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# A Laboratory-Developed TaqMan Array Card for Simultaneous Detection of 19 Enteropathogens

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**The TaqMan Array Card (TAC) system is a 384-well singleplex real-time PCR format that has been used to detect multiple infection targets. Here we developed an enteric TaqMan Array Card to detect 19 enteropathogens, including viruses (adenovirus, astrovirus, norovirus GII, rotavirus, and sapovirus), bacteria (*Campylobacter jejuni/C. coli*, *Clostridium difficile*, *Salmonella*, *Vibrio cholerae*, diarrheagenic *Escherichia coli* strains including enteroaggregative *E. coli* [EAEC], enterotoxigenic *E. coli* [ETEC], enteropathogenic *E. coli* [EPEC], and Shiga-toxigenic *E. coli* [STEC]), *Shigella*/enteroinvasive *E. coli* (EIEC), protozoa (*Cryptosporidium*, *Giardia lamblia*, and *Entamoeba histolytica*), and helminths (*Ascaris lumbricoides* and *Trichuris trichiura*), as well as two extrinsic controls to monitor extraction and amplification efficiency (the bacteriophage MS2 and phocine herpesvirus). Primers and probes were newly designed or adapted from published sources and spotted onto microfluidic cards. Fecal samples were spiked with extrinsic controls, and DNA and RNA were extracted using the QiaAmp Stool DNA minikit and the QuickGene RNA Tissue kit, respectively, and then mixed with Ag-Path-ID One Step real-time reverse transcription-PCR (RT-PCR) reagents and loaded into cards. PCR efficiencies were between 90% and 105%, with linearities of 0.988 to 1. The limit of detection of the assays in the TAC was within a 10-fold difference from the cognate assays performed on plates. Precision testing demonstrated a coefficient of variation of below 5% within a run and 14% between runs. Accuracy was evaluated for 109 selected clinical specimens and revealed an average sensitivity and specificity of 85% and 77%, respectively, compared with conventional methods (including microscopy, culture, and immunoassay) and 98% and 96%, respectively, compared with our laboratory-developed PCR-Luminex assays. This TAC allows fast, accurate, and quantitative detection of a broad spectrum of enteropathogens and is well suited for surveillance or clinical purposes.**

Diarrhea is the second cause of death worldwide in children under the age of five, with the majority of cases occurring in developing countries (1). Diarrheal diseases are also a public health problem in developed countries, particularly in the context of the food supply. Understanding the etiology of diarrhea is important to guide public health efforts and can be important for individual patient care. The conventional diagnosis for diarrheal diseases encompasses a variety of methods including bacterial culture, immunoassay for toxins and antigens of bacteria, viruses, and protozoa, and microscopy for parasites. PCR amplification has emerged as a useful tool to detect pathogen DNA or RNA. It can be performed in either singleplex or multiplex mode in combination with gel electrophoresis, probe hybridization, or real-time fluorescence for detection. Real-time PCR is found to be a sensitive and quantitative amplification/detection method, but its multiplexing capacity has been limited by the availability of fluorescent dyes and platforms. Recently, Life Technologies has started offering a TaqMan Array Card (TAC) platform enabling spatial multiplexing of up to 384 targets, which was originally intended for gene expression studies. The CDC pioneered the use of TAC for detection of 21 respiratory pathogens (2), while recently the United Kingdom's Defense Science and Technology Laboratory also demonstrated the feasibility of TAC for pathogen detection with an example of five biothreat agents (3). Here we report on a TaqMan Array card that we developed for simultaneous detection of 19 diarrhea-causing enteropathogens, including viruses (rotavirus, norovirus GII, adenovirus, astrovirus, and sapovirus), bacteria including diarrheagenic *Escherichia coli* (enterotoxigenic *E. coli* [ETEC], enteropathogenic *E. coli* [EPEC], enteroaggregative *E. coli* [EAEC], and Shiga-toxigenic *E. coli* [STEC]), *Shigella*/enteroinvasive *E. coli* (EIEC), *Salmonella*, *Campylobacter jejuni/Campylobacter coli*, *Vibrio cholerae*, and *Clostridium difficile*, and parasites (*Cryptosporidium*, *Giardia lamblia*, *Entamoeba histolytica*, *Ascaris lumbricoides*, and *Trichuris trichiura*).

## MATERIALS AND METHODS

**Specimens.** Reference strains purchased from the American Type Culture Collection (ATCC, Manassas, VA) or clinical isolates were used for analytical testing for *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio cholerae*, EAEC, STEC, EPEC, ETEC, EIEC, *C. difficile*, *Entamoeba histolytica*, and *Giardia lamblia*. *Cryptosporidium parvum* oocysts were purchased from Waterborne Inc. (New Orleans, LA). For RNA targets including rotavirus, norovirus GII, astrovirus, and sapovirus, *in vitro* transcripts were generated as described earlier and used for analytical testing (4). For adenovirus, *Ascaris lumbricoides*, and *Trichuris trichiura*, amplicons covering the targeted regions were generated. One hundred nine stool samples were

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**TABLE 1** Assay primer and probe sequences[a]

| Organism | Target | Sequence | Reference |
|---|---|---|---|
| Adenovirus | *Hexon*[b] | F, GCCACGGTGGGGTTTCTAAACTT<br>R, GCCCCAGTGGTCTTACATGCACATC<br>P, TGCACCAGACCCGGGCTCAG | 7 |
| Astrovirus | *Capsid* | F, CAGTTGCTTGCTGCGTTCA<br>R, CTTGCTAGCCATCACACTTCT<br>P, CACAGAAGAGCAACTCCATCGC | 4 |
| Norovirus GII | *ORF1-ORF2* | F, CARGARBCNATGTTYAGR TGGATGAG<br>R, TCGACGCCATCTTCATTCACA<br>P, TGGGAGGGCGATCGCAATCT | 8 |
| Rotavirus | *NSP3* | F, ACCATCTWCACRTRACCCTCTATGAG<br>R, GGTCACATAACGCCCCTATAGC<br>P, AGTTAAAAGCTAACACTGTCAAA | 9 |
| Sapovirus | *RdRp* | F, GAYCAGGCTCTCGCYACCTAC<br>F, TTTGAACAAGCTGTGGCATGCTAC<br>R, CCCTCCATYTCAAACACTA<br>P, CYTGGTTCATAGGTGGTRCAG<br>P, CAGCTGGTACATTGGTGGCAC | 10 (modified) |
| EAEC | *aaiC* | F, ATTGTCCTCAGGCATTTCAC<br>R, ACGACACCCCTGATAAACAA<br>P, TAGTGCATACTCATCATTTAAG | 11 (modified) |
| | *aatA* | F, CTGGCGAAAGACTGTATCAT<br>R, TTTTGCTTCATAAGCCGATAGA<br>P, TGGTTCTCATCTATTACAGACAGC | 11 |
| STEC | $stx_1$ | F, ACTTCTCGACTGCAAAGACGTATG<br>R, ACAAATTATCCCCTGWGCCACTATC<br>P, CTCTGCAATAGGTACTCCA | 12 (modified) |
| | $stx_2$ | F, CCACATCGGTGTCTGTTATTAACC<br>R, GGTCAAAACGCGCCTGATAG<br>P, TTGCTGTGGATATACGAGG | 12 |
| EPEC | *eae* | F, CATTGATCAGGATTTTTCTGGTGATA<br>R, CTCATGCGGAAATAGCCGTTA<br>P, ATACTGGCGAGACTATTTCAA | 13 (modified) |
| | *bfpA* | F, TGGTGCTTGCGCTTGCT<br>R, CGTTGCGCTCATTACTTCTG<br>P, CAGTCTGCGTCTGATTCCAA | 14 (modified) |
| ETEC | *LT* | F, TTCCCACCGGATCACCAA<br>R, CAACCTTGTGGTGCATGATGA<br>P, CTTGGAGAGAAGAACCCT | 12 |
| | *ST* | Fh, GCTAAACCAGYAGRGTCTTCAAAA<br>Fp, TGAATCACTTGACTCTTCAAAA<br>Rh, CCCGGTACARGCAGGATTACAACA<br>Rp, GGCAGGATTACAACAAAGTT<br>Ph, TGGTCCTGAAAGCATGAA<br>Pp, TGAACAACACATTTTACTGCT | 15 (modified) |
| EIEC/*Shigella* | *ipaH*[c] | F, CCTTTTCCGCGTTCCTTGA<br>R, CGGAATCCGGAGGTATTGC<br>P, CGCCTTTCCGATACCGTCTCTGCA | 16 |
| *Campylobacter jejuni/C. coli* | *cadF* | F, CTGCTAAACCATAGAAATAAAATTTCTCAC<br>R, CTTTGAAGGTAATTTAGATATGGATAATCG<br>P, CATTTTGACGATTTTTGGCTTGA | 17 |
| *Salmonella* | *invA* | F, TCGGGCAATTCGTTATTGG | 18 (modified) |

(Continued on following page)

**TABLE 1** (Continued)

| Organism | Target | Sequence | Reference |
|---|---|---|---|
| | | R, GATAAACTGGACCACGGTGACA | |
| | | P, AAGACAACAAAACCCACCGC | |
| *V. cholerae* | *toxR* | F, GTTTGGCGAGAGCAAGGTTT | 18 (modified) |
| | | R, TCTCTTCTTCAACCGTTTCCA | |
| | | P, CGCAGAGTCGAAATGGCTTGG | |
| *C. difficile* | *tcdB* | F, GGTATTACCTAATGCTCCAAATAG | 19 (modified) |
| | | R, TTTGTGCCATCATTTTCTAAGC | |
| | | P, CCTGGTGTCCATCCTGTTTC | |
| *Cryptosporidium* | *18S* | F, GGGTTGTATTTATTAGATAAAGAACCA | 20 (modified) |
| | | R, AGGCCAATACCCTACCGTCT | |
| | | P, TGACATATCATTCAAGTTTCTGAC | |
| *Giardia* | *18S* | F, GACGGCTCAGGACAACGGTT | 21 |
| | | R, TTGCCAGCGGTGTCCG | |
| | | P, CCCGCGGCGGTCCCTGCTAG | |
| *E. histolytica* | *18S* | F, ATTGTCGTGGCATCCTAACTCA | 21 |
| | | R, GCGGACGGCTCATTATAACA | |
| | | P, TCATTGAATGAATTGGCCATTT | |
| *Ascaris* | *ITS1* | F, GTAATAGCAGTCGGCGGTTTCTT | 22 |
| | | R, GCCCAACATGCCACCTATTC | |
| | | P, TTGGCGGACAATTGCATGCGAT | |
| *Trichuris* | *18S* | F, TTGAAACGACTTGCTCATCAACTT | This study |
| | | R, CTGATTCTCCGTTAACCGTTGTC | |
| | | P, CGATGGTACGCTACGTGCTTACCATGG | |
| PhHV | *gB* | F, GGGCGAATCACAGATTGAATC | 23 (modified) |
| | | R, GCGGTTCCAAACGTACCAA | |
| | | P, TATGTGTCCGCCACCATCT | |
| MS2 | *MS2g1* | F, TGGCACTACCCCTCTCCGTATTCAC | 24 |
| | | R, GTACGGGCGACCCCACGATGAC | |
| | | P, CACATCGATAGATCAAGGTGCCTACAAGC | |
| Bacterial 16S rRNA gene | | F, TCCTACGGGAGGCAGCA | 25 (modified) |
| | | R, GGACTACCAGGGTATCTAATCCTG | |
| | | P, CGTATTACCGCGGCTGCT | |

[a] F, forward primer; R, reverse primer; P, probe, labeled with FAM (6-carboxyfluorescein) at 5′ and MGB at 3′; h, STh; p, STp.
[b] The assay detected all adenovirus serotypes.
[c] The *ipaH* gene was targeted for detection of both *Shigella* and EIEC.

selected from studies in Haydom, Tanzania (2010 to 2011), and from the Mirpur region of Dhaka, Bangladesh (2008 to 2009), to validate the clinical performance of TAC with a goal of 15 samples positive by conventional methods for most pathogens. Tanzanian samples were tested through an ongoing 5-year study, Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED). Bangladeshi samples were selected from a birth cohort study (5). Stool samples were delivered to the laboratories within 3 h of collection while maintaining a cold chain, shipped to the University of Virginia on dry ice, and stored at −80°C prior to assays.

**Conventional tests.** Clinical samples were tested by enzyme-linked immunosorbent assay (ELISA) for *Cryptosporidium* spp., *Giardia lamblia*, *E. histolytica* (all from TechLab, Blacksburg, VA), *Campylobacter*, rotavirus, adenovirus, and astrovirus (all from Oxoid, Hampshire, United Kingdom). Samples were cultured on MacConkey, xylose lysine deoxycholate (XLD), and thiosulfate-citrate-bile salts-sucrose (TCBS) agars. *Salmonella*, *Shigella*, and *Vibrio cholerae* were identified biochemically. Colonies were pooled from MacConkey agar and tested for diarrheagenic *E. coli* strains including EAEC (targeting *aaiC* and *aatA*), EIEC (targeting *ipaH*), EPEC (targeting *eae* and *bfpA*), ETEC (targeting the heat-stable and heat-labile enterotoxin [ST and LT] genes), and STEC (targeting $stx_1$ and $stx_2$) using a 9-plex assay described previously (6). Samples were examined for *Ascaris* and *Trichuris* by microscopy.

**Nucleic acid extraction.** DNA was extracted from 200 mg of stool with a modified QiaAmp stool DNA extraction protocol (Qiagen, Valencia, CA). In brief, stool was first lysed with QiaAmp ASL buffer, beaten for 3 min with 212- to 300-μm glass beads (Sigma, St. Louis, MO), and boiled for 5 min, and we then proceeded according to the manufacturer's instructions. RNA was extracted from 50 mg of stool with the QuickGene RNA Tissue kit automated with QuickGene-810 (Fujifilm, Tokyo, Japan) (4). As extrinsic controls, $10^6$ copies of phocine herpesvirus (PhHV; a gift from Martin Schutten, Erasmus MC, Department of Virology, Rotterdam, The Netherlands) and $10^7$ MS2 bacteriophage per sample were spiked to the lysis buffers to monitor the efficiency/inhibition of extraction and amplification. The elution volume for DNA was 200 μl; for RNA, it was 50 μl, which was then supplemented with 50 μl of RNA storage

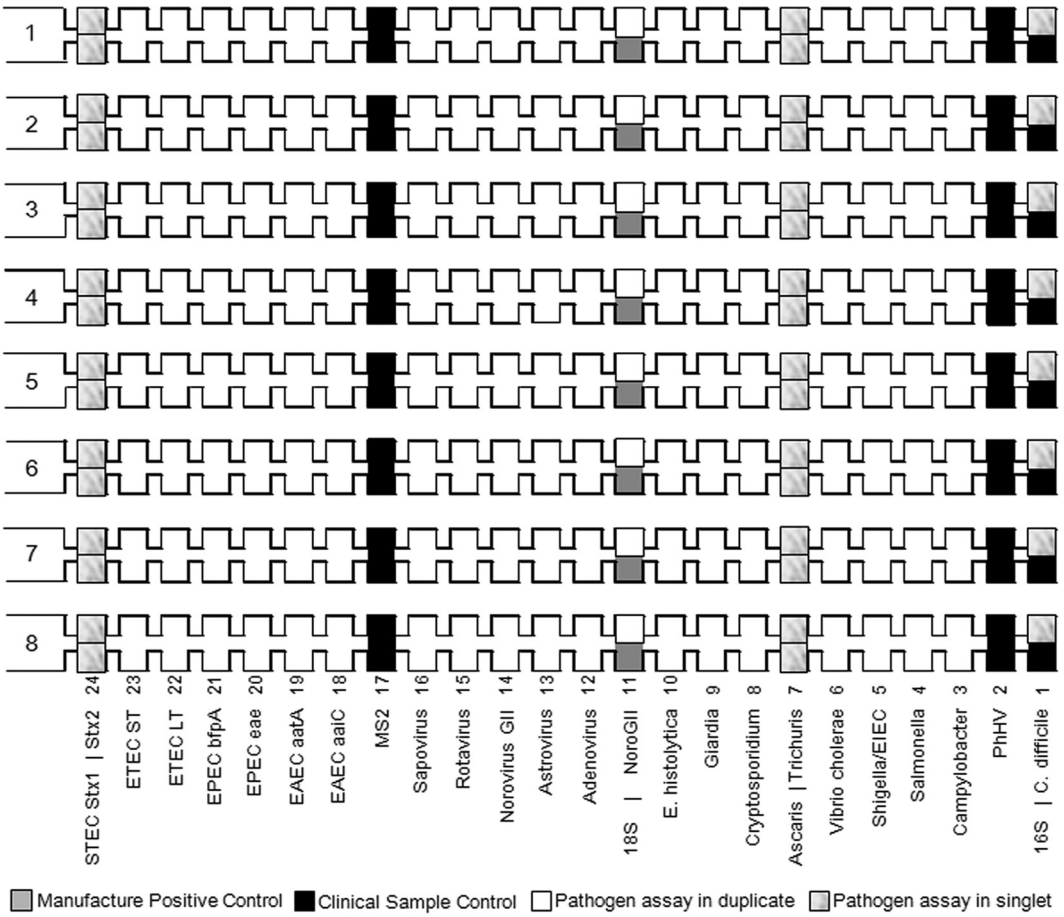


**FIG 1** Enteric TaqMan Array Card layout. Format 48 was used.

solution (Life Technologies, Carlsbad, CA). A no-template extraction control was included every week to exclude lab contamination.

**TAC design and procedure.** TAC assays were taken from our published assays and adapted from published sources when possible (Table 1). A single target was selected for 15 pathogens, while two targets were used for EPEC, STEC, ETEC, and EAEC. Assays were validated on plates based on the TaqMan array universal formula of a final primer concentration of 900 nM and a probe concentration of 250 nM. The Ag-Path-ID One-Step RT-PCR kit was used, and the cycling conditions were as follows: 45°C for 20 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The assay mixtures were spotted onto the microfluidic card as laid out in Fig. 1. Twenty assay mixtures were spotted in duplicate and six in singlet. For each sample, equal volumes (20 μl each) of DNA and RNA extracts were combined and then mixed with 50 μl of Ag-Path-ID RT-PCR buffer, 4 μl of enzyme mix, and 6 μl of water to a 100-μl final volume. After thorough mixing, the reaction mixture was loaded into each port of the card and centrifuged twice at 1,200 rpm for 1 min. The card was sealed, the loading ports were excised, and then the card was inserted into a ViiA7 instrument (Life Technologies) and run under the same cycling conditions as above. Upon receipt, this lot of cards was tested with a no-template control that confirmed no amplification.

**Combined positive controls.** Two combined positive controls, one for DNA targets and one for RNA targets, were designed according to the method of Kodani and Winchell (26). Plasmids were synthesized by GeneWiz (South Plainfield, NJ) (see the supplemental material for the sequences). For DNA targets, the plasmid was directly utilized, while the RNA template was generated by amplification of the insert portion with M13 primers and *in vitro* transcription with the AmpliScribe T7 High Yield Transcription kit (Epicentre, Madison, WI) (4).

**PCR-Luminex.** PCR-Luminex panels, including viral, bacterial, *E. coli*, protozoan, and helminth panels, were run according to previously established protocols (4, 6, 18, 27). Results were reported in units of median fluorescence intensity (MFI). To compare real-time PCR cycle threshold ($C_T$) values versus PCR-Luminex MFI values, relative fluorescence intensity was calculated as the percentile versus the highest fluorescence intensity observed for each target, i.e., the MFI cutoff for positivity was set as 0 and the maximal MFI as 100%.

**Analytical performance.** Linearity was tested with 10-fold serial dilutions of combined positive controls. For limit of detection (LOD) and precision (repeatability and reproducibility), positive materials (either whole organisms or the DNA/RNA templates as indicated above) were spiked into healthy donor stool, extracted, and then amplified. Repeatability was tested with eight repeats of two samples respectively spiked with a high and a low concentration of each target. Reproducibility was tested with 10 identically spiked samples for each concentration (two concentrations, high and low, were interrogated) that were extracted and assayed over 5 days. LOD was defined as the lowest concentration at which the target could be detected in all 10 spiked samples. Matrix inhibition was tested with combined positive controls spiked into three different lots of stool from healthy donors. Analytical accuracy was evaluated with reference samples. Nineteen pathogens were divided into three groups: A, consisting of adenovirus, astrovirus, norovirus GII, rotavirus, sapovirus, *Ascaris*, and *Trichuris*; B, consisting of *Campylobacter*, *Salmonella*, *Shigella*, *V. cholerae*, and *C. difficile*; and C, consisting of EAEC, STEC, EPEC, ETEC, *Cryptosporidium*, *Giardia*,

**TABLE 2** Analytical performance of enteric TaqMan Array Card[a]

| Organism | Linearity[b] $R^2$ (efficiency, %) | LOD (equiv. no. of copies)[c] | Low- and high-concn CV (%) for[d]: | | Accuracy (%)[d] | |
|---|---|---|---|---|---|---|
| | | | Repeatability | Reproducibility | Sensitivity[e] | Specificity |
| Adenovirus | 0.997 (99.7) | $10^6$ (100) | 1.1, 1.7 | 2.6, 4.8 | 100 | 100 |
| Astrovirus | 0.999 (93.0) | $5 \times 10^6$ (500) | 2.2, 2.6 | 3.2, 5.3 | 95 | 100 |
| Norovirus GII | 0.998 (99.2) | $5 \times 10^6$ (500) | 1.9, 1.1 | 3.5, 5.3 | 97 | 100 |
| Rotavirus | 0.993 (98.4) | $5 \times 10^6$ (500) | 1.5, 1.8 | 4.2, 7.5 | 100 | 100 |
| Sapovirus | 0.994 (94.9) | $5 \times 10^6$ (500) | 1.8, 2.9 | 5.8, 7.9 | 97 | 100 |
| EAEC | | | | | | |
| *aaiC* | 0.995 (99.7) | $10^5$ (10) | 1.4, 2.8 | 4.2, 6.6 | 100 | 100 |
| *aatA* | 0.998 (95.5) | $10^5$ (10) | 2.0, 3.0 | 6.3, 7.9 | 98 | 100 |
| STEC | | | | | | |
| $stx_1$ | 0.999 (96.3) | $10^5$ (10) | 0.9, 2.9 | 3.5, 5.8 | 100 | 100 |
| $stx_2$ | 0.999 (94.6) | $10^5$ (10) | 0.9, 1.8 | 4.2, 5.5 | 100 | 100 |
| EPEC | | | | | | |
| *eae* | 0.998 (100.2) | $10^5$ (10) | 1.2, 1.5 | 3.6, 4.9 | 100 | 100 |
| *bfpA* | 0.998 (96.7) | $10^5$ (10) | 0.9, 1.3 | 4.2, 5.2 | 100 | 100 |
| ETEC | | | | | | |
| LT | 0.998 (95.8) | $10^5$ (10) | 0.7, 1.3 | 2.5, 3.6 | 100 | 100 |
| ST | 0.998 (95.5) | $10^5$ (10) | 1.0, 1.1 | 3.1, 6.2 | 100 | 100 |
| EIEC/*Shigella* | 0.997 (104.2) | $10^5$ (10) | 1.2, 1.3 | 3.6, 5.2 | 100 | 100 |
| *Campylobacter* | 0.997 (95.9) | $10^6$ (100) | 0.9, 2.4 | 4.9, 7.2 | 100 | 100 |
| *Salmonella* | 0.997 (100.9) | $10^6$ (100) | 2.3, 2.1 | 7.2, 7.5 | 98 | 100 |
| *V. cholerae* | 0.997 (102.1) | $10^6$ (100) | 1.1, 1.7 | 7.6, 8.4 | 100 | 100 |
| *C. difficile* | 1.000 (99.4) | $10^6$ (100) | 2.2, 1.9 | 4.2, 5.1 | 97 | 100 |
| *Cryptosporidium* | 0.998 (100.4) | $10^3$ (0.1) | 2.0, 2.9 | 3.6, 6.3 | 100 | 100 |
| *Giardia* | 0.998 (101.4) | $10^3$ (0.1) | 1.7, 3.0 | 5.2, 4.7 | 100 | 100 |
| *E. histolytica* | 0.988 (93.0) | $10^3$ (0.1) | 2.0, 3.6 | 6.8, 6.7 | 100 | 100 |
| *Ascaris* | 0.993 (90.6) | $10^6$ (100) | 1.3, 2.5 | 4.5, 7.3 | 100 | 100 |
| *Trichuris* | 0.999 (94.6) | $10^6$ (100) | 0.7, 0.7 | 3.6, 5.2 | 100 | 100 |
| PhHV | 0.998 (98.3) | | 3.1, 1.8 | 4.2, 3.5 | | |
| MS2 | 0.999 (96.8) | | 1.4, 2.1 | 7.2, 8.4 | | |
| Bacterial 16S rRNA gene | 0.995 (91.0) | | 4.9, 4.2 | 5.1, 4.7 | | |

[a] Linearity was tested with combined positive controls. LOD and precision (repeatability and reproducibility) were tested with pooled positive materials spiked into healthy donor stool. LOD is the number of copies per gram of stool that were 100% detectable with 10 distinct extractions/amplifications.
[b] The linearity range was 10 to $10^6$ copy numbers per reaction for all the targets.
[c] LOD, copy number of an organism or artificial template per gram of stool; equiv. no. of copies, equivalent copy numbers per 1-μl volume of reaction mixture prior to extraction.
[d] Coefficients of variance (CVs) at both low (LOD) and high spiked concentrations are shown. High-concentration spikes were 1,000-fold higher for viral targets and 100-fold higher for bacterial and parasitic targets than LOD. The same concentrations were applied to accuracy tests.
[e] Sensitivity was calculated on the total number of PCRs for each target.

and *E. histolytica*. Combinations of three groups at different concentrations (negative, low, and high) were prepared and spiked into healthy donor stool. A total of 30 samples were prepared and assayed.

**Statistics.** Correlation was tested by regression analysis using the analysis of variance (ANOVA) test. $C_T$ values were compared by the *t* test using IBM SPSS software. All *P* values were two-tailed, and values of $<0.05$ were considered statistically significant.

## RESULTS

**Analytical performance.** We compared $C_T$ values between single template and pooled templates of all 19 targets, and no significant differences were observed (data not shown). Therefore, for development purposes the spiking materials for all 19 pathogens were mixed together and assayed as one sample. Under the universal conditions on the microfluidic card, all assays showed good linearity ($R^2 = 0.988$ to 1, $P < 0.05$) and 90 to 105% amplification efficiency within the tested range from 10 to $10^6$ organisms per reaction mixture (Table 2). The limit of detection of all the assays, defined as 100% detection among 10 distinct samples, ranged from $10^3$ to $10^7$ copies of organisms or nucleic acid template per gram of stool, equivalent to 0.1 to 500 copies (prior to extraction) per 1 μl of reaction mixture (Table 2). This LOD was within 10-fold of the cognate assays tested on plates (data not shown). The $C_T$ values of 23 pathogen assays had within-run variance from 0.7% to 3.6% (repeatability, $n = 8$) and between-run variance from 2.5% to 7.9% (reproducibility, $n = 10$) (Table 2). Stool is a difficult substrate to use for extraction and amplification because of the presence of a variety of inhibitors, which can vary between samples. We tested matrix inhibition using three lots of stool from healthy donors spiked with combined positive controls and extracted and amplified in duplicate. As shown in Fig. S1 in the supplemental material, no significant difference was observed among three lots for any of the assays.

**TABLE 3** Comparison of TAC results with conventional methods and PCR-Luminex results on clinical samples from Tanzania and Bangladesh[a]

| | Conventional assay positive | | Conventional assay negative | | PCR-Luminex positive | | PCR-Luminex negative | |
|---|---|---|---|---|---|---|---|---|
| Target | TAC+ | TAC− | TAC+ | TAC− | TAC+ | TAC− | TAC+ | TAC− |
| Adenovirus | 5 | 0 | 34 | 32 | 53 | 0 | 11 | 45 |
| Astrovirus | 6 | 5 | 3 | 58 | 14 | 0 | 1 | 94 |
| Rotavirus | 6 | 2 | 23 | 50 | 30 | 0 | 4 | 75 |
| *Campylobacter* | 22 | 13 | 5 | 28 | 44 | 2 | 5 | 58 |
| *Cryptosporidium* | 17 | 4 | 4 | 55 | 24 | 1 | 2 | 82 |
| *Giardia* | 17 | 3 | 11 | 49 | 32 | 0 | 8 | 69 |
| *E. histolytica* | 8 | 5 | 9 | 66 | 20 | 2 | 1 | 86 |
| *Salmonella* | 6 | 0 | 0 | 72 | 9 | 0 | 0 | 100 |
| *V. cholerae* | 8 | 0 | 0 | 72 | 9 | 0 | 0 | 100 |
| *Shigella*/EIEC | 1 | 0 | 12 | 59 | 37 | 0 | 0 | 72 |
| ETEC | | | | | | | | |
| ST | 2 | 0 | 20 | 46 | 38 | 1 | 7 | 63 |
| LT | 8 | 0 | 33 | 27 | 63 | 2 | 4 | 40 |
| EPEC | | | | | | | | |
| *eae* | 12 | 0 | 44 | 12 | 76 | 0 | 8 | 25 |
| *bfpA* | 7 | 0 | 22 | 39 | 34 | 0 | 9 | 66 |
| EAEC | | | | | | | | |
| *aaiC* | 17 | 1 | 22 | 28 | 57 | 2 | 5 | 45 |
| *aatA* | 26 | 0 | 28 | 14 | 77 | 4 | 5 | 23 |
| STEC | | | | | | | | |
| $stx_1$ | 0 | 0 | 6 | 62 | 11 | 0 | 0 | 98 |
| $stx_2$ | 0 | 0 | 9 | 59 | 10 | 0 | 1 | 98 |
| *Ascaris* | 8 | 0 | 1 | 79 | 9 | 0 | 0 | 100 |
| *Trichuris* | 8 | 0 | 1 | 79 | 16 | 0 | 0 | 93 |
| Norovirus GII | ND | ND | ND | ND | 31 | 0 | 3 | 75 |
| Sapovirus | ND | ND | ND | ND | 18 | 0 | 1 | 90 |
| Total | Sensitivity = 85% | | Specificity = 77% | | Sensitivity = 98% | | Specificity = 96% | |

[a] Values indicate the number of samples. TAC+, TAC-positive samples; TAC−, TAC-negative samples; ND, not done (including *C. difficile*).

**Analytical accuracy.** Combinations of pathogens or nucleic acid templates at different concentrations were spiked into stool samples from a healthy donor and assayed with TAC. Among a total of 1,260 reactions, all 23 targets spiked at high concentration were detected in all replicates. Twelve of 23 targets spiked at low concentration were detected in all replicates, 10 of 23 targets spiked at low concentration were detected in only one of the replicates, and the *C. difficile* low-concentration spike was not detected. Detection sensitivity and specificity for each target are listed in Table 2. Overall, this revealed 98.7% sensitivity and 100% specificity on these analytical specimens.

**Clinical performance.** Clinical samples from Tanzania and Bangladesh were tested with TAC and then compared with previously obtained results from conventional methods as well as our laboratory-developed PCR-Luminex assays (Table 3). Conventional tests included immunoassay (adenovirus, astrovirus, rotavirus, *Campylobacter*, *Cryptosporidium*, *Giardia*, and *E. histolytica*), culture (*Salmonella*, *V. cholerae*, and *Shigella*), culture with PCR of 5 picked colonies (ETEC, EIEC, EPEC, EAEC, and STEC), and microscopy (*Ascaris* and *Trichuris*). All samples that were positive by conventional methods for adenovirus, *Salmonella*, *V. cholerae*, EIEC, both ST and LT gene targets of ETEC, *eae* and *bfpA* of EPEC, *aatA* of EAEC, *Ascaris*, and *Trichuris* were identified with TAC, while some samples that tested positive by conventional methods for astrovirus, rotavirus, *Campylobacter*, *Cryptosporidium*, *Giardia*, *E. histolytica*, and the *aaiC* target of EAEC did not amplify in TAC (5/11, 2/8, 13/35, 4/21, 3/20, 5/13, and 1/18, respectively). However, all these TAC-negative results were confirmed with PCR-Luminex. Overall, sensitivity and specificity of TAC versus conventional results were 85% and 77%, respectively, while versus PCR-Luminex they were 98% and 96%, respectively. To confirm specific amplification among the TAC-positive/PCR-Luminex-positive/conventional-assay-negative specimens, amplicons from 81 of these PCRs were sequenced and all were confirmed to be the proper sequence ($n = 18$ targets).

We examined the correlation of $C_T$ values from TAC with those from the conventional assay results. Statistically, $C_T$ values of conventional-assay-positive and TAC-positive samples were significantly lower than those of conventional-assay-negative and TAC-positive samples for most targets (Fig. 2). Likewise, we examined the correlation of $C_T$ values from TAC with the PCR-Luminex MFI values. Samples that were discrepant for PCR-Luminex and TAC were generally lower-burden infections toward the lower detection limit (Fig. 3). Five samples were detected as *C. difficile* positive by TAC, all of which were confirmed with a secondary real-time PCR assay (28). Ultimately, use of this TAC led to detection of a greater number of infections in the clinical samples than did conventional methods, with an average of 5.9 pathogens per sample by TAC and 2.5 by conventional methods (Fig. 4, $P < 0.001$).

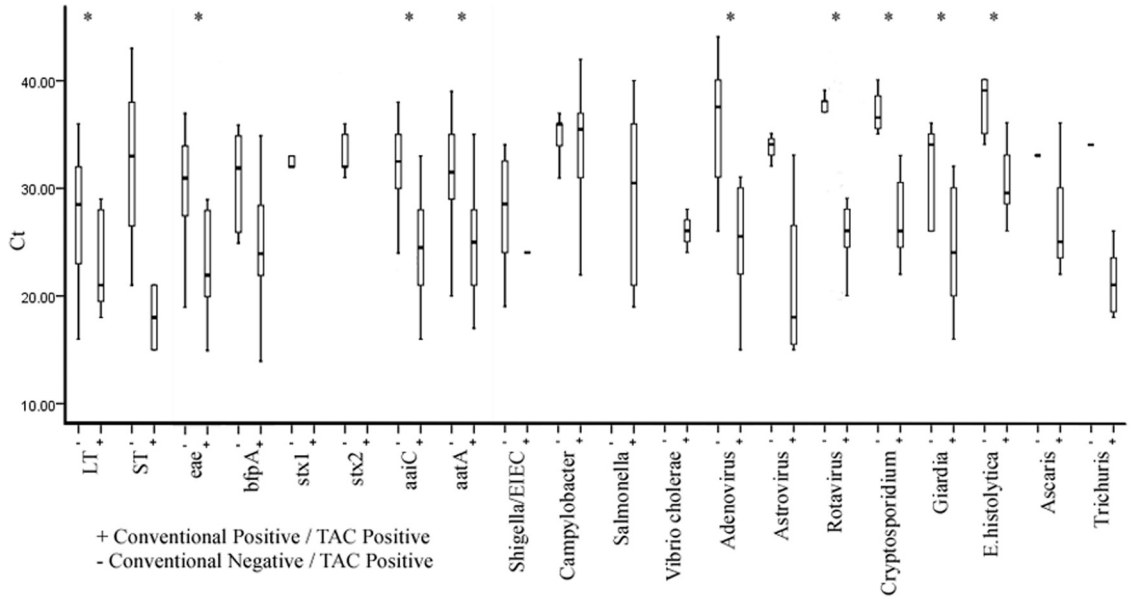


**FIG 2** Quantitative comparison of pathogen burdens in clinical samples that were positive or negative with conventional methods. Box plots with medians were generated with IBM SPSS software. Asterisks (*) indicate that $C_T$ was lower for conventional positive than for conventional negative samples ($P < 0.05$), including adenovirus ($P = 0.02$), rotavirus ($P = 0.01$), *Cryptosporidium* ($P < 0.0001$), *Giardia* ($P < 0.001$), *E. histolytica* ($P < 0.001$), LT of ETEC ($P = 0.04$), *eae* of EPEC ($P = 0.02$), and both *aaiC* and *aatA* of EAEC ($P < 0.001$ for both). A trend was observed for astrovirus ($P = 0.06$), ST of ETEC ($P = 0.08$), and *bfpA* of EPEC ($P = 0.14$), whereas correlation was poor between TAC and the *Campylobacter* enzyme immunoassay (EIA) ($P = 0.59$). Statistics were not applicable to $stx_1$ and $stx_2$ of STEC, *Shigella*/EIEC, *Salmonella*, *V. cholerae*, *Ascaris*, and *Trichuris*.

## DISCUSSION

This is the first described TaqMan Array Card for enteropathogens. We designed this assay to capture a wide range of enteropathogens relevant in developing country settings, where childhood diarrhea burden is highest. We developed the TaqMan Array Card as a singleplex real-time PCR platform, allowing for multitarget detection through spatial distribution. The singleplex format means a simpler assay design and better quantification and

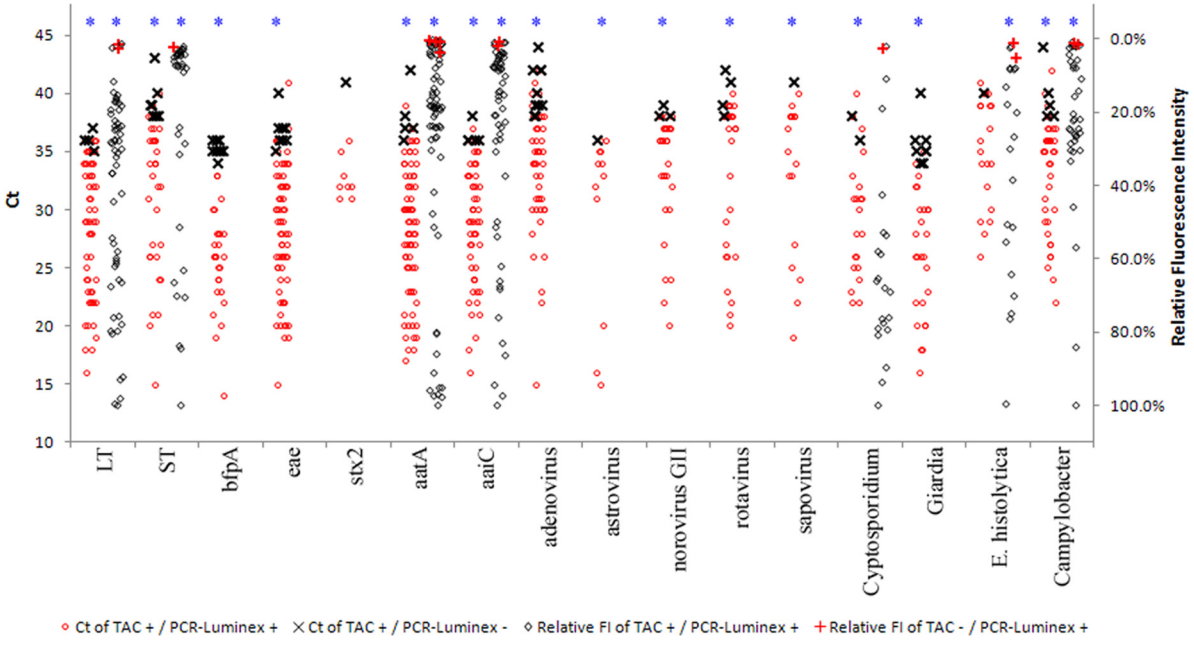


**FIG 3** Analysis of TAC and PCR-Luminex assays. $C_T$ values from TAC are shown for both PCR-Luminex-positive and -negative samples. Likewise, relative fluorescence intensities from PCR-Luminex are shown for both TAC-positive and -negative samples. To adjust for different Luminex fluorescence intensity scales, the relative fluorescence intensity of a PCR-Luminex-positive sample is shown as the percentile versus the highest fluorescence intensity observed. Asterisks (*) indicate that $C_T$ values were lower for PCR-Luminex-positive than for PCR-Luminex-negative samples and Luminex fluorescence intensity (FI) values were higher for TAC-positive than for TAC-negative samples ($P < 0.05$).

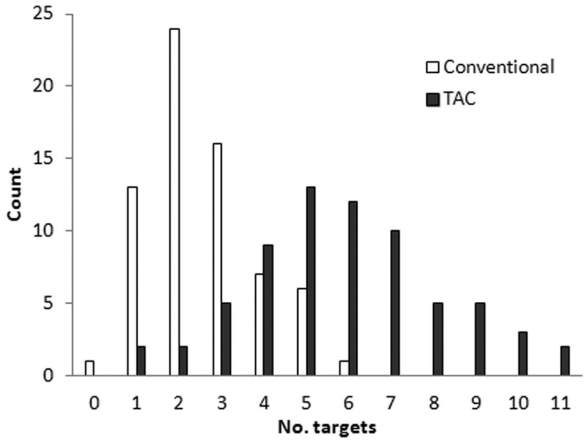


**FIG 4** Numbers of mixed infections detected with TAC versus conventional methods for 16 pathogens on samples from Tanzania (medians; $P < 0.001$).

sensitivity than multiplex real-time PCR; however, we did need to adapt most of the primers and probes, usually by modifying their length, so that they performed well under the universal conditions on the card.

We had to handle a broad range of enteropathogen types including both DNA and RNA genomes. Therefore, an RT-PCR protocol was applied to enable amplification of all targets. Since the targeted genes for parasites were rRNA genes, rRNA was far more abundant than genomic DNA, and this boosted the sensitivity of the assay by at least 2 log compared to regular PCR (data not shown). Panspecies or pangenus assays were usually chosen, and multicopy targets were utilized whenever possible to ensure sensitivity. We originally had some concern that the small sample volume might limit the lower-level detection of pathogens in clinical samples (3), so we maximized the sample volume (theoretically 0.4 μl) in each reaction and observed a <10-fold decrease of sensitivity compared with cognate assays performed on plates where 2 μl of sample was added. Our LODs, as shown in Table 2, were close to the theoretical detection limit of one single copy of template per reaction. This translates to an LOD adequate for most clinical cases, where the pathogen loads were reported as $10^3$ to $10^9$ CFU/g stool for bacteria, $10^3$ to $10^5$ CFU/g for protozoa, and $10^4$ to $10^{11}$ CFU/g for viruses.

Eighty-five percent of the positive samples by conventional detection were identified as positive with TAC; however, TAC detected an additional ~160% more positives, and this was particularly prevalent with the bacteria. We can envision several possible reasons for this. First, culture in stool is challenging, and bacteria can be in a viable but nonculturable state. Second, for *E. coli*, as is common, only five colonies were picked for typing, whereas the TAC assay examines stool in its entirety. Third, TAC could be detecting only nonviable nucleic acids. Another explanation for discrepancies in the case of *Campylobacter* is that the *Campylobacter* ELISA may detect a range of species (18) whereas we designed the TAC assay to be specific for *C. jejuni*/*C. coli*. In contrast to the discrepancies seen in comparison with conventional methods, the correlation between the molecular platforms of TAC and PCR-Luminex surpassed 96%, with only rare discrepancies, which were seen exclusively with low-burden samples. In general, TAC detected slightly more pathogens than PCR-Luminex, which we speculate reflects true low-burden infections picked up with the singleplex versus multiplex amplification methods.

With such sensitive and comprehensive molecular diagnostics, we were not surprised to find a high rate of mixed infections. Albert et al. found up to 5 pathogens in a Bangladeshi population using conventional methods (29). Lindsay et al. used a combination of culture, immunologic, and conventional PCR to detect 26 pathogens in India and found as many as six pathogens in a single specimen (30). Our findings with conventional methods were similar to the ones reported by those studies. We think, with the advent of highly sensitive molecular assays to interrogate multiple targets in stool, that we will detect even more pathogen nucleic acids in low socioeconomic settings (29–31). Understanding which infections are dominant or likely contributors and which ones are less likely playing a role will take a significant amount of work, ideally through prospective studies and probably interventional trials targeted at key pathogens. In our view, quantitative assays using a platform such as TAC, which minimizes target-to-target, panel-to-panel, and plate-to-plate variations, can serve as an important tool in this effort.

Quantitation of organisms in stool samples is challenging. We introduced two extrinsic controls to monitor the extraction and amplification inhibition and efficiency. We observed $C_T$ values for PhHV that ranged from 30 to 35 and for MS2 from 31 to 38, which represented up to 40- to 150-fold differences in DNA and RNA extraction/amplification efficiency, respectively. Furthermore, we included bacterial 16S rRNA gene (16S) assays as an indicator of total bacterial content in each sample. Our results showed that the $C_T$ values of the 16S assay ranged from 15 to 24, equivalent to a 500-fold difference. Normalization to 16S may be useful for accurate quantitation in the cases where it is difficult to measure the true sample mass, e.g., in watery stool. For best quantitation, should one be so inclined, we would propose first calculating the extraction/amplification efficiency by comparing the $C_T$ values of PhHV and MS2 in a given sample with the $C_T$ values of pure PhHV and MS2 at equivalent spiking concentrations. Next, take the $C_T$ value of a certain pathogen in that sample to calculate the copy number via a standard curve, and divide the copy number by the efficiency to yield the pathogen load per gram of stool. Finally, one could further normalize this to the copy number of 16S. An important caveat, however, is that for targets such as *Shigella*, *Cryptosporidium*, and *Giardia*, the quantitation may be less accurate due to the uncertainty of target copy numbers per cell (either plasmid or rRNA).

We would emphasize how molecular assays such as TAC greatly simplify the diagnostic process and procurement versus conventional methods. To detect 16 pathogens with conventional methods, we had to procure seven ELISA kits from two different companies, three types of bacterial culture media, biochemical identification reagents, a PCR gel electrophoresis system, and a microscope. At current prices, conventional reagent costs alone can run $200 per sample versus approximately $60 for TAC. The turnaround time of TAC is also significantly shorter than that of conventional methods.

There are limitations to the TAC method, however. Repeats are expensive, since one must run all the targets on the card even if only one or two require further investigation. Therefore, ideally one wants to have backup assays in plate format. We have found utility in spotting the TAC in duplicates, because in our work in developing countries there are often low-level infections at the

lower limit of detection, and duplicate results allow greater confidence. Finally, the quantitative PCR (qPCR) platform is costly. In sum, however, the TAC offers a sensitive, broad-range screen for enteropathogens that is useful for surveillance or clinical purposes at equipped sites. The assay is modular, so once the assay performance is established under the universal cycling conditions, different pathogen combinations can be spotted for the specific purpose of the end user.

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