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Simultaneous Detection of Six Diarrhea-Causing Bacterial Pathogens with an In-House PCR-Luminex Assay

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Diarrhea can be caused by a range of pathogens, including several bacteria. Conventional diagnostic methods, such as culture, biochemical tests, and enzyme-linked immunosorbent assay (ELISA), are laborious. We developed a 7-plex PCR-Luminex assay to simultaneously screen for several of the major diarrhea-causing bacteria directly in fecal specimens, including pathogenic *Aeromonas*, *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella*, *Shigella*, enteroinvasive *Escherichia coli* (EIEC), *Vibrio*, and *Yersinia*. We included an extrinsic control to verify extraction and amplification. The assay was first validated with reference strains or isolates and exhibited a limit of detection of 10^3 to 10^5 CFU/g of stool for each pathogen as well as quantitative detection up to 10^9 CFU/g. A total of 205 clinical fecal specimens from individuals with diarrhea, previously cultured for enteric pathogens and tested for *Campylobacter* by ELISA, were evaluated. Using these predicate methods as standards, sensitivities and specificities of the PCR-Luminex assay were 89% and 94% for *Aeromonas*, 89% and 93% for *Campylobacter*, 96% and 95% for *Salmonella*, 94% and 94% for *Shigella*, 92% and 97% for *Vibrio*, and 100% and 100% for *Yersinia*, respectively. All discrepant results were further examined by singleplex real-time PCR assays targeting different gene regions, which revealed 89% (55/62 results) concordance with the PCR-Luminex assay. The fluorescent signals obtained with this approach exhibited a statistically significant correlation with the cycle threshold (C_T) values from the cognate real-time PCR assays ($P < 0.05$). This multiplex PCR-Luminex assay enables sensitive, specific, and quantitative detection of the major bacterial causes of gastroenteritis.

Diarrheal disease is a leading cause of morbidity and mortality worldwide, particularly in children. It can be caused by a host of organisms, including viruses, parasites, fungi, and bacteria. Some of the major bacterial pathogens observed in communities throughout the world include *Aeromonas*, *Campylobacter*, diarrheagenic *Escherichia coli*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia*. In the United States, many such pathogens are reportable, and these data indicate that *Salmonella* and *Campylobacter jejuni/Campylobacter coli* are most frequent, followed by *Shigella* species, Shiga toxin-producing *E. coli*, *Yersinia*, *Listeria*, and *Vibrio* (9).

The microbiologic etiology of diarrhea is usually not clinically obvious; thus, laboratory diagnosis is important, since many of the bacteria mentioned, such as *Shigella*, *Vibrio*, and *Yersinia*, usually warrant specific antibiotic therapy (18). Conventional culture methods remain the norm for detection and identification of bacterial enteric pathogens in clinical laboratories, although studies have repeatedly noted poor yield and high cost (11, 18, 24, 31, 50). Moreover, culture detection and identification methods require significant skill, labor, and time, which can delay epidemiological investigation or treatment. Another complication is that many bacteria are inherently difficult to grow (1, 44), are greatly affected by prior antibiotics, and even may attain a so-called viable but nonculturable state (35), a phenomenon that has been described with *Vibrio cholerae* (12, 46), *Vibrio vulnificus* (45), *Salmonella enterica* serovar Enteritidis (40), *Shigella* (12, 38), and *Campylobacter jejuni* (8, 39). It is not surprising, therefore, that detection of bacterial enteropathogens by stool culture has been insensitive compared with that of enzyme-linked immunosorbent assay (ELISA) and PCR-based methods (5, 14, 17, 27).

Molecular diagnostic assays on fecal specimens have recently emerged for enteric pathogens, including several multiplex PCR approaches using real-time cyclers (13, 14, 19), gel electrophoresis (10, 16), or hybridization-based detection (34). Recently a rapid 4-plex real-time PCR assay detected *Campylobacter*, *Salmonella*, *Yersinia*, and *Shigella*, with results comparable to those of culture (13). There is also an EntericBio system (Serosep Ltd., Annacotty, Ireland) that amplifies and detects *Campylobacter*, *Shigella*, *Salmonella*, and *E. coli* O157 from overnight enrichment broths with line blot hybridization (34). We have performed PCR amplification followed by detection on Luminex beads for several enteric pathogens, including viruses and parasites (29, 41). This system offers expandability given that current platforms can detect 100 or more amplicon populations. Here, we present the same scheme to detect *Aeromonas*, *Campylobacter jejuni/Campylobacter coli*, *Salmonella*, *Shigella*, enteroinvasive *E. coli* (EIEC), *Vibrio*, and *Yersinia*. This panel can be added to other panels or used alone as a screen for these pathogens.

MATERIALS AND METHODS

Specimens. Two hundred and five clinical fecal specimens were obtained from patients from Kilimanjaro Christian Medical Centre, Tanzania, International Centre for Diarrheal Diseases and Research, Bangladesh

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(ICDDRB), and Aga Khan University Hospital, Pakistan. All work was approved by the respective and University of Virginia (UVA) institutional review boards. Samples were tested for *Campylobacter* using ELISA (ProSpecT *Campylobacter* microplate; Remel, Lenexa, KS) by following the manufacturer's protocol and for other bacterial pathogens using culture as described below. There were 24 specimens from these sites that were negative by all methods and were included in our evaluation of clinical samples. DNA was extracted with the QIAamp DNA stool minikit (Qiagen, Valencia, CA) by following the manufacturer's protocol. For spiking experiments, we utilized healthy volunteer stools from UVA and extracted with QuickGene DNA Tissue Kit S (Fujifilm, Tokyo, Japan) (41). Reference strains were obtained from the American Type Culture Collection (Manassas, VA), and extra *Salmonella* strains were from the *Salmonella* Genetic Stock Centre (University of Calgary, Alberta, Canada) (6). An exogenous phocine herpesvirus (PhHV-1), a gift from Martin Schutten, Erasmus MC, Department of Virology, Rotterdam, The Netherlands, was spiked into the lysis buffer for stool DNA extraction to monitor the efficiency/inhibition of extraction and amplification. The viral extract, estimated to be at a concentration of about 10^9 copies of genome per milliliter, was titrated to yield optimal signal, with 2,000 copies spiked into each fecal sample.

Conventional stool culture and biochemical test procedures. Two hundred milligrams of fecal specimen was preserved in Cary-Blair transport medium before culture. All culture media and reagents were obtained from BD Biosciences (Franklin Lakes, NJ). MacConkey agar was used to identify non-lactose-fermenting colonies for further testing. Xylose lysine deoxycholate (XLD) agar and thiosulfate citrate bile sucrose agar (TCBS) identified suspected *Salmonella* and *Vibrio* species, respectively. Blood agar was used to distinguish *Aeromonas* colonies from *Vibrio* colonies. Suspicious colonies were selected for further identification using biochemical methods and confirmed with serotyping as appropriate.

Design of a 7-plex PCR-Luminex assay. We aimed to simultaneously detect the pathogenic strains of the six diarrhea-causing bacteria: *Aeromonas*, *Campylobacter*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia*. We chose gene targets and evaluated an inclusivity panel of 129 strains as indicated in Table S2 in the supplemental material. Targeted genes included *aerolysin* for *Aeromonas* species, including *A. hydrophila*, *A. caviae*, and *A. veronii* (23, 37, 49) (see Table S1 in the supplemental material). An assay targeting *invA*, a gene widely used to detect *Salmonella* species (21, 30), was designed. The *ipaH* assay detected all four *Shigella* species, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, as well as EIEC without any differentiation (43). A conserved region in the *toxR* gene was used for both *Vibrio cholerae* and *Vibrio parahaemolyticus*. Assays targeting *cadF* and *lysP* were adapted from the literature for *Campylobacter* (*C. jejuni* and *C. coli*) and pathogenic *Yersinia* species, respectively (13).

Multiplex PCR. Primers (see Table S1 in the supplemental material) were sourced from the published literature where possible, but with some modifications (13, 33, 41, 43). Forward primers were labeled with biotin-TEG (tetraethyleneglycol, 15 atoms) at 5′ ends. Primer concentration was titrated for optimal sensitivity according to the methods of Gunson et al. (20), and then 7-plex primer mix was prepared to have final concentrations of 3.1, 1.6, 12.5, 6.3, 12.5, 6.3, 12.5, 3.1, 12.5, 6.3, 3.1, 12.5, 1.6, 3.1, and 3.1 μM for forward and reverse primers of *aerolysin*-, *cadF*-, *invA*-, *ipaH*-, *toxR* (two reverse primers)-, *lysP*-, and *gB*-targeting regions, respectively. A 25-μl multiplex reaction mixture typically contained 1× Qiagen Multiplex PCR Master Mix, 2 μl of Q-solution, 1 μl of primer mix, and 2 μl of DNA sample. PCR was performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: initial denaturation at 95°C for 15 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final 10-min extension at 72°C. Positive samples and a negative control (nuclease-free water) were included on each 96-well plate.

Microsphere-based Luminex detection. Oligonucleotide capture probes (see Table S1 in the supplemental material) were modified with an amino-C12 linker at the 5′ ends to enable coupling to the carboxylated fluorescent microspheres (Bio-Rad). Bead coupling and hybridization were performed according to published protocols (4, 28). Samples were analyzed on the BioPlex 200 system (Bio-Rad). One hundred microspheres of each set were analyzed, and the results were reported as microsphere-specific median fluorescent intensity (MFI). Amplification reactions for positive and negative controls were included in the assay. Corrected MFI (cMFI) was calculated as follows: $\text{cMFI} = (\text{MFI}_{\text{analyte}} - \text{MFI}_{\text{negative control}})/\text{MFI}_{\text{negative control}}$. All positive-control cMFIs were greater than 10.

Singleplex real-time PCR. The same designs (see Table S1 in the supplemental material) were used for real-time PCR assays, except that forward primers were not biotinylated and the capture oligonucleotides were converted into 6-carboxyfluorescein (FAM)-labeled TaqMan-MGB (minor groove binder) probes (Life Technologies, Carlsbad, CA). Singleplex quantitative PCR (qPCR) was carried out with a CFX96 real-time system (Bio-Rad) in a 20-μl reaction mixture containing 10 μl of Quantitect PCR Master Mix (Qiagen), 10 pmol of each forward and reverse primer, 4 pmol of TaqMan probe, and 2 μl of nucleic acid sample under the following conditions: initial denaturation at 95°C for 15 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The secondary assays targeting different genes (see Table S1 in the supplemental material) were run in a 20-μl reaction mixture containing 10 μl of SYBR IQ supermix (Bio-Rad), 4 pmol of each forward and reverse primer, and 2 μl of nucleic acid under the following conditions: initial denaturation at 95°C for 3 min and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting-curve analysis which revealed single amplicons of an appropriate melting temperature.

Performance on analytical specimens. Bacteria were grown in tryptic soy broth, except for *Vibrio*, which was grown in nutrient broth with 3% NaCl. Overnight cultures were serially diluted with phosphate-buffered saline (PBS), plated onto blood agar plates, and incubated overnight to determine the number of CFU. The same dilutions were spiked into healthy donor fecal samples, extracted, and then amplified and detected with the 7-plex PCR-Luminex assay. Genomic DNA was purified from bacterial culture, and serial dilutions were prepared to evaluate linearity.

Statistics. Receiver operating characteristic (ROC) analysis performed with PASW Statistics software was used to define cMFI cutoffs using culture/ELISA results as the gold standard. Correlation among bacterial copy numbers, qPCR C_T values, and cMFI from PCR-Luminex assay were tested by regression analysis using the analysis of variance (ANOVA) test. All P values were two-tailed, and values <0.05 were considered statistically significant.

RESULTS

Performance on analytical specimens. The assay was validated as follows (7). As shown in Table S2 in the supplemental material, the assay was tested and positive on diverse reference strains or clinical isolates for *Aeromonas* ($n = 7$), *Campylobacter* ($n = 4$), *Salmonella* ($n = 95$), *Shigella* ($n = 11$), *Vibrio* ($n = 8$), and *Yersinia* ($n = 3$). An exclusivity panel of 25 pathogenic and nonpathogenic organisms that can be present in feces was also tested and negative (see Table S2 in the supplemental material). Reference strains of *Aeromonas hydrophila*, *Campylobacter jejuni*, *C. coli*, *Salmonella enterica* serovar Enteritidis, *Shigella sonnei*, *Vibrio cholerae*, *V. parahaemolyticus*, and *Yersinia enterocolitica* were cultured, and genomic DNA was extracted and quantified. Ten to 10^7 genomic copies were amplified and detected with the 7-plex PCR-Luminex assay, and linearity for each analyte was assessed as shown in Fig. 1 (*V. cholerae*), Fig. S1 in the supplemental material (the other bacteria), and Table 1 (summary). As we observed with our previous PCR-Luminex assays (29), the cMFI statistically correlated with copy numbers, but second-order polynomial regression was tighter than linear fit ($R^2 = 0.85$ to 0.96), and R^2 values were not as high as qPCR values using the same primers and probes ($R^2 =$

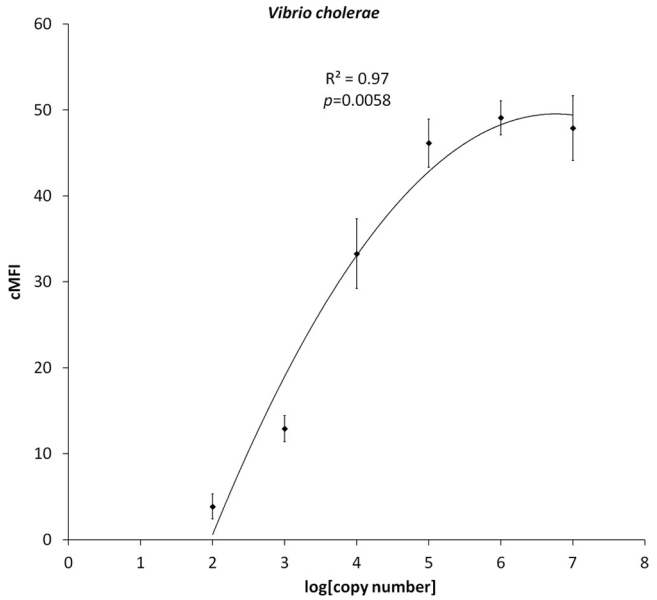

FIG 1 Correlation between template copy numbers and detection by multiplex PCR-Luminex assay (cMFI) for *Vibrio cholerae*. Best-fit lines and regression (R^2) were extrapolated. The other bacteria are presented in Fig. S1 in the supplemental material.

0.995 to 1.000; $P < 0.001$). The limit of detection was determined by spiking healthy volunteer pathogen-negative stool and was found to be 10^3 to 10^5 CFU/g depending on the pathogen (Table 1). Precision was examined with 10 fecal samples spiked with 10^5 CFU of bacterial culture, and the coefficient of variation (CV) ranged from 4.7% to 11.9% within runs and from 6.0% to 19.7% between runs over 5 days.

Validation with clinical samples. Two hundred and five clinical diarrheal samples were obtained from Tanzania, Pakistan, and Bangladesh, and 181 of them were previously tested positive for bacterial pathogens via ELISA for *Campylobacter* and via culture for the rest. ROC analysis was performed to ascribe the cMFI cutoffs that would maximize sensitivity/specificity against the conventional methods. This yielded an overall sensitivity and specificity for the PCR-Luminex assay of 91.4% and 95.6%, respectively (Table 2). Most of the discrepancies (46/62) were Luminex-positive/conventional-method-negative results. Generally, the Luminex-positive/conventional-method-negative results had lower fluorescence intensities than the Luminex-positive/conventional-method-positive counterparts, although this was only statistically significant for *Salmonella* (21.0 ± 15.7 versus 5.5 ± 2.4; $P = 0.01$). qPCRs using the same designs were performed on all 205 specimens, and results were 100% concordant with the PCR-Luminex results (data not shown). Additionally, secondary qPCR targeting a different gene for each analyte (see Table S1 in the supplemental material) was performed to evaluate the 62 discrepant results. These secondary qPCR results supported the Luminex results in 89% (55/62) of instances (Table 3). If we used 2 out of 3 concordant results as a gold standard for positivity or negativity, the PCR-Luminex result offered 93 to 100% sensitivity and 98 to 100% specificity across targets. The number of positive *Yersinia* specimens was small; thus, our sensitivity confidence interval was broad.

Mixed infections. With the conventional methods, only four samples were found to be positive for multiple pathogens: two for *Campylobacter* and *Vibrio*, one for *Campylobacter* and *Salmonella*, and one for *Campylobacter* and *Aeromonas*. In contrast, the PCR-Luminex assay detected these 4 mixed infections plus an additional 23. The most common pathogens observed in mixed infections were, in descending order, *Campylobacter*, *Shigella*, *Salmonella*, *Vibrio*, and *Aeromonas*.

Quantitation with the 7-plex PCR-Luminex assay. These mixed infections raised the need for quantitation of enteropathogens; thus, we explored this topic in detail. We already noted in Fig. 1 and Fig. S1 in the supplemental material that in analytical specimens the template copy numbers correlated quantitatively with the PCR-Luminex fluorescence. We also noted in Fig. 2 that the C_T obtained by real-time PCR using the same designs statistically correlated with the PCR-Luminex result ($P < 0.0005$ for *Aeromonas*, *Campylobacter*, *Salmonella*, *Shigella*, and *Vibrio*). These observations, however, do not account for stool-sample-to-stool-sample variability in DNA extraction or PCR amplification. Therefore, an extrinsic control, phocine herpesvirus (PhHV), was spiked into each sample during extraction to ensure extraction/amplification and normalization for quantitation (31). Sixteen different stool specimens were each spiked with 2,000 copies of PhHV and 10^3 to 10^8 CFU of *Salmonella enterica* serovar Enteritidis and examined for *Salmonella* cMFI or *Salmonella*/PhHV cMFI ratio (see Fig. S2 in the supplemental material). The ratio yielded a tighter correlation with spiked CFU than *Salmonella* cMFI alone

TABLE 1 Performance characteristics of the PCR-Luminex assay to detect bacterial enteropathogens

Pathogen	Strain	Second-order polynomial regression: Reportable range (copies/PCR)	Second-order polynomial regression: R^2	Limit of detection[a] (CFU/g stool)	Precision[b] (% CV): Within run	Precision[b] (% CV): Between runs
Aeromonas hydrophila	Clinical isolate	10^2–10^7	0.98	10^4	11.12	15.82
Campylobacter jejuni	ATCC 33291	10^2–10^7	0.96	10^3	9.58	16.27
Campylobacter coli	ATCC 43473	10^3–10^7	0.91	10^5	11.10	18.63
Salmonella Enteritidis	SARB no. 16	10^2–10^7	0.95	10^3	7.02	12.09
Shigella sonnei	Clinical isolate	10^2–10^7	0.92	10^3	10.51	15.30
Vibrio cholerae	Clinical isolate	10^2–10^7	0.97	10^4	6.20	14.78
Vibrio parahaemolyticus	ATCC 17802	10^3–10^7	0.89	10^5	11.77	19.73
Yersinia enterocolitica	ATCC 23715	10^2–10^7	0.98	10^4	4.72	6.07

[a] Limit of detection was determined with control fecal samples spiked with the lowest amount of bacterial culture that could be detected in 10/10 samples.
[b] Within-run precision and between-run (over 5 days) precision were both tested on 10 samples.

TABLE 2 Performance on clinical specimens

Characteristic[a]	Value					
	Aeromonas	*Campylobacter*	*Salmonella*	*Shigella*	*Vibrio*	*Yersinia*
No. of positive samples	35	52	23	48	25	2
No. of negative samples	170	153	182	157	180	203
cMFI cutoff	0.49	1.49	1.97	0.83	0.46	1.27
% sensitivity (95% CI)	89 (74–96)	89 (77–95)	96 (79–99)	94 (83–98)	92 (75–98)	100 (34–100)
% specificity (95% CI)	94 (89–96)	93 (88–96)	95 (91–97)	94 (89–97)	97 (93–98)	100 (98–100)
% PPV	74	81	71	83	79	100
% NPV	97	95	99	98	99	100

[a] CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

($R^2 = 0.92$ versus 0.71). Thus, the target/control ratio can be used to accurately extrapolate bacterial burden. Another concern with mixed infections is that one target may preferentially amplify over another target. We evaluated this by cospiking 10^7 copies of targets into the same PCR across 15 combinations and comparing amplification with 10^7 copies of a single target alone (see Table S3 in the supplemental material). The combinations revealed that *Aeromonas*, *Salmonella*, *Shigella*, and *Yersinia* were amplified efficiently regardless of the presence of any of the other targets (P values of $>$ 0.05 between cMFIs of one target and two targets). In contrast, *Campylobacter* detection remained but was diminished in the presence of *Salmonella* or *Vibrio*. These spiked amounts represent high burden templates, and no loss of signal was detected when only 10^4 copies of *Salmonella* or *Vibrio* were cospiked (119.5 $\pm$ 5.8 for *Campylobacter* alone versus 104.9 $\pm$ 18.5 and 109.1 $\pm$ 13.2 in the presence of 10^4 copies of *Salmonella* and *Vibrio*, respectively; $P > 0.05$).

TABLE 3 Testing of Luminex PCR/conventional test-discrepant samples, with qPCR targeting a second gene

qPCR targeting a second gene	No. of samples		% correlation between secondary qPCR and Luminex
	Luminex PCR positive/ conventional test negative	Luminex PCR negative/ conventional test positive	
Aeromonas 16S qPCR positive	10	0	93
Aeromonas 16S qPCR negative	1	4	93
Campylobacter 16S qPCR positive	11	4[a]	76
Campylobacter 16S qPCR negative	0	2	76
Salmonella ompC qPCR positive	9	0	100
Salmonella ompC qPCR negative	0	1	100
Shigella virA qPCR positive	7	0	83
Shigella virA qPCR negative	2	3	83
Vibrio hlyA/tlh qPCR positive	6	0	100
Vibrio hlyA/tlh qPCR negative	0	2	100

[a] These four *Campylobacter* 16S products were sequenced, and three were *C. upsaliensis* and one was *C. hyointestinalis*.

DISCUSSION

The value of this diagnostic assay is a molecular screen for several common bacterial enteropathogens that can be used directly on stool specimens. The assay has been validated for linearity, limit of detection, precision, as well as sensitivity and specificity compared to those of conventional culture/ELISA methods. It was tested against clinical samples from around the world, including Pakistan, Tanzania, and Bangladesh. We propose its use for epidemiologic studies. For example, in the context of recent severe gastrointestinal outbreaks of *V. cholerae* in Haiti, such an assay could be useful as an early positive/negative indicator that drives the workup forward. The assay provides quantitative information for these pathogens, which we postulate may be useful in the setting of mixed infections and potentially to evaluate severity. It may also find use in clinical settings. The assay is modular, such that additional panels could be added to include viruses, diarrheagenic *E. coli*, or protozoa. Likewise, if some species are rare, as is often the case with *Vibrio* and *Yersinia*, these reagents can be withheld.

Previous PCR-based assays to detect a broad range of enteric bacteria have generally used several PCRs. Some have multiplexed the reactions, but usually to only four targets (2, 3). We were able to achieve a 7-plex assay with a sensitivity of 89 to 100% and specificity of 93 to 100% for all targets. Most discrepancies were due to additional PCR-Luminex detections, which we generally interpret as being true positives, where the PCR-Luminex assay was more robust and sensitive. Indeed the analytical sensitivity of our assay (10^3 to 10^5 CFU/g of stool) was below the bacterial burden (10^3 to 10^9 CFU/g) often reported in symptomatic patients as measured by culture (17, 26, 32). Furthermore, a secondary PCR targeting a different gene region yielded the PCR-Luminex result in 93.5% of the Luminex-positive/conventional-method-negative samples. There were also small numbers of PCR-negative/conventional-method-positive results, which could reflect degraded nucleic acid since the PCR assays were performed on stored frozen stool, while culture was performed fresh. There were 4 Luminex-negative/*Campylobacter* ELISA detections which we identified as non-*C. jejuni*/*C. coli* infections, species which we deliberately did not target.

The assay detected an additional 14 to 39% of infections for *Aeromonas*, *Campylobacter*, *Salmonella*, *Shigella*, or *Vibrio* in the clinical specimens beyond what was ascertained by conventional means. We think this is highly plausible based on the prevalence of these infections in Pakistan, Tanzania, and Bangladesh. We also observed several mixed pathogens in these diarrhea cases. Such mixed infections make elucidating the most important etiologies a vexing problem (36). There are a few basic biological possibilities

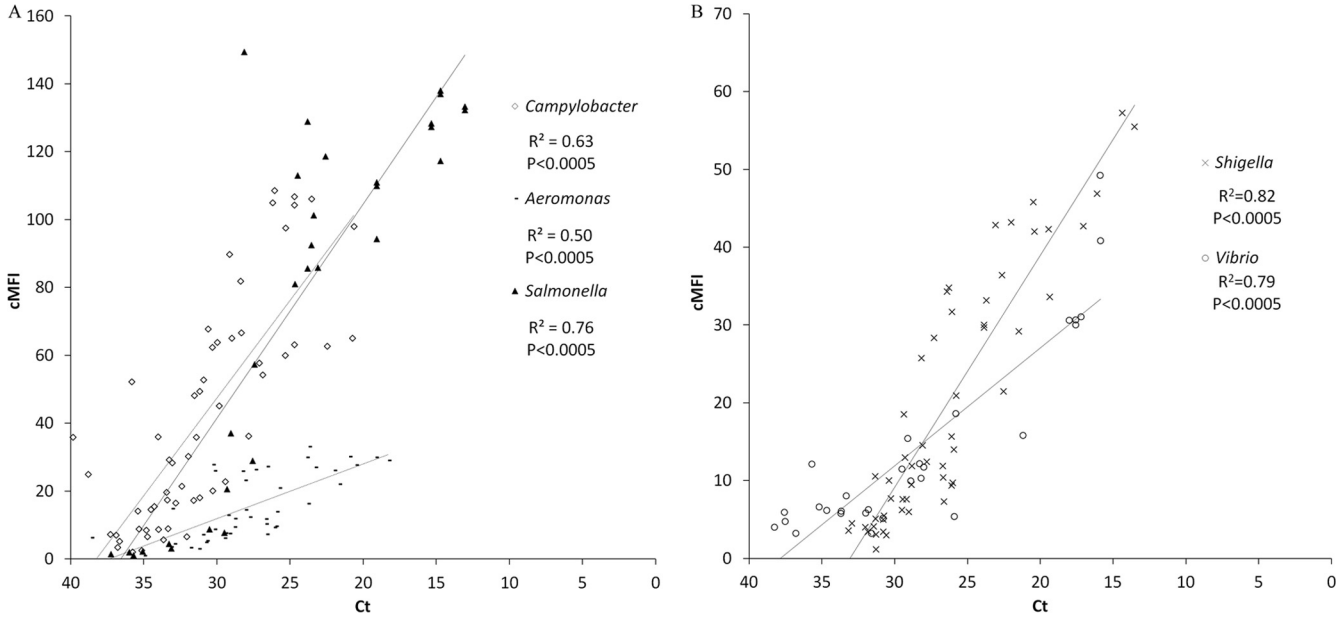

FIG 2 Correlation of PCR-Luminex assay cMFI with real-time PCR C_T values on clinical samples. Data are shown for fecal samples infected with *Aeromonas*, *Campylobacter*, and *Salmonella* (A) and *Shigella* and *Vibrio* (B) that were positive by both PCR-Luminex assay and real-time PCR using the same primers and probe designs.

to mixed infections: both could be contributing to diarrhea, one could be the main pathogen, while another asymptomatic or a colonizer, or both could be colonizers. We feel that quantitation is an important first step toward understanding this phenomenon, under the hypothesis that high burden pathogens are more likely contributors of disease. This represents a strength of this molecular approach over conventional approaches that are only qualitative. In this work, we were able to calculate the genome copy number of each pathogen in a sample from the standard curves generated with a known copy number of genomic DNA, followed by normalization for the particular stool sample's extraction/amplification efficiency as monitored by extrinsic control under the same principle as that demonstrated in our previous study (29). This normalization improved quantitation, and a simple ratio of target cMFI to extrinsic control cMFI yielded a number predictive of bacterial load. Our results showed that the mixed infections usually had a predominant organism with up to 4 logs more bacteria than the other organism. Relevant to quantitation is the fact that most of our targets were single-copy chromosomal genes (*aerolysin*, *cadF*, *toxR*, *lysP*, and *invA*) (15, 22, 25, 48). The *ipaH* target is likely to be less useful, because its copy numbers vary between species (42, 47). Additionally, the quantitation of most targets was generally unaffected by the presence of other pathogens.

In our hands, the assay yielded results within 8 h, with about $9 in reagent costs per sample, including extraction, amplification, and detection. It is implementable in any research or clinical laboratory with Luminex platforms, or the primers and probes can be adapted to other technologies, such as standard real-time PCR or microarray.

There were limitations to this work. First, our assay detects only a limited number of clinically significant pathogens and does not detect the diarrheagenic *E. coli*, protozoa, or viral targets. As mentioned, we have intended this assay to be used in concert with other multiplexed methods should those targets be considered. For the *Campylobacter* assay, if one is considering rare species such as *Campylobacter upsaliensis* or *Campylobacter hyointestinalis*, then one should use another method, such as the 16S assay, since we noted four cases of *C. upsaliensis* or *C. hyointestinalis* where the *cadF* PCR-Luminex was negative. Additionally, when *Campylobacter* is detected together with high burdens of *Salmonella* or *Vibrio*, the quantitation for *Campylobacter* is less accurate. Another limitation is that the Luminex assay requires a post-PCR step, which adds a potential contamination risk compared with closed platforms. Finally, our study did not include any prospective testing or correlation of quantitative results with clinical symptoms or outcomes. The latter will take significant work, and we hope to examine this at the Tanzania, Bangladesh, and Pakistan field sites in the near future.

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