEC) (44). In this study, *Shigella* was detected, with $C_T$ values below 30, in 20% of children with diarrhea. Our results confirm that *Shigella* is an important cause of childhood diarrhea (43) and they indicate that PCR may improve detection, compared with bacterial culture (13, 15, 19).

Previous studies have shown that *Cryptosporidium* is an important cause of gastroenteritis in children in developing countries (45–47); in the recent GEMS, around 10% of diarrhea cases in children younger than 2 years were attributed to *Cryptosporidium* (3). In our study, *Cryptosporidium* was detected in 30% of children with diarrhea versus 11% of asymptomatic children, and 25% of patients had *Cryptosporidium* detected with $C_T$ values below the cutoff of 35, indicating a causative role. This is a remarkably large proportion, which might reflect the seasonality of this infection or contamination of drinking water (48).

*Campylobacter* was the second most common pathogen but was found as frequently among healthy children (33%) as among sick children (35%). There was a weak association between $C_T$ values and disease ($P = 0.03$), but an independent association could not be confirmed in the multivariate analysis. Previous studies in sub-Saharan Africa also reported similar detection rates for children with and without diarrhea, but in general the rates were lower than we observed (37, 38, 49), a difference that might be explained by the lower sensitivity of culture versus PCR (11).

Norovirus GII was common (19%) and was strongly associated with symptomatic infections and with younger age, as recently observed by others (50), with a remarkably high rate (24%) among sick children younger than 1.5 years. Both rotavirus and norovirus GII were rare among asymptomatic children; therefore, an association between the viral load ($C_T$ value) and symptomatic infection, as reported by others (17, 18), could not be properly investigated.

The younger age of the patients versus the controls is a limitation of the study that might bias the analysis of associations between pathogens and disease. When age was included in the multivariate analysis, however, the main associations between pathogen detection and diarrhea remained or were stronger. We did not use bacterial culture as a comparative method, since such cultures could not be performed at the site of the study. As a result,

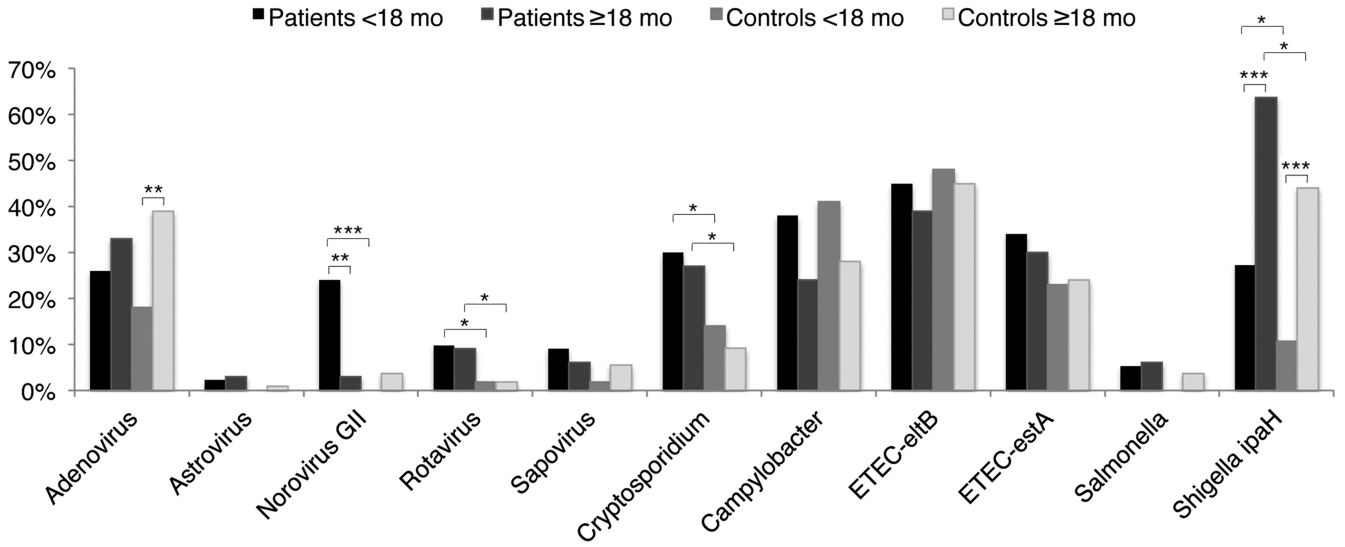


**FIG 2** Proportions of patients and asymptomatic controls younger or older than 18 months who tested positive by PCR. *, $P < 0.10$; **, $P < 0.01$; ***, $P < 0.001$.

we cannot evaluate whether and how much molecular analysis improved the sensitivity of detection. However, it is likely that the rates of detection were greater than would have been observed with nonmolecular methods, particularly among asymptomatic subjects, for whom the pathogen loads were generally lower.

Our results suggest that pathogen quantification in feces may be clinically useful. In order to compare different studies and to implement quantification in diagnostic testing, standardization would be important but also difficult. Whereas real-time PCR quantification in blood can be accurately performed as copies/ml, a corresponding unit is neither available nor relevant for feces, mainly because 1 volume unit of watery feces is not comparable to 1 volume unit of feces with normal consistence. An option for standardization might be to relate the $C_T$ value for each bacterial target to the $C_T$ value determined by PCR targeting a conserved part of the gene coding for 16S rRNA (51). We plan to evaluate this type of normalization with respect to the total bacterial content in the sample, which would be relevant also when rectal swabs are used for molecular testing. Even if such standardization is desirable, it should be kept in mind that the differences in fecal contents between samples (which differ by a factor of 10 or less in most cases, corresponding to 3 cycles in $C_T$ values) are considerably smaller than the differences in pathogen loads between samples (which may correspond to 20 cycles or more in $C_T$ values). We realize that $C_T$ cutoff values may differ between laboratories. However, the key finding was not the cutoff values but rather the finding that pathogen loads were relevant and that the application of cutoff values increased specificity. These observations encourage further studies on the importance of pathogen loads in feces and how to standardize quantification.

In conclusion, this investigation of acute diarrhea in children younger than 5 years in rural Zanzibar demonstrates the utility of broad molecular diagnostic testing for studying the etiology of acute gastroenteritis and underlines the necessity to include healthy controls in such studies. In addition to high analytical sensitivity and specificity, real-time PCR provided quantitative information that appears to be useful for data interpretation, at least for some agents. Thus, for *Cryptosporidium*, ETEC carrying an *estA* gene, and *Shigella*, $C_T$ cutoff values could be identified by comparing data from patients and controls, and the application of these cutoffs improved specificity and allowed stricter interpretation of the results, which we believe provided a more correct description of the diarrheal etiology, with a smaller proportion of cases classified as being caused by multiple pathogens. The most common causative agents were *Shigella*, *Cryptosporidium*, ETEC, and norovirus GII. In comparison with previous studies, rotavirus was rarer and *Cryptosporidium* more frequent, differences that may be related to the limited time period for this study and the seasonality of these infections.

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