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Diarrhoeagenic microbes by real-time PCR in Rwandan children under 5 years of age with acute gastroenteritis

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Abstract

Acute gastroenteritis is a main cause of disease and death among children in low-income countries. The causality rates and pathogenic characteristics of putative aetiological agents remain insufficiently known. We used real-time PCR targeting 16 diarrhoeagenic agents to analyse stool samples from children ≤5.0 years old with acute diarrhoea in Rwanda. Among the 880 children (median age 14.2 months; 41% female) at least one pathogen was detected in 92% and two or more agents in 63% of cases. Rotavirus was detected in 36.9%, adenovirus in 39.7%, enterotoxigenic *Escherichia coli* (ETEC) with genes for labile (*eltB*) or stable (*estA*) toxin in 31.3% and 19.0%, *E. coli* with *eae* or *bfpA* genes in 25.2% and 14.2%, *Shigella* in 17.5% and *Cryptosporidium* in 7.8%. Rotavirus and ETEC-*estA* were associated with more severe dehydration than diarrhoea due to other causes. *Shigella* was associated with bloody stools and higher CRP. Microbial loads (C_t values) of rotavirus, ETEC-*estA* and *Shigella* were associated with severity of symptoms. Rotavirus, *ETEC-estA* and *E. coli* with *bfpA* were associated with younger age, *Shigella* with older age. Antibiotic treatment was given to 42% and was associated with dehydration, fever and CRP, but not with pathogen. We conclude that rotavirus and ETEC-*estA* were the most important causes of diarrhoea with dehydration, that *Shigella* caused bloody diarrhoea but less severe dehydration, that microbial loads of rotavirus, ETEC-*estA* and *Shigella* were associated with severity of symptoms, and that antibiotic use was frequent and in poor agreement with microbiological findings.

Keywords: Diarrhoea, epidemiology, faeces, gastroenteritis, real-time PCR
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Background

Acute diarrhoeal disease is the second most common cause of death world-wide in children under 5 years of age [1]. Most of the morbidity occurs in low-income countries, where the aetiologies and epidemiology of gastroenteritis remain incompletely understood. A recent global multicentre study provided strong evidence that rotavirus, enterotoxigenic *Escherichia coli* (ETEC), *Cryptosporidium* and *Shigella* are the main causative agents [2], but it is important to obtain more information about the relative importance of different aetiologies and their clinical characteristics. Improvements of hygiene, sanitary standards, food handling, nutrition status and healthcare are general goals for low-income countries that may reduce the burden of diarrhoeal disease. However, decisions regarding more specific interventions such as vaccination against rotavirus [3], *Shigella* or ETEC [4] and use of antibiotics [5] are important and require data from studies that investigate the majority of putative aetiologies, preferably using methods with a similar diagnostic performance for all causative agents. New multi-targeting molecular methods should be useful for such investigations because they allow detection of essentially all diarrhoeagenic pathogens with high specificity and sensitivity [6–8].

Identification of causative agents is typically based on comparison between patients and controls. In the present study, we instead investigated pathogenicity by relating severity of symptoms to, firstly, the mere detection of essentially all putative diarrhoeagenic agents, and secondly, to pathogen load. By these approaches we found that rotavirus, ETEC carrying a gene for heat stable enterotoxin and *Shigella* were the main pathogens, and observed novel associations between pathogen load and symptoms.

Methods

Patients and samples

We included in this study children seeking care at any of three health centres, two district hospitals or one university teaching hospital in Kigali, or any of two health centres, one district hospital or one university teaching hospital in Butare, Rwanda, during repeated study periods from November 2009 to June 2012, so as to cover both rainy and dry seasons. The inclusion criteria were age ≤5.0 years and diarrhoea with duration of <96 h (with or without vomiting or fever), and exclusion criteria were non-enteric acute infections, severe malnutrition and AIDS. The specimen type was faeces only in 535 cases, rectal swab only in 159 cases and both faeces and rectal swab in 184 cases. In the latter 184 cases, the lowest C_t (threshold cycle) value (or the only C_t value if only one was PCR positive) was recorded.

Clinical characteristics

The following clinical parameters were registered: body temperature (axillary); history of vomiting, stool frequency and blood in faeces (according to parent/carer); degree of dehydration; type of rehydration (oral or intravenous) or antibiotic treatment (for patients who were sent home as well as for those who stayed in hospital). Dehydration was scored by the study nurse on arrival according to WHO guidelines as severe (reduced consciousness, lack of urine output, cool, moist extremities, rapid and feeble pulse, low or undetectable blood pressure, pale skin), moderate (thirst, restlessness, irritable behaviour, decreased skin elasticity, sunken eyes), mild or none (absence of these symptoms). For patients who remained at a district or university hospital the duration of hospitalization and survival were recorded; no follow-up was performed for patients who were sent home (their outcome was recorded as survival).

Stool sample collection

Stool samples were collected as a rectal swab (Copan Regular Flocked Swab 502CS01, Copan Italia Spa, Brescia, Italy) in a tube with 1 mL of sterile saline, or as 2 mL of faeces [9]. The samples were sent to a local laboratory for storage at −80°C until transport to the Department of Infectious Diseases at University of Gothenburg, Sweden, where molecular testing was performed.

Microbial agents and target sequences

The targets for real-time PCR are described in Table S1. Amplified regions of viruses were located to conserved genomic regions, using established primers and probes [10–13]. Samples reactive for adenovirus were tested by an additional PCR targeting only types 40/41. Assays for bacteria and protozoa were developed by adapting traditional PCR to real-time PCR [14–23]. For ETEC, both heat labile toxin (*eltB*) and heat stable toxin (*estA*) genes were targeted [22,23], as were genes coding for intimin (*eae*) and bundle-forming pilus (*bfpA*), considered to be markers for enteropathogenic *E. coli* (EPEC) [20,21]. *Shigella* was identified by amplification of the invasion plasmid antigen H gene (*ipaH*, which also may be present in enteroinvasive *E. coli* (EIEC). The target gene was fibronectin-binding protein for *Campylobacter*, enterotoxin Yst precursor for *Yersinia enterocolitica*, cholera toxin A subunit for *Vibrio cholerae*, outer membrane protein C for *Salmonella*, and oocyst wall protein for *Cryptosporidium*.

Sample preparation and nucleic acid extraction

Approximately 250 μL of faeces were dissolved in 4.5 mL of saline and centrifuged for 5 min at 750 *g*. Then, 250 μL of dissolved faeces or 250 μL of rectal swab fluid were mixed with 2 mL of lysis buffer, and this volume was used for extraction of total nucleic acid in an EasyMag instrument (Biomerieux, Marcy l'Étoile, France). The nucleic acids were eluted in 110μL and 5 μL of this were used for real-time PCR. These procedures correspond to an approximate dilution of faeces to 1:10 prior to PCR. The dilution of rectal swab samples depends on the specimen volume contained in the swab, but typically was 1:10 to 1:100 [9]. These dilutions effectively prevent the potential impact of factors that might inhibit amplification [9].

Real-time PCR

Amplification was performed in an ABI7900 instrument (Applied Biosystems, Foster City, CA, USA) in 11 parallel 20-μL reactions containing oligonucleotides (Table S1) and Taqman Fast Virus 1-step Mastermix (ABI, for RNA targets) or Universal Mastermix (ABI, for DNA targets). A two-step amplification (15 s 95°C, 60 s 56°C) was run for 45 cycles after an initial 10 min denaturation at 95°C and 30 min reverse transcription at 46°C. Plasmids (pUC57) containing the target regions for all agents were amplified in parallel with patient specimens to verify the performance of each target PCR (mastermix control).

The result from PCR was recorded as detected or not (a qualitative result) and as the C_t (threshold cycle) value, a quantitative parameter that has a negative linear relationship to the logarithm of the concentrations of the pathogen in faeces.

C-reactive protein

CRP levels in blood samples drawn on admission were measured at a local laboratory in Rwanda by the NycoCard assay (Medinor, Lidingö, Sweden) according to the manufacturer's instructions. Briefly, 5 μL of capillary blood were diluted and 50 μL of diluted samples were added to a reaction device, followed by the addition of one drop of conjugate and after 30 s one drop of washing solution, and measurement in NycoCard Reader II.

Ethical committee approval

The study was approved by the regional ethical review board in Gothenburg and by the ethical committee at the National University of Rwanda. An informed consent was obtained from the parents/carers of each child included in the study.

Statistics

Fisher's exact test was used for analysing associations between pathogen detection (yes/no) and symptoms. Threshold cycle (C_t) values were compared by logistic regression, age with *t*-test in univariate analysis. Multiple logistic regression analysis was performed with vomiting, degree of dehydration or intravenous fluid therapy as dependent variables, and age (continuous) in combination with PCR reactivity (yes/no) or C_t values (continuous) as independent variables. The JMP software (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

Results

General

Patient characteristics of the 880 patients are shown in Table 1. The majority (72%) had a history of vomiting, 13% had signs of severe dehydration and 34% no or mild dehydration. Nearly all patients (97%) received oral rehydration solution, almost half receiving intravenous fluids, and 42% received antibiotics.

Detection frequencies

At least one pathogen was detected in 92% of the cases. As shown in Table 2, the most frequent agents were adenovirus (39.7%), rotavirus (36.9%), ETEC-*eltB* (31.3%) and EPEC *eae* (25.2%). In most cases, these agents were found together with other pathogens, and infection with two or more agents was seen in 63% (n = 558) of all cases (Fig. 1a). Among infections with only one agent (n = 251), rotavirus was the most common (37.1%), followed by adenovirus (14.3%), *Shigella* (9.2%) and ETEC-*eltB* (8.8%). Rotavirus was detected at a similar frequency among co-infected patients; other agents were more often present as co-infections (Fig. 1b). Only 16.2% (35/216) of the cases with adenovirus were identified as types 40 or 41. *Escherichia coli* with the EPEC markers *eae* and *bfpA* were detected at different rates (25.2% and 14.2%), as was also seen for ETEC (31.3% with *eltB* and 19.0% with *estA* genes).

TABLE 1. Clinical information for 880 children with diarrhoea

Age and gender	
Months of age, median (IQR)	14.2 (9.1–23.8)
Age <12/12–24/>24 months	350/312/218 (40%/35%/25%)
Female/male	362/518 (41%/59%)
Clinical presentation	
Duration of symptoms, mean (SD)	2.4 (0.80) days
Vomiting: yes/no	630/249 (72%/28%)
Frequency of diarrhoea, >6/<6 per day	288/592 (33%/67%)
Temperature, ≥38.0/<38.0°C	402/475 (46%/54%)
Dehydration: severe/moderate/no or mild	110/470/300 (13%/53%/34%)
CRP, >50/10–50/<10 mg/L	137/248/388 (18%/32%/50%)
Clinical care	
Level of care: health centre/district hospital/university hospital	419/421/40 (48%/48%/4%)
Clinical decision: hospitalized/referred/sent home	388/59/433 (44%/7%/49%)
Mean duration (SD) in hospital for patients not sent home	2.87 (1.50) days
ORS therapy: yes/no	851/28 (97%/3%)
Intravenous rehydration: yes/no	405/473 (46%/54%)
Zinc treatment: yes/no	695/185 (79%/21%)
Antibiotic treatment: yes/no	373/506 (42%/58%)
Outcome: recovered/died	875/5 (99.4%/0.6%)

IQR, interquatile range (25–75%); ORS, oral rehydration solution.
Total number was <880 for some variables due to missing data.

Detection frequencies and clinical parameters

The frequency of symptoms in cases positive or negative for each pathogen is shown in Table 2. For rotavirus, ETEC-*estA* and *Shigella* associations with vomiting, dehydration, intravenous fluid treatment and CRP remained when age was included in the analysis, as shown in Table 3. Rotavirus and ETEC-*estA* were also associated with more frequent diarrhoea (mean 6.06 vs. 5.74 stools/day, p 0.005, and 6.23 vs. 5.78 stools/day, p 0.052).

Shigella was associated with higher CRP levels (p 0.0005) and presence of blood in faeces (p 0.015), but of the 154 patients with *Shigella*, only 13 (8.4%) had bloody diarrhoea (OR = 2.47, p 0.015), and 32 (21%) had CRP > 50 mg/L (OR = 1.3). *Cryptosporidium* (OR = 1.68, p 0.044), *Campylobacter* (OR = 1.49, p 0.030) and EPEC-*bfpA* (OR = 1.50, p 0.041) were associated with intravenous fluid therapy.

Pathogen load (C_t values) and symptoms

In order to study the potential association between disease and concentration of pathogen in faeces, C_t values were related

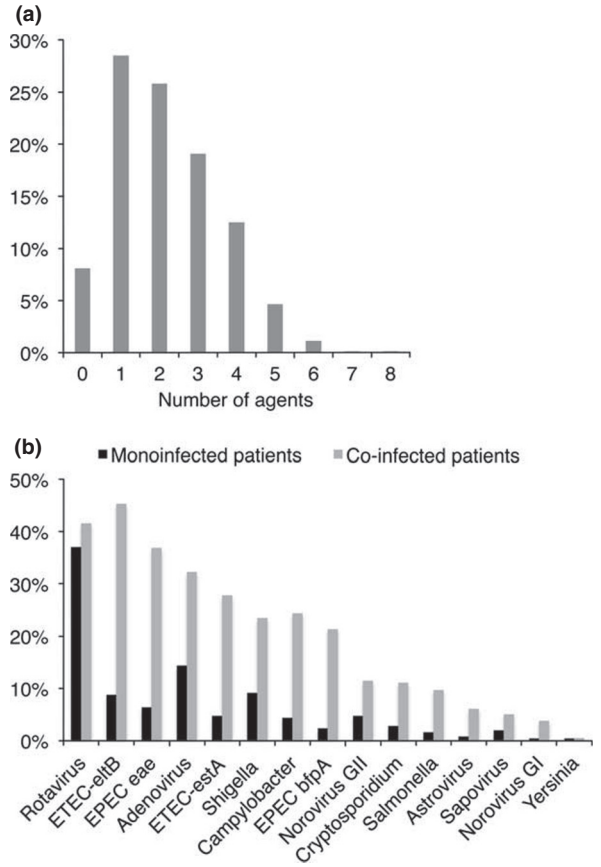

FIG. 1. (a) Number of microbial agents detected by PCR in faeces from 880 children with diarrhoea. (b). Detection frequency for each agent among 251 patients with mono-infection and 558 patients with co-infections (≥2 detected agents).

to clinical parameters. As shown in Table 3, higher viral load (lower C_t) of rotavirus was associated with vomiting, more severe dehydration and intravenous fluid therapy. Similarly, a higher concentration of ETEC-*estA* was associated with vomiting and intravenous fluid therapy, and higher load of *Shigella* was associated with dehydration and intravenous fluid therapy.

Importance of age

As shown in Fig. 2, rotavirus, ETEC-*estA* and EPEC-*bfpA* were more frequent (p <0.005) in younger patients (observed in 50%, 23% and 21% of children <12 months, as compared with 18%, 12% and 7.3% of those >24 months of age). Conversely, *Shigella* was more common (p 0.0005) in older children (24% in children >24 months, 5.1% in those <12 months of age).

Younger age was associated with severe dehydration, hospitalization and intravenous fluid therapy irrespective of infecting agent (p 0.0001), and to some extent the associations between pathogen detection or concentration and clinical parameters mentioned above were related to age. However, significant associations between detection of rotavirus or ETEC-*estA* and more severe dehydration or need for intravenous fluids, were observed also when age was taken into account (Table 3, upper part). The significant associations between clinical markers (vomiting, dehydration and intravenous fluid therapy) and pathogen load of rotavirus, ETEC-*estA* and *Shigella* also remained when age was taken into account (Table 3, lower part).

CRP and pathogen detection

The only agent associated with higher CRP levels was *Shigella* (p 0.002), as shown in Table 1. CRP was associated with blood in faeces (p 0.031), blood or mucus in faeces (p 0.0008) and higher body temperature (p 0.0059).

Prescription of antibiotics

Antibiotics were given to 373 patients (42%). Cotrimoxazol was used in 37%, amoxicillin or ampicillin in 25%, ampicillin and gentamycin in 20%, cefotaxim in 12%, ciprofloxacin in 2% and erythromycin in 2% of these cases.

There was no significant association between any pathogen and antibiotic treatment. Among cases with only one detected pathogen (n = 251), antibiotics were given to 45.8% of non-bacterial and to 42.1% of bacterial cases. Neither age nor presence of blood in faeces influenced the use of antibiotics. However, antibiotics were more often given to children with body temperature above 38°C (p <0.0001) or with elevated CRP levels (59%, 43% or 39% if CRP was ≥50, 10–50 or <10 mg/L; p 0.0059), or if dehydration was significant (58%, 44% or 35% if dehydration was severe, moderate or mild/none; p 0.0001).

Discussion

The present study demonstrates the utility of a broad real-time PCR panel for investigating causes of acute gastroenteritis in children in a low-income country. Using this technique, at least one diarrhoeagenic agent was detected in 92% of 880 children with diarrhoea for <96 h, and two or more agents were detected in 63%. A control group of healthy children was not included in this study, so we could not assess the burden of disease attributed to each pathogen. However, by relating detection frequencies and pathogen load estimates (C_t values) to clinical parameters several associations were identified.

Firstly, detection of rotavirus or ETEC-*estA* was associated with higher rate of vomiting, more frequent diarrhoea, more severe dehydration and more need for intravenous fluid

TABLE 2. Real-time PCR results related to clinical parameters by univariate analysis

	Adenovirus	Astrovirus	NoV GI	NoV GII	Rotavirus	Sapovirus	*Cryptosp.*	*Campylob.*	ETEC-*eltB*	ETEC-*estA*	EPEC *bfpA*	EPEC *eae*	*Salmonella*	*Shigella*
Number of PCR positive (%)														
Median C_t value (IQR)	216 (39.7%)	36 (4.1%)	22 (2.5%)	76 (8.6%)	325 (36.9%)	33 (3.8%)	69 (7.8%)	147 (16.7%)	275 (31.3%)	167 (19.0%)	125 (14.2%)	222 (25.2%)	58 (6.6%)	154 (17.5%)
	31.4 (30.1–39.3)	26.4 (24.0–29.3)	30.2 (28.0–35.4)	27.8 (23.4–33.5)	21.5 (18.9–26.3)	29.4 (23.8–34.4)	36.2 (32.5–38.8)	30.6 (26.6–35.3)	32.8 (27.3–36.7)	25.5 (22.3–36.6)	31.6 (23.1–37.5)	34.7 (30.0–38.4)	41.4 (39.7–42.3)	29.0 (25.2–34.0)
Vomiting														
Yes/no	145/71	23/13	18/4	52/24	272/53	21/9	48/21	108/39	198/76	127/39	98/27	153/69	40/18	100/54
OR	0.77	0.69	1.80	0.84	2.80	0.68	0.90	1.11	1.04	1.36	1.51	0.84	0.87	0.68
p[a]	0.18	0.34	0.35	0.51	<0.0001	0.33	0.68	0.62	0.81	0.15	0.086	0.30	0.65	0.049
C_t value (vomiting, yes/no)	36.2/36.1	26.7/24.7	30.2/31.7	27.7/28.6	21.2/22.3	29.4/29.2	36.2/36.2	30.0/34.4	32.7/32.7	24.7/33.3	30.9/33.7	34.3/35.9	41.7/40.4	28.9/29.2
p[b]	0.91	0.06	0.91	0.83	0.035	0.54	0.54	0.017	0.22	0.0087	0.28	0.85	0.27	0.66
Dehydration														
Severe/ moderate /mild or no	24/92/100	5/19/12	1/14/7	7/38/31	63/210/52	2/18/13	9/41/19	20/86/41	37/149/89	33/93/41	13/72/40	22/123/77	3/33/22	12/71/71
OR[c]	0.59	1.13	0.33	0.69	2.49	0.44	0.51	1.13	1.13	2.03	0.79	0.76	0.36	0.54
p[d]	0.014	0.85	0.77	0.16	<0.0001	0.32	0.31	0.12	0.41	0.0003	0.92	0.30	0.21	0.0003
C_t value (severe /moderate /mild or no)	36.1/36.5/35.9	26.0/26.7/24.6	34.0/29.4/31.0	30.4/27.2/28.4	20.5/ 21.5/ 22.8	39.1/28.6/26.4	34.5/36.0/38.0	29.5/30.5/34.4	31.7/32.8/33.0	25.5/24.7/32.1	25.4/30.9/33.1	33.6/34.9/35.0	41.7/41.7/40.2	25.6/28.3/30.9
p[c]	0.73	0.87	0.69	0.54	0.0085	0.13	0.09	0.37	0.063	0.28	0.038	0.3	0.79	0.012
Intravenous fluid														
Yes/no	75/141	21/15	14/8	28/48	215/109	13/20	40/29	80/67	128/146	95/72	68/56	89/132	22/36	48/105
OR	0.70	1.67	2.08	0.66	3.78	0.75	1.68	1.49	1.03	1.71	1.50	0.73	0.69	0.47
p[a]	0.049	0.17	0.13	0.093	<0.0001	0.48	0.044	0.030	0.83	0.0025	0.041	0.051	0.22	p <0.0001
C_t value i.v. fluid, yes/no	32.5/31.5	26.7/24.9	30.2/28.3	27.0/28.5	20.8/23.0	30.6/26.4	35.5/38.0	29.9/32.3	36.2/37.0	24.7/31.6	28.4/33.6	34.0/35.4	41.7/41.2	27.9/30.5
p[b]	0.77	0.45	0.37	0.32	0.0005	0.23	0.042	0.26	0.056	0.032	0.0011	0.11	0.42	0.0083
Bloody faeces														
Yes/no	8/208	2/34	0/22	2/74	6/318	1/32	3/66	6/141	15/260	6/161	4/121	10/212	1/57	12/142
OR	1.11	1.28	0	0.56	0.29	0.67	0.98	1.30	1.39	0.77	0.68	1.02	0.36	2.47
p[a]	0.82	0.67	0.62	0.57	0.0036	1.00	1.00	0.90	0.38	0.68	0.64	1.00	0.51	0.015
Diarrhoea frequency														
>6/<6 per day	61/157	11/25	7/15	22/54	120/205	8/25	26/43	48/99	93/182	65/102	47/78	72/150	17/41	57/97
OR	0.95	0.90	0.96	0.82	1.35	0.65	1.27	1.00	1.07	1.40	1.29	0.98	0.84	1.26
p[a]	0.84	0.86	1.0	0.52	0.045	0.35	0.35	1.00	0.64	0.07	0.22	0.93	0.66	0.22
CRP														
>10/<10 mg/L	89/102	20/13	12/7	37/30	138/123	21/9	37/27	57/71	122/125	66/74	62/55	102/104	24/27	58/92
OR	0.97	0.64	0.58	0.80	0.63	0.19	0.72	1.31	1.05	1.16	0.88	1.04	1.14	1.79
p[a]	0.93	0.29	0.35	0.44	0.32	0.04	0.24	0.18	0.82	0.46	0.55	0.87	0.66	0.002

OR, odds ratio; NoV, norovirus.
Total number was 878 or 879 for some variables due to missing data. Adenovirus was analysed in 544 samples (not in samples collected in 2009–2010). CPR was missing for 107 patients.
[a]p value by Fisher's exact test.
[b]p value by Mann–Whitney *U*-test.
[c]OR by comparing severe vs. not severe.
[d]p value by linear regression with 0 for no or mild, 1 for moderate and 2 for severe dehydration.

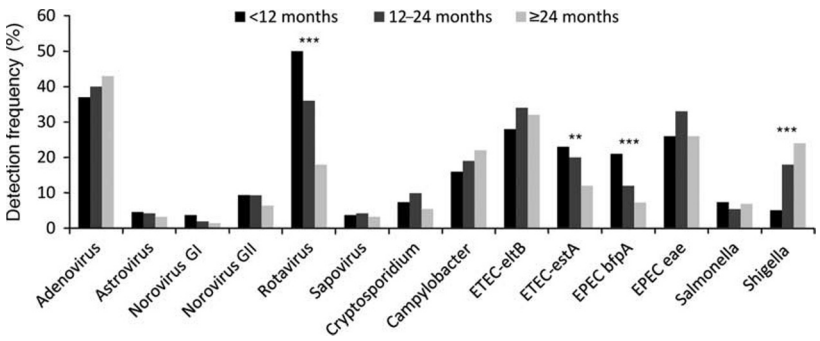

FIG. 2. Detection frequencies among 880 children with diarrhoea by age group. ** p <0.01, *** p <0.001, by *t*-test comparing age as continuous variable.

TABLE 3. Upper part shows multiple logistic regression analysis including age and detection (yes or no) of rotavirus, ETEC-*estA* and *Shigella* as independent variables, and vomiting, dehydration or intravenous fluid treatment as dependent variables. Lower part shows age-adjusted associations between C_t value and vomiting, dehydration or intravenous fluid therapy

		Vomiting yes/no		Severe dehydration yes/no		Intravenous fluid yes/no	
		OR (CI)	p value	OR (CI)	p value	OR (CI)	p value
Rotavirus		2.28 (1.60–3.29)	<0.0001	1.91 (1.25–2.94)	0.0031	2.79 (2.05–3.80)	<0.0001
ETEC-estA		1.13 (0.76–1.72)	0.54	1.79 (1.13–2.82)	0.012	1.42 (0.99–2.05)	0.060
Shigella		0.94 (0.64–1.40)	0.78	0.78 (0.39–1.45)	0.45	0.73 (0.48–1.08)	0.12
Age		1.36 (1.19–1.56)	<0.0001	1.48 (1.16–1.92)	0.0025	1.67 (1.43–1.95)	<0.0001
Rotavirus C_t	n = 325	1.57 (1.04–2.33)	0.032	1.47 (0.94–2.44)	0.09	2.18 (1.54–3.11)	<0.0001
Campylobacter C_t	n = 147	2.21 (1.09–4.63)	0.03	1.90 (0.82–4.66)	0.13	1.64 (0.90–3.02)	0.11
ETEC-estA C_t	n = 167	1.74 (1.08–2.84)	0.024	0.97 (0.59–1.60)	0.89	1.81 (1.20–2.75)	0.004
Shigella C_t	n = 154	1.10 (0.61–1.99)	0.75	3.89 (1.23–15.0)	0.02	2.29 (1.21–4.55)	0.01

OR, odds ratio.
OR in upper part compares positive vs. negative by PCR, and ratio per 1 year decrease for age. OR in lower part shows ratio per 10 cycles of C_t value decline (≈ per 3 $\log_{10}$ increase in pathogen concentration).

therapy than when not detected (i.e. when diarrhoea had another cause). These results indicate that these two agents are not only the most frequent causes of diarrhoea in low-income countries, along with *Cryptosporidium* and *Shigella* [2], but also more likely than other agents to cause severe dehydration. The finding that ETEC-*estA* was more pathogenic than ETEC-*eltB* agrees with previous studies [2,24,25], and points to the need for an ETEC-*estA* vaccine. The pathogenicity of *Cryptosporidium* could not be well addressed because the detection frequency was relatively low. *Shigella* was the only agent associated with enhanced CRP levels or bloody faeces, although the latter was seen in only 8.4% of the *Shigella* cases. A limitation of our study is the lack of *Shigella* species identification, and that identification by the ipaH gene does not distinguish *Shigella* and enteroinvasive *E. coli*. Still, the results support the observations in previous studies that *Shigella* is an important cause of childhood diarrhoea [26,27]. The lower degree of dehydration as compared with rotavirus and ETEC-*estA* might have a bearing on recommendations for the use antibiotics, and calls for further studies.

By relating real-time PCR C_t values to markers for disease, we investigated whether microbial concentrations in faeces were linked to disease. We found that lower C_t values (higher microbial concentrations) for rotavirus were associated with more vomiting, more severe dehydration and intravenous fluid therapy. Likewise, lower C_t values of ETEC-*estA* were associated with vomiting and intravenous fluid therapy, and lower C_t values of *Shigella* were associated with more severe dehydration and intravenous fluid therapy. Previous studies have reported that quantification of rotavirus [28], ETEC-*estA* [29], norovirus GII [29], *Cryptosporidium* [29] or *Shigella* [29,30] may serve to distinguish symptomatic and asymptomatic infections, but to our knowledge this is the first study that associates faecal pathogen load estimates (C_t values) of rotavirus, ETEC-*estA* and *Shigella* with severity of symptoms.

Age was associated with clinical symptoms and also closely related to detection frequencies for rotavirus, ETEC-*estA*, EPEC-*bfpA* and *Shigella*, with 50% and 18%, 23% and 12%, 21% and 7.3%, and 5% and 24% of cases below 12 and above 24 months of age, in agreement with previous reports [2].

Thus, differences in age were present for the same agents that were associated with disease markers, indicating that the latter might be attributed to age rather than to microbial pathogenicity. To some extent this was true, because younger age was strongly associated with dehydration and need for hospitalization. However, in multiple logistic regression analysis including age, the associations between disease and detection or C_t values of rotavirus and ETEC-*estA* remained.

Salmonella was detected in 6.6%, but with high C_t values, using a target gene (ompC) presumed to be present in all Salmonella types. Complementary testing of 36 samples using a real-time PCR targeting the InvA gene (considered to be specific for enteritic types) was reactive in only four cases (not shown). The significance of the Salmonella ompC gene detection in the other cases is uncertain, but might be due to low levels of non-enteritic Salmonella, possibly reflecting exposure from animals in the environment.

Antibiotics were given to 42% of all children. They were more often given to children with fever, elevated CRP or severe dehydration, but not more often if blood was present in faeces, and were as commonly prescribed for viral as bacterial infections. These findings argue against the use of antibiotics for acute diarrhoea in children, because most treated patients had viral infections and many patients with *Shigella* or other bacterial infections that might benefit from treatment were not treated. Also, the value of treatment is uncertain even if diarrhoea has bacterial aetiology, in particular as antibiotic resistance is frequent and increasing. The milder degree of dehydration in patients with *Shigella* also may argue against the use of antibiotics, which, however, might be justified in children with bloody diarrhoea.

In summary, a broad real-time PCR panel was useful for studying microbial agents in faeces from children with diarrhoea, revealing at least one pathogen in 92% of cases. Rotavirus and ETEC-*estA* were the most important aetiologies, and for these agents, as well as for *Shigella*, clinical symptoms were associated with microbial load.

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Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers and probes targeting RNA or DNA of diarrhoeagenic agents.

References

1. Liu L, Johnson HL, Cousens S *et al.* Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012; 379: 2151–2161.
2. Kotloff KL, Nataro JP, Blackwelder WC *et al.* Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case–control study. *Lancet* 2013; 382: 209–222.
3. Steele AD, Patel M, Parashar UD *et al.* Rotavirus vaccines for infants in developing countries in Africa and Asia: considerations from a World Health Organization-Sponsored consultation. *J Infect Dis* 2009; 200: S63–S69.
4. Barry EM, Wang J, Wu T, Davis T, Levine MM. Immunogenicity of multivalent *Shigella*-ETEC candidate vaccine strains in a guinea pig model. *Vaccine* 2006; 24: 3727–3734.
5. Shah D, Choudhury P, Gupta P *et al.* Promoting appropriate management of diarrhea: a systematic review of literature for advocacy and action: UNICEF-PHFI series on newborn and child health, India. *Indian Pediatr* 2012; 49: 627–649.
6. Liu J, Gratz J, Amour C, *et al.* A laboratory-developed TaqMan array card for simultaneous detection of 19 enteropathogens. *J Clin Microbiol* 2013; 51: 472–480.
7. de Boer RF, Ott A *et al.* Improved detection of five major gastrointestinal pathogens by use of a molecular screening approach. *J Clin Microbiol* 2010; 48: 4140–4146.
8. Sinha A, Sengupta S, Guin S *et al.* Culture-independent real-time PCR reveals extensive polymicrobial infections in hospitalized diarrhoea cases in Kolkata, India. *Clin Microbiol Infect* 2011; 28: 173–180.
9. Kabayiza J-C, Andersson ME, Welinder-Olsson C *et al.* Comparison of rectal swabs and faeces for real-time PCR detection of enteric agents in Rwandan children with gastroenteritis. *BMC Infect Dis* 2013; 13: 447.
10. Oka T, Katayama K, Hansman GS *et al.* Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. *J Med Virol* 2006; 78: 1347–1353.
11. Gustavsson L, Westin J, Andersson L-M, Lindh M. Rectal swabs can be used for diagnosis of viral gastroenteritis with a multiple real-time PCR assay. *J Clin Virol* 2011; 51: 279–282.
12. Heim A, Ebnet C, Harste G, Pring-Akerblom P. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. *J Med Virol* 2003; 70: 228–239.
13. Pang XL, Lee B, Boroumand N *et al.* Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. *J Med Virol* 2004; 72: 496–501.
14. Konkel ME, Gray SA, Kim BJ, Garvis SG, Yoon J. Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. *J Clin Microbiol* 1999; 37: 510–517.
15. Sethabutr O, Venkatesan M, Yam S *et al.* Detection of PCR products of the ipaH gene from *Shigella* and enteroinvasive *Escherichia coli* by enzyme linked immunosorbent assay. *Diagn Microbiol Infect Dis* 2000; 37: 11–16.

16. Haque R, Roy S, Siddique A *et al.* Multiplex real-time PCR assay for detection of *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. *Am J Trop Med Hyg* 2007; 76: 713–717.
17. Alvarez J, Sota M, Vivanco AB *et al.* Development of a multiplex PCR technique for detection and epidemiological typing of salmonella in human clinical samples. *J Clin Microbiol* 2004; 42: 1734–1738.
18. Ibrahim A, Liesack W, Griffiths MW, Robins-Browne RM. Development of a highly specific assay for rapid identification of pathogenic strains of *Yersinia enterocolitica* based on PCR amplification of the Yersinia heat-stable enterotoxin gene (yst). *J Clin Microbiol* 1997; 35: 1636–1638.
19. Shirai H, Nishibuchi M, Ramamurthy T *et al.* Polymerase chain reaction for detection of the cholera enterotoxin operon of *Vibrio cholerae*. *J Clin Microbiol* 1991; 29: 2517–2521.
20. Beaudry M, Zhu C, Fairbrother JM, Harel J. Genotypic and phenotypic characterization of *Escherichia coli* isolates from dogs manifesting attaching and effacing lesions. *J Clin Microbiol* 1996; 34: 144–148.
21. Gunzburg ST, Tornieporth NG, Riley LW. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *J Clin Microbiol* 1995; 33: 1375–1377.
22. Victor T, du Toit R, van Zyl J, Bester AJ, van Helden PD. Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin A subunit. *J Clin Microbiol* 1991; 29: 158–161.
23. Stacy-Phipps S, Mecca JJ, Weiss JB. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. *J Clin Microbiol* 1995; 33: 1054–1059.
24. Qadri F, Svennerholm A-M, Faruque ASG, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev* 2005; 18: 465–483.
25. Steinsland H, Valentiner-Branth P, Perch M *et al.* Enterotoxigenic *Escherichia coli* infections and diarrhea in a cohort of young children in Guinea-Bissau. *J Infect Dis* 2002; 186: 1740–1747.
26. Thiem VD, Sethabutr O, von Seidlein L *et al.* Detection of *Shigella* by a PCR assay targeting the ipaH gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. *J Clin Microbiol* 2004; 42: 2031–2035.
27. Kotloff KL, Winickoff JP, Ivanoff B *et al.* Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ* 1999; 77: 651–666.
28. Dung TTN, Phat VV, Nga TVT *et al.* The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting Rotavirus A and Norovirus. *J Virol Methods* 2013; 187: 138–143.
29. Elfving K, Andersson M, Msellem MI *et al.* Real-time PCR threshold cycle (C_t) cut-offs help to identify agents causing acute childhood diarrhea in Zanzibar. *J Clin Microbiol* 2014; 52: 916–923.
30. Lindsay B, Ochieng JB, Ikumapayi UN *et al.* Quantitative PCR for detection of *Shigella* improves ascertainment of *Shigella* burden in children with moderate-to-severe diarrhea in low-income countries. *J Clin Microbiol* 2013; 51: 1740–1746.