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MAJOR ARTICLE

Giardia/Cryptosporidium QUIK CHEK Assay Is More Specific Than Quantitative Polymerase Chain Reaction for Rapid Point-of-care Diagnosis of Cryptosporidiosis in Infants in Bangladesh

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Background. *Cryptosporidium* is a major cause of childhood diarrhea. Current modes of cryptosporidiosis diagnosis involve procedures that are costly and require both a well-equipped laboratory and technical expertise. Therefore, a cost-effective, user-friendly, and rapid method for point-of-care detection of *Cryptosporidium* is desirable.

Methods. A total of 832 diarrheal stool specimens collected from 200 children aged <2 years were tested by *Giardia/Cryptosporidium* QUIK CHEK, enzyme-linked immunosorbent assay (ELISA), and quantitative polymerase chain reaction (qPCR) to compare the performance of the individual techniques. We also tested for the presence of other diarrheal pathogens in qPCR-positive samples with a TaqMan Array Card (TAC) to assess whether *Cryptosporidium* was the sole causative agent for the diarrheal episodes.

Results. Of 832 samples, 4.4% were found positive for *Cryptosporidium* by QUIK CHEK, 3.6% by ELISA, and 8.8% by qPCR. Using TAC-attributed *Cryptosporidium* diarrhea as the gold standard, the sensitivities of QUIK CHEK, ELISA, and qPCR were 92.3%, 71.8%, and 100%, respectively; the specificities were 97.1%, 94.3%, and 0%, respectively. Analysis of the qPCR-positive and QUIK CHEK–negative samples by TAC identified other enteropathogens as more likely than *Cryptosporidium* to be the causative agents of diarrhea.

Conclusions. QUIK CHEK was more sensitive and specific than ELISA. While qPCR detected *Cryptosporidium* in more samples than QUIK CHEK, most of these were instances of qPCR detecting small quantities of *Cryptosporidium* DNA in a diarrheal episode caused by another enteropathogen. We concluded that QUIK CHEK was comparable in sensitivity and superior in specificity to qPCR for the diagnosis of *Cryptosporidium* diarrhea.

Keywords. cryptosporidiosis; childhood diarrhea; rapid diagnostic test; diagnostic performance; Bangladesh.

Diarrhea is one of the most widely recognized diseases and a major cause of childhood mortality [1], with intestinal protozoan infections being important causes of acute and persistent diarrhea [2]. *Cryptosporidium* species is the leading protozoal cause of diarrhea worldwide in immunocompetent and immunocompromised subjects [3–5], with 8%–19% of cases attributed to *Cryptosporidium* species in low-income nations, and it has a significant effect on mortality [6, 7]. Among the diarrheagenic protozoan pathogens, *Cryptosporidium* species results in the most deaths among children <5 years of age, while the other 2 enteric protozoan parasites, *Giardia* species and *Entamoeba histolytica*, also contribute, but to a lesser extent [8, 9]. Although several *Cryptosporidium* species have been identified in humans, *Cryptosporidium hominis* and *Cryptosporidium parvum* cause >90% of human cases of cryptosporidiosis, while other species that are less commonly associated with human infection include *Cryptosporidium meleagridis*, *Cryptosporidium cuniculus*, *Cryptosporidium felis*, and *Cryptosporidium canis*; this is, however, dependent on setting [10–12]. The parasite infects the microvillous region of epithelial cells in the digestive and respiratory tract of humans, ultimately causing infectious diarrhea [13, 14]. The Global Enteric Multicenter Study identified *Cryptosporidium* as one of the 4 major contributors to moderate-to-severe diarrheal diseases during the first 2 years of life and showed *Cryptosporidium* as a key pathogen in diarrheal disease, even among otherwise healthy children. In fact, *Cryptosporidium* is second only to rotavirus as an agent of moderate to extreme diarrhea in children <2 years of age. There is a 2–3 times higher risk of death among children aged 12–23 months with cryptosporidiosis

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than in subjects of a similar age group without diarrhea [8, 15]. Enteric infection caused by *Cryptosporidium* in children can have devastating consequences by affecting intestinal absorption of nutrients and perturb childhood development [16]. This parasite is transmitted through fecal–oral routes either by consumption of contaminated food or water, or by person-to-person (anthroponotic) or animal-to-human (zoonotic) transmission [17].

Current methods of diagnosis of *Cryptosporidium* species include identification of *Cryptosporidium* oocysts by microscopy, antigen detection by enzyme-linked immunosorbent assay (ELISA), and DNA detection by polymerase chain reaction (PCR). Stool microscopy has low sensitivity in detecting *Cryptosporidium* species [18], and acid-fast staining is a prerequisite to differentiate the *Cryptosporidium* oocysts from other parasites and also from the yeast cells that are frequently present in stool [19]. The diagnostic methods becoming more widely used therefore are based on either fecal antigen detection or parasite DNA, but both require considerable technical expertise. Quantitative PCR (qPCR) is considered the most sensitive method, but because of this sensitivity may detect clinically insignificant amounts of cryptosporidial DNA in a diarrheal episode due to another pathogen. qPCR is also expensive and requires skilled personnel, which limits its use [20, 21]. Rapid detection techniques of fecal antigen have the potential to provide easy and cost-effective diagnosis of this pathogen in resource-limited settings.

Rapid antigen device tests are now available for the detection of *Cryptosporidium* include ImmunoCard STAT! *Cryptosporidium*/*Giardia* (Meridian Bioscience), Xpect *Giardia*/*Cryptosporidium* (Remel), and *Giardia*/*Cryptosporidium* QUIK CHEK (TechLab) [22–24]. Using ELISA as the reference standard, the QUIK CHEK had a sensitivity and specificity of 100% and 100%, respectively, for *Cryptosporidium*, which compared favorably to the result of ImmunoCard STAT! and Xpect test [24, 25].

Here, we report the performance of a rapid membrane immunoassay, QUIK CHEK, for the qualitative detection of *Cryptosporidium* species causing diarrheal episodes. In this study, the performance of this rapid antigen point-of-care test for detection of *Cryptosporidium* species was compared to ELISA and qPCR. The TaqMan Array Card (TAC), which provides quantitative results for a broad panel of enteropathogens, was used to attribute the cause of diarrhea and was considered the gold standard for diagnosis of *Cryptosporidium* diarrhea.

MATERIALS AND METHODS

Ethics Approval

The study was approved by the research and ethical review committees of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). Informed written consent was obtained from parents/guardians for the participation of their child in the study.

Study Area, Population, and Sampling

The study was conducted using 832 diarrheal stool specimens from a cohort of 200 children (aged up to 2 years) from November 2014 to July 2016 in Mirpur, Dhaka, Bangladesh. All of the fresh fecal specimens were tested by QUIK CHEK (TechLab) at the field site (Mirpur, Dhaka) and then transported to the Emerging Infections and Parasitology Lab, icddr,b, maintaining a cold chain and thereafter stored at –80°C. Then, ELISA (*Cryptosporidium* II test, TechLab) and (after DNA extraction) parasite-specific qPCR assays were performed with all of the stool samples in batches. The 74 samples found to be positive for *Cryptosporidium* by qPCR (37 QUIK CHEK positive and 37 QUIK CHEK negative) were further tested by TAC assay, which can detect the other enteropathogens that are common in Bangladeshi infants.

Rapid Antigen Point-of-care Test (QUIK CHEK)

The rapid antigen point-of-care test or QUIK CHEK assay was used in accordance with the manufacturer's instructions. All reagents and specimens were brought to room temperature prior to testing. Twenty-five microliters of fresh fecal specimens was used for testing. Then, 0.5 mL of diluent was added to the prelabeled test tube containing the fecal specimen. One drop of conjugate was then added. Tubes were then inverted and vortexed to ensure adequate suspension; 0.5 mL of the sample-conjugate mixture was then transferred into the sample well of the test device. Following the incubation of the test device for 15 minutes, 300 µL of wash buffer was added to the reaction window, followed by 2 drops of substrate, after which the device was left for 10 minutes at room temperature. Test results were read immediately after the final incubation [24].

Cryptosporidium Oocyst Antigen Test (ELISA)

The Cryptosporidium II kit (TechLab) was used for the *Cryptosporidium* antigen detection in samples according to the principles of ELISA. The kit was used in accordance with the manufacturer's instructions [26]. Interpretation of the assay was based on optical density (OD) readings at a single wavelength of 450 nm with the OD values ≥0.15 being considered positive for *Cryptosporidium*. Positive and negative controls were run with each batch of test specimens.

Extraction of Nucleic Acid From Fecal Specimens

Total nucleic acid extraction was performed using the modified QIAamp fast stool DNA extraction protocol, which incorporates a 3-minute bead-beating step to lyse the *Cryptosporidium* oocysts [27]. Total DNA was purified with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) from fresh or frozen stool samples. An internal control, PhHV (phocine herpesvirus) was used for inhibition control with each sample during extraction, which was then measured by qPCR reaction. In addition, 1 negative control (purified water instead of stool) was included in each batch (24 samples usually) as an extraction blank to control for potential carryover contamination.

Multiplex qPCR

The *Cryptosporidium* species real-time PCR assay was performed as part of a multiplex assay including *Giardia intestinalis* and *E. histolytica*. The multiplex qPCR also included an internal control (PhHV) to determine efficiency of the qPCR and detect inhibition in the sample. Positive and negative controls were used in each run of qPCR. Amplification consisted of 15 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C [28]. The 16S small-subunit ribosomal RNA gene primers and TaqMan probes for *Cryptosporidium* species, *G. intestinalis*, *E. histolytica*, and PhHV were used [28, 29]. Amplification, detection, and data analysis were performed with the CFX96 real-time detection system (Bio-Rad). Fluorescence was measured during the annealing step of each cycle.

TAC Assay

Seventy-four samples found to be positive for *Cryptosporidium* by qPCR were tested by TAC assay according to the protocol as described by Liu et al [30] for the identification of other enteric pathogens in the diarrheal episodes. We attributed pathogens as the cause of diarrhea if they were diarrhea-associated pathogens present at the diarrhea-associated quantities described by Liu et al [27].

Data Analysis

Descriptive statistics including mean and percentage were explored. To assess our experimental diagnostic test (QUIK CHEK) in comparison with reference standards (ELISA and qPCR), we used accuracy measurements which included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa (κ) coefficient. The *diagti* and *kap* commands in Stata 13.0 were used to calculate these accuracy measurements, and the 95% confidence interval (CI) for those accuracy measurements was calculated using the exact binomial distribution approach. A 2-tailed P value ≤.05 was considered significant.

RESULTS

A total of 832 diarrheal stool samples were tested by QUIK CHEK, *Cryptosporidium* II ELISA, and qPCR. Of these 832 diarrheal stool samples, 4.4% (n = 37) were found positive for *Cryptosporidium* by QUIK CHEK assay, 3.6% (n = 30) by ELISA assay, and 8.9% (n = 74) by qPCR testing (Table 1). Thirty-seven of the 832 samples had been found to be both QUIK CHEK and qPCR positive, in which the average qPCR quantification cycle (Cq) value was 23. Among these 37 samples, 29 were found to be *Cryptosporidium* positive by all the 3 techniques with an average qPCR Cq value of 22 and ELISA OD value of 1.34 (range, 0.16–2.87). On the other hand, the mean Cq value of qPCR-positive but QUIK CHEK–negative samples was 31, suggesting that these samples contained substantially less parasite DNA.

Agreement between QUIK CHEK and ELISA was 86.0% (κ = 0.860 and $P < .0001$), with sensitivity of 96.7% and specificity of 99.0% and PPV and NPV of 78.4% and 99.9%, respectively (ELISA as the gold standard). Likewise, agreement between QUIK CHEK and qPCR was 64.6% (κ = 0.646 and $P < .0001$), with sensitivity of 50% and specificity of 100%; the PPV and NPV had been 100% and 95.3%, respectively (qPCR as the gold standard). When using qPCR as the reference, the measured agreement, sensitivity, specificity, PPV, and NPV of ELISA were 55.4%, 40.5%, 100%, 100%, and 94.5%, respectively (Table 2).

We further assessed the burden of pathogens in all 74 qPCR-defined *Cryptosporidium*-positive samples by TAC assay. The QUIK CHEK–positive samples had lower TAC Cq (mean Cq value = 19) values (ie, more parasite DNA) than QUIK CHEK–negative samples (mean Cq value = 29) (Figure 1).

The TAC assay was used on all cryptosporidia qPCR-positive samples to test for the presence of other potential causes of diarrhea. The TAC assay was designed with a total of 76 enteropathogen targets including the positive controls and different strains. The major pathogens identified in the diarrheal stool samples are given in Table 3. We found that 39 of the 74 samples had a *Cryptosporidium* quantity that was highly diarrhea associated per a large multisite study (Cq ≤ 24) [27]. Thus, the TAC assay demonstrated that those 39 diarrheal episodes were caused by this high *Cryptosporidium* burden as well as categorized to "TAC-attributed *Cryptosporidium* diarrhea" (Figure 2A). For the rest (35/74) of the samples, the *Cryptosporidium* quantity was lower and not highly diarrhea associated (Cq > 24) and there were other diarrhea-associated pathogens present; those were categorized as "mixed infections" (Figure 2B). Interestingly, 92.3% (36 of 39) of the samples categorized by TAC as "*Cryptosporidium* diarrhea" were revealed positive for *Cryptosporidium* by the QUIK CHEK assay. For the diagnosis of *Cryptosporidium* causing diarrhea, the sensitivity

Table 1. Sample Testing Profile for the Evaluation of Different Diagnostic Techniques for *Cryptosporidium* Detection

	QUIK CHEK Assay		ELISA		TAC Assay	
qPCR Testing (n = 832)	Positive	Negative	Positive	Negative	Positive	Negative
Positive: n = 74 (8.8%)	37 (50)	37 (50)	30 (40.5)	44 (59.5)	74 (100)	0 (0)
Negative: n = 758 (91.1%)	0 (0)	758 (100)	0 (0)	758 (100)	Not tested	Not tested

All 832 samples were tested by QUIK CHEK, ELISA, and qPCR. Only the qPCR-positive samples were tested by TAC.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; TAC, TaqMan Array Card.

Table 2. Performance Comparison of the Techniques (QUIK CHEK®, ELISA and qPCR) in Detecting *Cryptosporidium* spp.

Methods Comparison (n = 832)	Sensitivity	Specificity	PPV	NPV	Agreement Analysis
QUIK CHEK vs ELISA[a]	96.7%	99.0%	78.4%	99.9%	$\kappa = 0.860$ ($P < .0001$)
QUIK CHEK vs qPCR[a]	50.0%	100.0%	100%	95.3%	$\kappa = 0.646$ ($P < .0001$)
ELISA vs qPCR[a]	40.5%	100.0%	100%	94.5%	$\kappa = 0.554$ ($P < .0001$)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; NPV, negative predictive value; PPV, positive predictive value; qPCR, quantitative polymerase chain reaction.
[a]Gold standard.

and specificity of the QUIK CHEK assay had been calculated to be 92.3% and 97.1%, respectively (Table 4); sensitivity and specificity of ELISA were found to be 71.8% and 94.3%, respectively (Table 4); and for qPCR those were 100% and 0%, respectively (considering "TAC-attributed *Cryptosporidium* diarrhea" as the gold standard); see Table 4.

The average TAC Cq value of *Cryptosporidium* target in diarrhea attributed to *Cryptosporidium* parasite was 20 ± 3.43 and average TAC Cq of *Cryptosporidium* for diarrhea caused by other pathogens was 29 ± 2.92. Notably, a total of 37 samples, including 36 *Cryptosporidium* diarrheal episodes, were detected positive by the QUIK CHEK assay; those were also positive by qPCR. Moreover, there were another 37 samples found to be positive by qPCR but designated as negative by QUIK CHEK. All of the 37 diarrheal samples those were QUIK CHEK negative but qPCR positive for cryptosporidia were also evaluated by TAC to determine the causative agents according to Liu et al [27]. Analysis of qPCR-positive but QUIK CHEK–negative samples revealed, 92% (34/37) had lower quantities of *Cryptosporidium* DNA and other enteropathogens identified as the probable cause of diarrhea (mixed infections) by TAC assay.

DISCUSSION

This study shows the potential of the QUIK CHEK assay for rapid point-of-care diagnosis of highly diarrhea-associated *Cryptosporidium*. Using the TAC as the gold standard to identify *Cryptosporidium*-attributable diarrhea, the QUIK CHEK was more specific than qPCR, which is the most important finding of this present study.

For detection of *Cryptosporidium* in diarrheal samples, TAC assay and qPCR showed similar results, whereas only 50% (n = 37) of those positive samples were detected by the QUIK CHEK assay. Thirty-six of these 37 QUIK CHEK–positive samples were, however, cryptosporidia causing diarrheal episodes, demonstrated by TAC assay, whereas TAC assay revealed 39 of the 74 qPCR-positive samples as highly diarrhea-associated *Cryptosporidium*. Among 39 highly diarrhea-associated *Cryptosporidium* diarrheal samples detected by TAC assay, in 24 samples *Cryptosporidium* was the only infection detected. In addition, 28 of the 30 ELISA-positive samples were revealed as *Cryptosporidium* causing diarrheal episodes by TAC analysis. Therefore the present study demonstrated that the QUIK CHEK is more specific than qPCR and ELISA for the detection of *Cryptosporidium* as a cause of diarrhea.

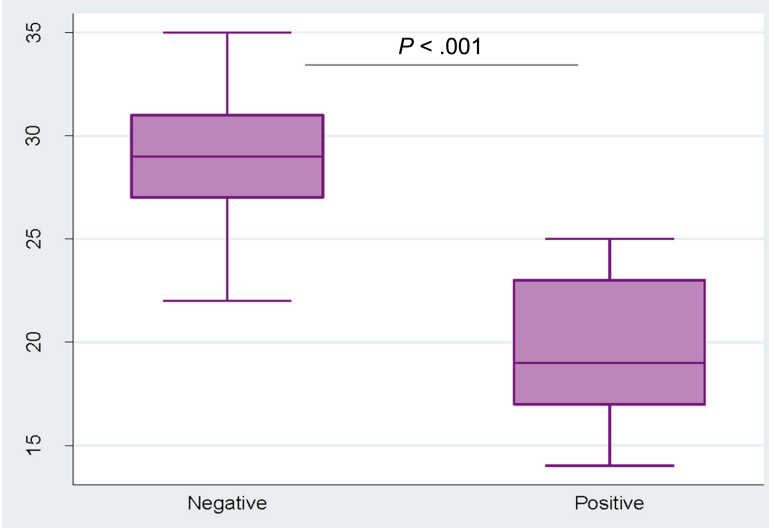

Figure 1. Distribution of QUIK CHEK–positive and –negative samples (quantification cycle) among the samples tested by TaqMan Array Card assay. Abbreviations: Ct, cycle threshold; TAC, TaqMan Array Card.

Table 3. Major Enteropathogens Other Than *Cryptosporidium* Detected by TaqMan Array Card Assay in the Diarrheal Samples

Serial	Pathogen	Prevalence Rate, %
1	*Campylobacter jejuni*	59.4
2	EPEC-bfpa and ETEC-STh	45.9
3	*Shigella*	44.6
4	Sapovirus	37.8
5	Adenovirus 40/41	36.5
6	*Giardia*	31.1
7	Norovirus GII	27.0
8	Rotavirus	10.8
9	Astrovirus	8.1
10	*Vibrio cholerae*	2.7

Abbreviations: bfpa, bundle-forming pilus A; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; STh, heat-stable toxin.

In this study, the relation between QUIK CHEK–positive results and higher pathogen load, as expressed by lower Cq values, is particularly noteworthy. To our knowledge, this is the only report thus far where TAC assay has been performed to specifically differentiate the *Cryptosporidium* causing diarrheal infections from other causes. Ninety-two percent of the *Cryptosporidium* causing diarrheal episodes was successfully detected by QUIK CHEK in this study, which is close to qPCR and higher than ELISA.

The performance (sensitivity and specificity) of diagnostic tests in different epidemiological and pathogenic scenarios has practical implications for the design of surveillance and/or control programs for *Cryptosporidium*. In previous studies, sensitivity rates reported for various commercially available

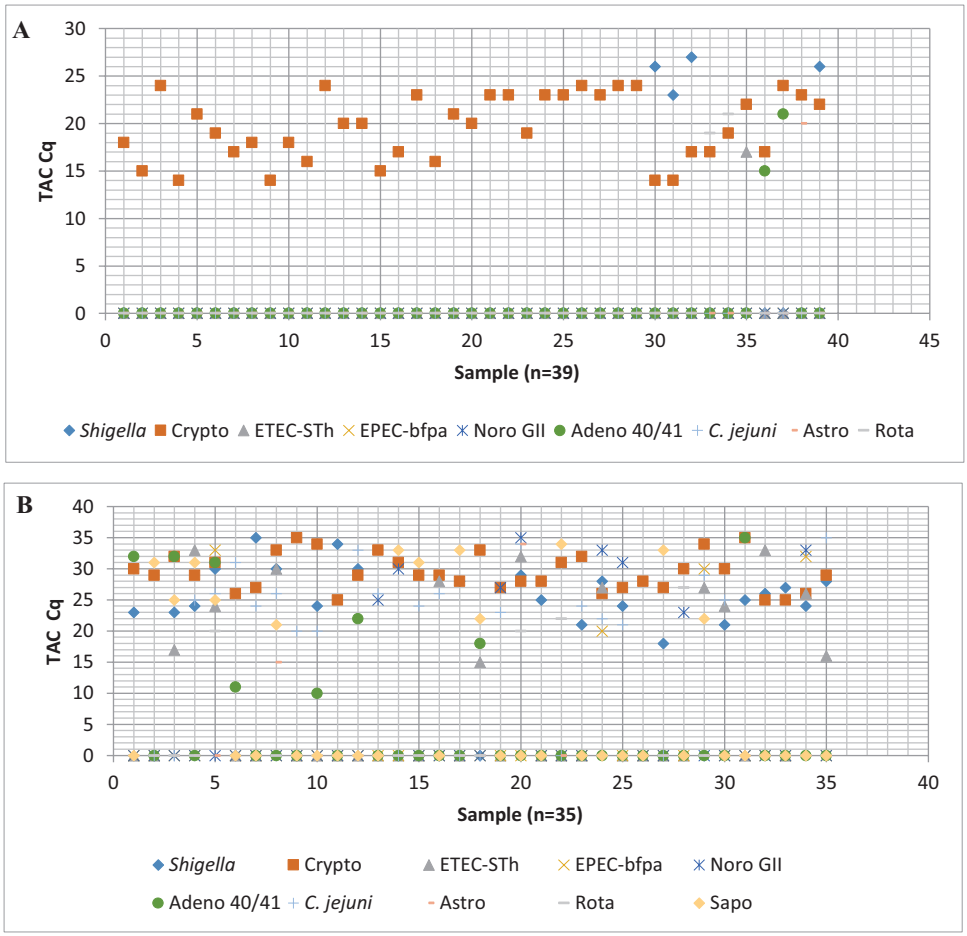

Figure 2. *A*, Diarrheal episodes (n = 39) with highly diarrhea-associated *Cryptosporidium* (quantification cycle [Cq] ≤24) revealed by TaqMan Array Card (TAC) assay and categorized as diarrhea attributed to *Cryptosporidium*. The y-axis indicates the highly diarrhea-associated Cq of different pathogens including *Shigella*, *Cryptosporidium* species (Crypto), enterotoxigenic *Escherichia coli* (ETEC-STh), enteropathogenic *E. coli* (EPEC-bfpa), norovirus GII (Noro), adenovirus 40/41 (Adeno), *Campylobacter jejuni*, astrovirus (Astro), and rotavirus (Rota). The x-axis indicates 39 highly diarrhea-associated *Cryptosporidium* samples (1–39). For most of the cases, "0" in the TAC Cq line indicates "not detected" by the TAC assay, although in some cases, "0" defines the pathogen's Cq value that exceeds the highly diarrhea-associated quantity (Cq) denoted by Liu et al [27]. *B*, Diarrheal episodes (n = 35) caused by mixed infections with various enteropathogens including lower *Cryptosporidium* burden (Cq >24). The y-axis indicates the diarrhea-associated Cq of different pathogens including *Shigella*, *Cryptosporidium* species, ETEC-STh, EPEC-bfpa, norovirus GII, adenovirus 40/41, *C. jejuni*, astrovirus, rotavirus, and sapovirus (Sapo). The x-axis indicates 35 samples with lower *Cryptosporidium* burden (1–35). The symbol "0" in the TAC Cq line indicates "not detected" by the TAC assay.

Table 4. Performance of QUIK CHEK, Enzyme-linked Immunosorbent Assay, and Quantitative Polymerase Chain Reaction in Detecting Diarrhea Attributed to *Cryptosporidium*

Diagnostic Technique	TAC-attributed *Cryptosporidium* Diarrhea	Diarrhea Attributed to Other Pathogens	Total
QUIK CHEK (n = 74)			
Positive	36	1	37
Negative	3	34	37
Total	39	35	74
ELISA (n = 74)			
Positive	28	2	30
Negative	11	33	44
Total	39	35	74
qPCR (n = 74)			
Positive	39	35	74
Negative	0	0	0
Total	39	35	74

Abbreviations: ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; TAC, TaqMan Array Card.

immunochromatographic and enzyme immunoassays had a detection range from 63% to 100% [31–34].

Sensitive and specific detection of *Cryptosporidium* infection is required to ensure that patients receive appropriate treatment. The clinical symptoms seen in the cases of mixed infections are due to complex interactions involving some or all of the other enteric pathogens present. If the clinicians start to use newer molecular methods such as TAC or qPCR for detection of enteropathogens in diarrheal stool samples, they will be able to identify the burden of enteropathogens responsible for the diarrhea. Based on the results of new molecular assays and the signs and symptoms of the patients, clinician will be able to figure out the causative agent of any diarrheal episode. Because molecular diagnosis such as qPCR/TAC and ELISA remain expensive and require skilled technicians, the clinician can use the QUIK CHEK test for rapid diagnosis of *Cryptosporidium* diarrhea as an alternative to molecular techniques. In our study, the QUIK CHEK's specificity revealed results that were better than that of ELISA and qPCR, whereas the sensitivity is close to qPCR and higher than ELISA in detecting *Cryptosporidium* diarrhea. In comparison with ELISA and qPCR, the rapid point-of-care test QUIK CHEK is more feasible due to its enhanced convenience for use in field sites in remote areas, its cost-effectiveness, and its practicality, as it does not require highly skilled technicians or well-equipped laboratory settings.

In conclusion, the *Giardia/Cryptosporidium* QUIK CHEK has a comparable sensitivity and higher specificity than that of qPCR for the diagnosis of *Cryptosporidium*-attributable diarrhea. Conversely, qPCR was more sensitive yet less specific. Therefore, rapid point-of-care antigen testing provides a robust means with which to diagnose *Cryptosporidium*-attributable diarrhea.

Notes

Author contributions. R. H., W. A. P., C. A. G., and A. S. G. F. conceived and designed the study. Field work, data gathering, and laboratory experiments at icddr,b were performed by E. A., S. A., M. K., B. H. and M. A., with supervision of R. H. and A. S. G. F. M. K. and E. A. performed statistical analyses and also wrote the manuscript with input from R. H., E. R. H., M. T., and W. A. P. All authors contributed to revisions and approved the final version.

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Potential conflicts of interest. W. A. P. is a consultant for TechLab and reports grants to his institution from Perrogo Nutritionals and payments for expert testimony for lectures from the University of California, Los Angeles and the University of Florida; patents and royalties from the University of Virginia; and travel expenses from the University of Virginia and the Bill & Melinda Gates Foundation. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed. S. A., M. A., E. A., R. H., B. H., A. F. and M. K. are employed by icddr,b and received grants from NIH. M. T., C. G., and E. H. employed by University of Virginia and received grants from NIH.

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