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Real-Time PCR Threshold Cycle Cutoffs Help To IdentifyAgents 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 \( \geq 37.5^\circ C \) 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/docus/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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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<sub>T</sub> 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<sub>T</sub> 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<sub>T</sub> values were also lower for Cryptosporidium and ETEC-estA, which were both more common among patients. C<sub>T</sub> 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<sub>T</sub> cutoff value was established. Comparing patients and controls with C<sub>T</sub> 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<sub>T</sub> cutoff values were considered (as described in Materials and Methods), were Cryptosporidium (25% with C<sub>T</sub> values of <35), Shigella (20% with C<sub>T</sub> values of <30), norovirus GII (19% with any C<sub>T</sub> value), ETEC-estA (16% with any C<sub>T</sub> values of <31), and rotavirus (9.1% with any C<sub>T</sub> 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>
<thead>
<tr>
<th>Pathogen</th>
<th>Mixture</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
<th>Fluorophore</th>
<th>Target gene/region</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus GII</td>
<td>1</td>
<td>TGGAYTTTAYTGGCCACG</td>
<td>CGACGCCATCTCCATTCAC</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-TAMRA</td>
<td>Pol-capsid junction</td>
<td>22</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>1</td>
<td>AACCACCTCACACATGAAACCTATAG</td>
<td>GGTCACAATACGCGGAATGCCC</td>
<td>CAACTAGTTAAAAGCTACACGATTACAG</td>
<td>FAM-MGB</td>
<td>Pol-capsid junction</td>
<td>23</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>2</td>
<td>GAYGCAATCTCATTGGCTTCAAG</td>
<td>CCGCTGCAATCTTCCTGAGCC</td>
<td>TGGGAGAGAGAGGAGGATGGGCC</td>
<td>VIC-MGB</td>
<td>Pol-capsid junction</td>
<td>24</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>2</td>
<td>TTGGCCTGCCCCCCACTTAC</td>
<td>CCACTGCAATCTTCCATTCCTG</td>
<td>TGGGAGAGAGAGGAGGATGGGCC</td>
<td>VIC-MGB</td>
<td>Pol-capsid junction</td>
<td>25</td>
</tr>
<tr>
<td>Norovirus GI</td>
<td>3</td>
<td>TGGCCAGGCCATGTTCCGCT</td>
<td>TTTGGGTGGGGCTTCACTG</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Pol-capsid junction</td>
<td>26</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>4</td>
<td>ATGCAAACATATAAGTGTTCACC</td>
<td>ATGCGATATGTGATATGAGAATC</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Enterotoxin Yst precursor</td>
<td>27</td>
</tr>
<tr>
<td>Yersinia</td>
<td>5</td>
<td>GCTKGATTGTCAGGAGTTGGTTGGTGTC</td>
<td>ATCCCAATGAAATGAAATGAAATGC</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Enterotoxin Yst precursor</td>
<td>28</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>6</td>
<td>CCACTTATGGAATGTTCCACATATGTA</td>
<td>ATGCGATATGTGATATGAGAATC</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Cholera toxin A subunit</td>
<td>29</td>
</tr>
<tr>
<td>Salmonella</td>
<td>7</td>
<td>CGGCTGCGCACAGATGTTGGT</td>
<td>TGAATATGCGAGGCATAGTCATG</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Outer membrane protein C</td>
<td>30</td>
</tr>
<tr>
<td>ETEC-estA</td>
<td>7</td>
<td>AACCACGAATAGTGAATTCTGCTG</td>
<td>TTAATAGCAGCCCATAGCCAGAGA</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Heat-stable enterotoxin</td>
<td>31</td>
</tr>
<tr>
<td>ETEC-eltB</td>
<td>8</td>
<td>TGGGCGAGGAGATGTTGTTGCC</td>
<td>CGATGCTGCTGGGACATTTTGG</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Heat-labile enterotoxin</td>
<td>32</td>
</tr>
<tr>
<td>Shigella</td>
<td>8</td>
<td>ACCGGTCGCGCGCAGTGGT</td>
<td>CGATGCTGCTGGGACATTTTGG</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Invasion plasmid antigen H</td>
<td>33</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>9</td>
<td>CAAATTGATACCGTTTGTCCTTCTG</td>
<td>TGGTGGCGGTATTCGTTGCG</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Oocyst wall protein</td>
<td>34</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>10</td>
<td>GCCAGCGGCGGTGGTCTGCTTTT</td>
<td>GCCAGCGGCGGTGGTCTGCTTTT</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Hexon</td>
<td>35</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>10</td>
<td>GCCAGCGGCGGTGGTCTGCTTTT</td>
<td>GCCAGCGGCGGTGGTCTGCTTTT</td>
<td>AGCCAGATGGCGATGGCCC</td>
<td>VIC-MGB</td>
<td>Hexon</td>
<td>36</td>
</tr>
</tbody>
</table>

\*FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGB, minor groove binding; BQ1, black hole quencher 1; JOE, 4,5-dichloro-3-indole-3-carboxyfluorescein; 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.
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 \( (P = 0.07, P = 0.03, \text{and } P = 0.01, \text{respectively}) \) or above \( (P = 0.08, P = 0.02, \text{and } P = 0.07, \text{respectively}) \) 18 months.

**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 controls.

### TABLE 2 Comparison between patients and controls with respect to crude PCR detection rates and \( C_T \) values

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. (%) of patients</th>
<th>No. (%) of controls</th>
<th>OR</th>
<th>( P^a )</th>
<th>Median ( C_T ) for patients</th>
<th>Median ( C_T ) for controls</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>45 (27)</td>
<td>53 (32)</td>
<td>0.79</td>
<td>0.40</td>
<td>38.2</td>
<td>39.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Adenovirus 40/41</td>
<td>10 (6.1)</td>
<td>6 (3.6)</td>
<td>1.71</td>
<td>0.44</td>
<td>36.6</td>
<td>35.0</td>
<td>0.66</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>4 (2.4)</td>
<td>1 (0.6)</td>
<td>4.07</td>
<td>0.37</td>
<td>19.9</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>Norovirus G1</td>
<td>1 (0.6)</td>
<td>1 (0.6)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norovirus GII</td>
<td>33 (20)</td>
<td>4 (2.4)</td>
<td>10.1</td>
<td>&lt;0.0001</td>
<td>25.1</td>
<td>26.9</td>
<td>0.28</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>16 (10)</td>
<td>3 (1.8)</td>
<td>5.80</td>
<td>0.003</td>
<td>24.4</td>
<td>26.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>14 (8.5)</td>
<td>7 (4.2)</td>
<td>2.09</td>
<td>0.18</td>
<td>25.6</td>
<td>28.3</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter</td>
<td>58 (35)</td>
<td>54 (33)</td>
<td>1.11</td>
<td>0.73</td>
<td>31.8</td>
<td>33.3</td>
<td>0.12</td>
</tr>
<tr>
<td>Vibrio cholera</td>
<td>1 (0.6)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC-eltB</td>
<td>72 (44)</td>
<td>76 (46)</td>
<td>0.91</td>
<td>0.74</td>
<td>31.3</td>
<td>34.6</td>
<td>0.002</td>
</tr>
<tr>
<td>ETEC-estA</td>
<td>55 (33)</td>
<td>39 (24)</td>
<td>1.62</td>
<td>0.07</td>
<td>32.6</td>
<td>37.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>Salmonella</td>
<td>9 (5.5)</td>
<td>4 (2.5)</td>
<td>2.32</td>
<td>0.26</td>
<td>42.2</td>
<td>40.6</td>
<td>0.22</td>
</tr>
<tr>
<td>Shigella</td>
<td>33 (20)</td>
<td>4 (2.4)</td>
<td>10.1</td>
<td>&lt;0.0001</td>
<td>25.1</td>
<td>26.9</td>
<td>0.28</td>
</tr>
<tr>
<td>Yersinia</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>49 (30)</td>
<td>18 (11)</td>
<td>3.45</td>
<td>&lt;0.0001</td>
<td>32.1</td>
<td>36.8</td>
<td>0.0009</td>
</tr>
<tr>
<td>Negative</td>
<td>10 (6)</td>
<td>27 (16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) Fisher’s exact test.
\( b \) Mann-Whitney U test for agents with a total of >10 positive samples.

### TABLE 3 Multivariate logistic regression analysis and discrimination by \( C_T \) cutoff values

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Coefficient (SE)</th>
<th>( P )</th>
<th>( C_T ) value</th>
<th>OR (CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus GII</td>
<td>0.18 (0.04)</td>
<td>&lt;0.0001</td>
<td>45</td>
<td>10.1 (3.5–29.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>0.12 (0.04)</td>
<td>0.002</td>
<td>45</td>
<td>5.8 (1.7–20.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>0.16 (0.04)</td>
<td>&lt;0.0001</td>
<td>35</td>
<td>8.5 (3.5–20.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ETEC-estA</td>
<td>0.08 (0.03)</td>
<td>0.008</td>
<td>31</td>
<td>10.1 (3.0–34.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Shigella</td>
<td>0.16 (0.03)</td>
<td>&lt;0.0001</td>
<td>30</td>
<td>4.3 (2.0–9.4)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\( a \) 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.

**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.
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 CT values as markers for pathogen loads in the interpretation of results. This was done first by including CT values when analyzing the associations between PCR results and disease and then by setting and applying CT cutoff values for some agents, in an effort to improve the distinction of causative agents.

Thus, when CT 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, CT cutoff values improved the distinctions between symptomatic and asymptomatic children. When CT 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 CT 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 multicity 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 CT 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 CT 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 CT 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 (CT 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,
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 CT value for each bacterial target to the CT 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 CT values) are considerably smaller than the differences in pathogen loads between samples (which may correspond to 20 cycles or more in CT values). We realize that CT 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, CT 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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