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Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis

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ABSTRACT

Background: Several viruses can cause diarrheal disease, a leading cause of morbidity and mortality worldwide. Existing diagnostic methods include ELISA and nucleic acid amplification, usually performed individually.
Objectives: (1) To develop a multiplexed assay for simultaneous detection of major enteric viral pathogens. (2) Quantitation of viral load by normalizing with an extrinsic control.
Study design: A simple protocol combining a one-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) with microsphere-based fluorescence detection was developed for norovirus GI and GII, rotavirus, astrovirus, sapovirus, and adenovirus. An extrinsic control, bacteriophage MS2, was spiked into each fecal sample before nucleic acid extraction to normalize between samples for the efficiency of nucleic acid extraction and amplification.
Results: The fluorescent results were quantitative and nearly as sensitive as the corresponding singleplex real time RT-PCR (qRT-PCR) assay on analytic samples. Upon testing 229 fecal samples from inpatients with diarrhea in Tanzania the assay yielded between 88% and 100% sensitivity and specificity for all analytes. The difference in fluorescence intensities of MS2 between samples indicated variable extraction efficiency and was used to better refine the viral load of each specimen.
Conclusions: This one-step nucleic acid-based assay enables rapid, sensitive and specific detection of the major viral causes of gastroenteritis. The quantitation yielded by the assay is informative for clinical research particularly in the context of mixed infections.

1. Background

Gastroenteritis is the second most common illness after the common cold. Globally diarrhea accounts for approximately 2 million deaths in children less than 5 years old, or 19% of total child deaths.[1,2] Diarrheal pathogens include viruses, bacteria and parasites. Several studies have found at least one viral agent in about 43% of diarrheal specimens, with multiple agents seen in 11%.[3] Although rotavirus is generally the most common enteric pathogen in children worldwide, the role of other enteric viruses is less understood because of laboratory challenges and the need for multiple individual assays. However caliciviruses (norovirus genogroup GI and GII and sapoviruses), astroviruses, and adenoviruses have been increasingly appreciated using newer molecular tools.

2. Objectives

We sought to develop a single multiplex nucleic acid amplification test for these major viral enteropathogens, with an added desire that the reaction be quantitative to better understand mixed infections.

3. Study design

3.1. Specimen collection

Fecal samples were collected from inpatients with diarrhea from Kilimanjaro Christian Medical Centre and referral hospitals

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, real time reverse transcription polymerase chain reaction; MFI, median fluorescence intensity; pfu, plaque forming unit; ROC, receiver-operating characteristic; PBS, phosphate buffered saline.

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Table 1
Primer and probe design for the 7-plex RT-PCR-Luminex assay.

		Target region	Primer/Probe Sequence[a,b]	Location of 5' (accession no.)	Reference
Adenovirus	Hexon	F	GCCACRGTGGGRTTTCTCAACTT	18,988 (L19443 and M21163)	This study
		R	GCCGCAATGGTCTTACATGCACATC	18,857 (L19443 and M21163)	This study
		P	TGCACCAGGCCCGGGCTCAG	18,925 (L19443 and M21163)	This study
Astrovirus	Capsid	F	CAGTTGCTTGCTGCGTTCA	4174 (AY720892)	This study
		R	CTTGCTAGCCATCACACTTCT	4345 (AY720892)	This study
		P	CACAGAAGAGCAACTCCATCGC	4284 (AY720892)	This study
Norovirus GI	ORF1–ORF2	F	CGYTGGATGCGNTTYCATGA	5291 (M87661)	5
		R	CTTAGACGCCATCATCATTYAC	5375 (M87661)	5
		P	AGATCGCAATCTTCTGCCCG	5329 (M87661)	This study
Norovirus GII	ORF1–ORF2	F	CARGARBCNATGTTYAGR TGGATGAG	5003 (AF145896)	5
		R	TCGACGCCATCTTCATTCACA	5100 (AF145896)	5
		P	TGGGAGGGCGATCGCAATCT	5048 (AF145896)	5
Rotavirus	NSP3	F	ACCATCTWCACRTRACCCTCTATGAG	963 (X81436)	8
		R	GGTCACATAACGCCCCTATAGC	1049 (X81436)	8
		P	AGTTAAAAGCTAACACTGTCAAA	995 (X81436)	8
Sapovirus	RdRp-capsid	F	GAYCAGGCTCTCGCYACCTAC	5078 (AY237420)	6
			TTGGCCCTC GCCACCTAC	707 (U73124)	6
			TTTGAACAAGCTGTGGCATGCTAC	5112 (AY646856)	6
		R	CCCTCCATYTCAAACACTA	5181 (AY237420)	6
		P	CYTGGTTCATAGGTGGTRCAG	5103 (AY237420)	This study
			CAGCTGGTACATTGGTGGCAC	5138 (AY646856)	This study
Extrinsic control	MS2g1	F	TGGCACTACCCCTCTCCGTATTCAC	289 (NC_001417)	7
		R	GTACGGGCGACCCCACGATGAC	387 (NC_001417)	7
		P	CACATCGATAGATCAAGGTGCC	330 (NC_001417)	This study

[a] Mixed bases are as follows: Y, C or T; R, A or G; B, not A; N, any.
[b] F, R and P represent forward primer, reverse primer and capture oligonucleotide probe, respectively.

in Moshi, Tanzania from February 2008 to June 2009, transported within 2 h, and stored at −80 °C prior to assay. There were no additional tests performed to detect enteric viruses other than those described here.

3.2. Nucleic acid extraction from fecal specimens

Total nucleic acid extraction was performed using the QuickGene RNA tissue kit SII. Briefly, the fecal sample was suspended in phosphate buffered saline (PBS) and centrifuged at 4000 × *g* for 20 min. 200 μl of supernatant was added to 250 μl of LRT lysis solution supplemented with 1% β-mercaptoethanol, vortexed, and 146 μl of SRT solubilization solution added prior to incubation at room temperature for 10 min. 250 μl of 70% ethanol was added and the samples were processed using QuickGene-810 system with a RNA Tissue protocol.

3.3. One-step RT-PCR amplification

Primers and probes (Table 1) were sourced from these literatures where possible,[4–8] but with some modifications. For the RT-PCR-Luminex assay, reverse primers were labeled with biotin-TEG at 5′ ends. Multiplex reactions typically contained 1× Qiagen OneStep RT-PCR buffer, 0.5 mM $MgCl_2$, 0.4 mM dNTP, 12, 2, 8, 8, 8, 8 and 4 pmol of each of the adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus, sapovirus and MS2 primers, 0.8 μl of enzyme mix, 16 units of RNase inhibitor and 1 μl of extracted nucleic acid sample. RT-PCR was performed with the following conditions: 50 °C for 30 min; 95 °C for 15 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; a final 10 min extension at 72 °C. Positive samples and negative control (nuclease free water) were included on each 96-well plate. The singleplex qRT-PCR was carried out in a 20-μl reaction containing 10 μl of Quantitect Probe RT-PCR Master Mix (Qiagen, Valencia, CA), 20 pmol of each forward and reverse primers, 4 pmol of Taqman probe, 0.2 μl of RT-mix, and 1 μl of nucleic acid sample under the following conditions: 50 °C for 30 min; 95 °C for 15 min, 40 cycles of 95 °C for 10 s, 56 °C for 1 min. For qRT-PCR the same primer and probe sets in Table 1 were used for adenovirus, norovirus GII, rotavirus, sapovirus and MS2. For norovirus GI, the probes were from Kageyama et al.[5] For astrovirus, we used the primer and probe sequences of Logan et al. because they had undergone validation.[9] For the multiplex RT-PCR-Luminex assay the norovirus GI probe and the astrovirus sequences were redesigned.

3.4. Microsphere-based Luminex detection

Oligonucleotide capture probes (Table 1) were modified with an amino-C12 linker at 5′ ends for covalent linkage to carboxylated microspheres (BioRad, Hercules, CA). Bead coupling and hybridization were performed according to published protocols.[10,11] Samples were analyzed on the BioPlex 200 system (BioRad) with high RP1. Results were reported as median fluorescent intensity (MFI). ΔMFI was calculated as $\Delta MFI = MFI_{Analyte} - MFI_{NegativeControl}$.

3.5. Extrinsic control

Bacteriophage MS2 (15597-B1™, ATCC, Manassas, VA) plaque forming units (pfu) were determined by plaque assays. PBS was used to make dilution to 10^7 pfu/μl, and 1 μl was added to each sample before extraction.

3.6. Preparation of RNA transcript

PCR products of each targeted region were cloned into pCR®2.1 TA vector (Invitrogen, Carlsbad, CA). Insert sections were amplified using M13 forward (−20) and M13 reverse primers and in vitro transcribed using AmpliScribe T7 Transcription Kit (EPICENTRE, Madison, WI). A serial dilution of RNA transcript was made with either RNA storage solution (Ambion, Austin, TX) or nucleic acid extract from an uninfected fecal sample. Each sample was tested by both singleplex qRT-PCR and 7-plex RT-PCR-Luminex assays.

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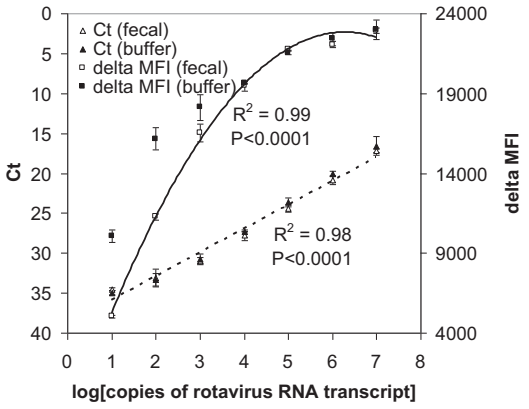

Fig. 1. Correlation between template copy number, detection by singleplex qRT-PCR (Ct), and multiplex RT-PCR-Luminex assay (median fluorescence intensity – negative control). A serial dilution of T7 RNA transcript for was prepared either alone in 1 mM sodium citrate (buffer) or spiked into a common fecal nucleic acid extract. Materials were then interrogated by both singleplex qRT-PCR and the multiplex RT-PCR-Luminex assay. Best fit lines and regression (R^2) were extrapolated. The rest of the analytes are presented in supplemental Figure S1.

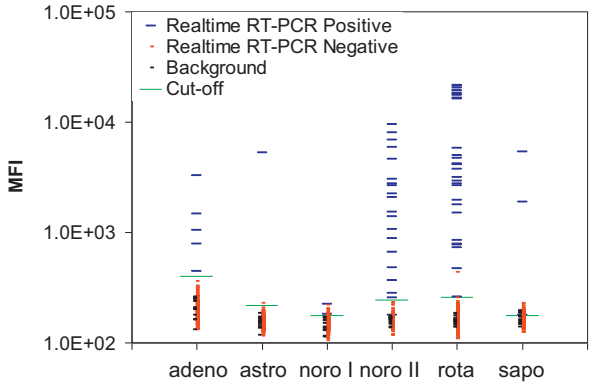

Fig. 2. Correlation between singleplex qRT-PCR results and the multiplex RT-PCR-Luminex assay on clinical specimens. Two hundred and twenty nine diarrheal specimens from inpatients in Tanzania underwent nucleic acid extraction and testing by both singleplex qRT-PCR and the multiplex RT-PCR-Luminex assay. Specifically, these included 29 qPCR positive specimens for rotavirus, 20 for norovirus GII, five for adenovirus, three for sapovirus, two for norovirus GI and 1 for astrovirus. Cut-off threshold values were determined by ROC analysis (see Table 2).

3.7. Statistics

Receiver-operating characteristic (ROC) analysis was performed with PASW Statistics Software and used to define a cut-off in the ΔMFI values to attribute positivity to viral pathogens in diarrhea cases, using singleplex qRT-PCR as gold standard. Data shown as mean ± SD unless otherwise indicated. Correlation among viral copy number, qRT-PCR Ct values and ΔMFI from RT-PCR-Luminex assay was tested by regression analysis using Pearson's test.

4. Results

4.1. Viral gastroenteritis panel design

We started our development with multiplex qRT-PCR; however, it became technically arduous to multiplex in a single reaction with dozens of primers and probes. Thus we transitioned to a flow cytometry based Luminex assay that separated the multiplex PCR and detection steps. Because fecal samples are heterogeneous and rich in extraction and amplification inhibitors, an RT-PCR assay for MS2, an RNA bacteriophage, was developed and 10^7 pfu was spiked into each sample as an extrinsic control[12] to monitor the combined efficiency of extraction and amplification. MS2 was chosen because it has been used as internal control in similar studies and has not been found in human feces.[7,13,14,15]

4.2. 7-plex RT-PCR-Luminex assay is sensitive and quantitative

The sensitivity of the assay was first tested with analytical samples (rotavirus shown in Fig. 1 and the rest in supplemental Figure S1). For this we used in vitro transcripts for RNA viruses and plasmid DNA for DNA virus [4,16–18] and performed serial dilutions both in buffer and in a common fecal nucleic acid extract. Ct values from qRT-PCR showed an expected decrease with each log of virus copy number (R^2 = 0.96–1.00). The 7-plex RT-PCR-Luminex assay also exhibited linear fluorescence, however the fit was not as tight as qRT-PCR (R^2 = 0.82–0.95, P values between 0.0048 and 0.0002, curves not shown). Polynomial regression yielded a better fit for the RT-PCR-Luminex assay (Figs. 1 and S1, R^2 = 0.96–0.99) and was used to generate a standard curve for each analyte. The limit of detection of the multiplex RT-PCR-Luminex assay correlated to ~10^2–10^4 viral copies per gram of stool depending on the virus, whereas singleplex amplifications, either detected by Luminex or qRT-PCR usually detected 5–50 times lower levels.

4.3. Performance in clinical specimens from patients with diarrhea

We then evaluated the assay on fecal specimens from 229 inpatients across all ages with diarrhea from Tanzania. Singleplex qRT-PCR was used here as the gold standard. By singleplex qRT-PCR, 53 (23.1%) were found to be positive for at least one of the 6 viral pathogens, of which more than half (54.7%) were rotavirus infection while 37.7% were norovirus GII. The incidences of the other viruses were much lower: 9.4% were adenovirus, 5.7% were sapovirus, 3.8% were norovirus GI and 1.9% was astrovirus. Seven mixed infections were detected: two for adenovirus and rotavirus, one for astrovirus and rotavirus, one for norovirus GI and GII, two for norovirus GII and rotavirus, and one for rotavirus and sapovirus. ROC analysis was performed to assign Luminex ΔMFI cutoff values which yielded sensitivity and specificity of between 88% and 100% for each virus (Figs. 2 and 3, Table 2). The cutoff values for norovirus GI, adenovirus, astrovirus, and

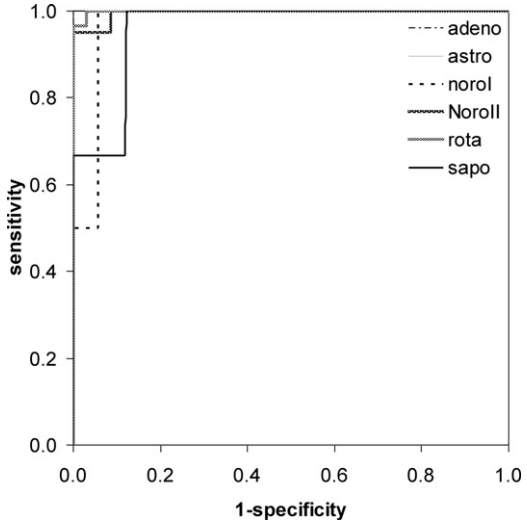

Fig. 3. Receiver-operating characteristic (ROC) analysis for the multiplex RT-PCR-Luminex assay versus singleplex qRT-PCR as the gold standard.

Table 2
Summary of receiver-operating characteristic analysis.

Pathogen interrogated	ΔMFI cut-off	Sensitivity	Specificity	Youden's index	Sample size	
					Positive	Negative
Adenovirus	170	1.00	1.00	1.00	5	224
Astrovirus	47	1.00	1.00	1.00	1	228
Norovirus GI	30	1.00	0.94	0.94	2	227
Norovirus GII	77	0.95	1.00	0.95	20	209
Rotavirus	46	1.00	0.97	0.97	29	200
Sapovirus	21	1.00	0.88	0.88	3	226

Youden's index is an overall measure of diagnostic test accuracy and = sensitivity + specificity − 1, where 1.00 is perfect.

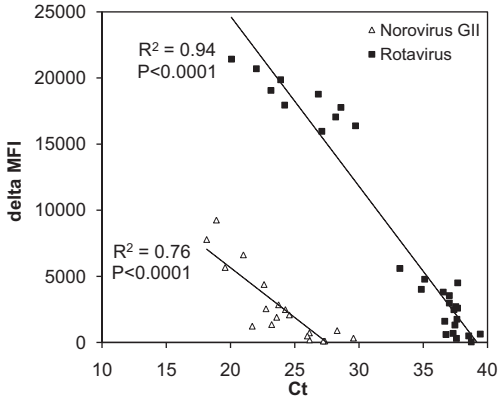

Fig. 4. Correlation between singleplex qRT-PCR Ct and the multiplex RT-PCR-Luminex assay MFI values on clinical specimens. Best fit lines and regression (R^2) were extrapolated.

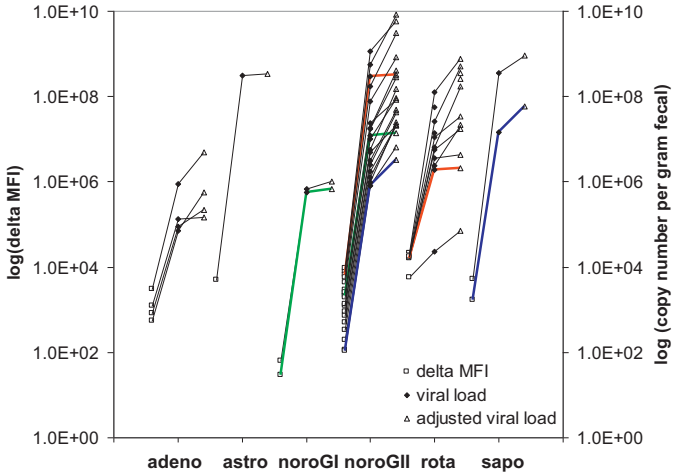

Fig. 5. Quantitation of viral load and normalization for the sample's extraction and amplification efficiency. The viral load was estimated from the multiplex RT-PCR-Luminex positive samples based on the standard curves of Figs. 1 and S1, then multiplied by the assay dilution factor to yield viral copies per gram of stool. This viral load was then adjusted for the sample's nucleic acid extraction and RT-PCR amplification efficiency. Specifically, for each sample the loss of detection of the extrinsic control was estimated by comparing the expected amount of spiked MS2 versus the detected amount of MS2. Three cases of co-infection were included and shown in color, i.e. one norovirus GI and GII coinfection in green, one norovirus GII and rotavirus coinfection in red, one norovirus GII and sapovirus coinfection in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sapovirus are less certain because of the low number of positive samples.

4.4. Quantitation of the 7-plex RT-PCR-Luminex assay

We next examined the correlation between the ΔMFI and the qRT-PCR Ct. This revealed a statistically significant correlation for norovirus GII and rotavirus (Fig. 4). Data could not be evaluated for adenovirus, astrovirus, norovirus GI and sapovirus because of small sample size (n = 1–5). We then used the ΔMFI values to approximate the viral loads based on the equations of Figs. 1 and S1 (note, viral loads were similar when the qRT-PCR Ct equations were used, data not shown). These viral load data for the norovirus GII samples are shown in Table 3. Finally, by estimating the detected viral load of MS2 bacteriophage, and comparing this to the expected amount of spiked MS2 (calculated from ΔMFI values of PBS samples spiked with the same amount of MS2), we were able to calculate an efficiency of nucleic acid extraction/amplification. This revealed significant variation in the efficiency of extraction/amplification between samples as others have reported.[7] We then adjusted the estimated viral load according to this efficiency (Fig. 5 and Table 3). This refinement led to a significant shift in the order of viral loads, for example samples 11 and 12 had similar viral loads based on ΔMFI but when adjusted for their efficiency sample 11 had nearly 1 log more norovirus.

We postulated that accurate quantitation of viral loads may be useful to predict etiology in mixed infections (Fig. 5). Specifically we show three co-infections involving which revealed greater norovirus GII loads (vs. norovirus GI and rotavirus) and greater sapovirus loads (vs. norovirus GII). Finally, since standard curves with in vitro transcripts are impractical for field use, we examined whether a simple formula could predict the adjusted viral load and found a tight correlation between the ΔMFI ratio and standard curve-derived viral load (Fig. 6).

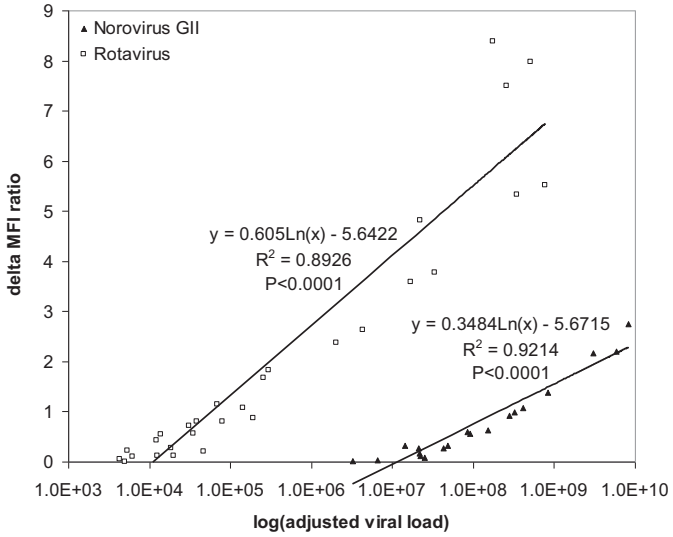

Fig. 6. Simplified estimation for viral load. The ratio of viral ΔMFI value to extrinsic control ΔMFI ($\Delta MFI_{Analyte}/\Delta MFI_{ExtrinsicControl}$) is shown versus the adjusted viral load values of Fig. 5. Norovirus GII and rotavirus are shown.

Table 3
Variation in nucleic acid extraction and amplification efficiency among clinical samples. The data for norovirus GII are shown as an example ($n = 2$). The estimated load of norovirus GII and of the extrinsic control were extrapolated from the standard curves of Fig. S1. The expected versus detected load of the extrinsic control allowed a measure of the efficiency of nucleic acid extraction and amplification. Adjusted viral burden ranks were thus changed.

Viral ΔMFI	Estimated viral load	Initial viral burden rank	MS2 ΔMFI	Efficiency	Adjusted viral load	Adjusted viral burden rank
8884 ± 368	(9.94 ± 1.76)E+08	1	4044 ± 167	0.179 ± 0.021	(5.50 ± 0.33)E+09	2
7401 ± 382	(4.70 ± 0.94)E+08	2	2685 ± 140	0.061 ± 0.008	(7.60 ± 0.60)E+09	1
6531 ± 93	(2.87 ± 0.15)E+08	3	6574 ± 149	0.871 ± 0.073	(3.31 ± 0.11)E+08	6
5382 ± 284	(1.48 ± 0.26)E+08	4	2536 ± 80	0.053 ± 0.004	(2.75 ± 0.29)E+09	3
4140 ± 242	(6.49 ± 1.09)E+07	5	3024 ± 127	0.082 ± 0.009	(7.88 ± 0.50)E+08	4
3009 ± 160	(2.76 ± 0.36)E+07	6	4790 ± 65	0.298 ± 0.013	(9.21 ± 0.79)E+07	10
2602 ± 53	(1.94 ± 0.09)E+07	7	2361 ± 7	0.045 ± 0.000	(4.30 ± 0.17)E+08	5
2365 ± 120	(1.58 ± 0.17)E+07	8	2701 ± 12	0.062 ± 0.001	(2.56 ± 0.25)E+08	7
1937 ± 148	(1.06 ± 0.15)E+07	9	6488 ± 56	0.852 ± 0.027	(1.28 ± 0.15)E+07	17
1885 ± 20	(9.93 ± 0.18)E+06	10	3322 ± 77	0.104 ± 0.006	(9.60 ± 0.39)E+07	9
1461 ± 105	(6.38 ± 0.75)E+06	11	2300 ± 135	0.043 ± 0.006	(1.49 ± 0.02)E+08	8
1120 ± 112	(4.27 ± 0.60)E+06	12	4332 ± 76	0.219 ± 0.011	(1.95 ± 0.18)E+07	16
827 ± 73	(2.88 ± 0.29)E+06	13	2704 ± 61	0.062 ± 0.003	(4.64 ± 0.22)E+07	11
791 ± 65	(2.74 ± 0.26)E+06	14	2678 ± 57	0.060 ± 0.003	(4.52 ± 0.20)E+07	12
574 ± 82	(1.98 ± 0.26)E+06	15	3105 ± 119	0.087 ± 0.008	(2.26 ± 0.08)E+07	15
283 ± 49	(1.18 ± 0.12)E+06	16	2485 ± 142	0.051 ± 0.007	(2.31 ± 0.08)E+07	14
258 ± 62	(1.12 ± 0.14)E+05	17	2274 ± 72	0.042 ± 0.003	(2.67 ± 0.15)E+07	13
194 ± 82	(9.78 ± 1.73)E+05	18	3752 ± 213	0.146 ± 0.023	(6.70 ± 0.15)E+06	18
175 ± 68	(9.38 ± 1.43)E+05	19	4563 ± 69	0.256 ± 0.012	(3.65 ± 0.39)E+06	19

Norovirus GII viral load: $\Delta MFI = 611 \times \log^2(Cv) - 2227 \times \log(Cv) + 1766$, where Cv the copy numbers of norovirus GII.
Extrinsic control load: $\Delta MFI = 673 \times \log^2(Cs) - 2407 \times \log(Cs) + 2339$, where Cs the copy numbers of extrinsic control.

5. Discussion

Viral pathogens are responsible for a significant proportion of the diarrheal disease burden. Thus a broad enteroviral diagnostic can be important for epidemiologic research on causes of diarrhea as well as clinical care.

Our entire assay can be performed in less than 6 h and is currently being used by our collaborators in Tanzania, Gambia, Thailand, and Bangladesh. Results are consistent with other reports using molecular tests.[3,19] For example, one review of childhood diarrhea from several countries found, in descending order, rotavirus, caliciviruses, adenoviruses, and astroviruses.[3] In our study, 42 samples were from children under 5 years of age, among which 31% were positive for rotaviruses, 26% for caliciviruses, and 5% for adenoviruses.

An important feature of our assay is quantitation. We anticipate this will be useful for discerning the etiology of diarrhea in mixed infections or where asymptomatic carriage is common. Such asymptomatic carriage is frequent in both developing and developed countries.[20–22] We reported a significant correlation between severity and rotavirus quantity (measured by Ct).[21] Phillips et al.[23] found that up to 16% of healthy individuals may be qRT-PCR positive for noroviruses and argued that a lower qRT-PCR Ct value could be used to determine when norovirus was the likely cause of diarrhea. In terms of mixed infections, one report found that most norovirus GI and GII coinfections had higher GII viral load than GI (fold changes from 4 to 452),[24] as observed in our study.

More information on the expected range of enteroviral loads is needed to better interpret viral loads. In our samples we found $\sim 10^5$–10^6/g for adenovirus and norovirus GI and $\sim 10^8$/g for astrovirus, rotavirus, norovirus GII, and sapovirus. These numbers fit within the wide ranges estimated in the literature,[4,17,24–27] but data are limited. None of these estimates corrected for the extraction or amplification efficiency of the sample, a variable we found important to control for in clinical fecal specimens. With this assay a quick formula using the ratio ($\Delta MFI_{Analyte}/\Delta MFI_{ExtrinsicControl}$) was statistically adequate for this purpose and is easily calculated with the data from each run. Thus, our assay can deliver fast and quantitative data to facilitate diagnosis of viral gastroenteritis and better understand etiology.

Conflict of interest

None.

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Ethical approval

All studies were approved by the institutional review boards.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2010.12.009.

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