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METHODS

Dead-End Ultrafiltration and DNA-Based Methods for Detection of *Cyclospora cayetanensis* in Agricultural Water

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ABSTRACT *Cyclospora cayetanensis* is a protozoan parasite that causes foodborne and waterborne diarrheal illness outbreaks worldwide. Most of these outbreaks are associated with the consumption of fresh produce. Sensitive and specific methods to detect *C. cayetanensis* in agricultural water are needed to identify the parasite in agricultural water used to irrigate crops that have been implicated in outbreaks. In this study, a method to detect *C. cayetanensis* in water by combining dead-end ultrafiltration (DEUF) with sensitive and specific molecular detection was developed and evaluated. Triplicates of 10-liter agricultural water samples were seeded with 200, 100, 25, 12, and 6 *C. cayetanensis* oocysts. Surface water samples were also collected in the Mid-Atlantic region. All water samples were processed by DEUF and backflushed from the ultrafilters. DNA was extracted from concentrated samples and analyzed by quantitative PCR (qPCR) targeting the *C. cayetanensis* 18S rRNA gene. All water samples seeded with 12, 25, 100, and 200 oocysts were positive, and all unseeded samples were negative. Samples seeded with 6 oocysts had a detection rate of 66.6% (8/12). The method was also able to detect *C. cayetanensis* isolates in surface water samples from different locations of the Chesapeake and Ohio Canal (C&O Canal) in Maryland. This approach could consistently detect *C. cayetanensis* DNA in 10-liter agricultural water samples contaminated with low levels of oocysts, equivalent to the levels that may be found in naturally incurred environmental water sources. Our data demonstrate the robustness of the method as a useful tool to detect *C. cayetanensis* from environmental sources.

IMPORTANCE *Cyclospora cayetanensis* is a protozoan parasite that causes foodborne and waterborne outbreaks of diarrheal illness worldwide. These foodborne outbreaks associated with the consumption of fresh produce and agricultural water could play a role in the contamination process. In this study, a method to detect *C. cayetanensis* in agricultural water by combining a robust filtration system with sensitive and specific molecular detection was developed and validated by the FDA. The results showed that this approach could consistently detect low levels of *C. cayetanensis* contamination in 10 liters of agricultural water, corresponding to the levels that may be found in naturally occurring environmental water sources. The method was also able to detect *C. cayetanensis* in surface water samples from a specific location in the Mid-Atlantic region. Our data demonstrate the robustness of the method to detect *C. cayetanensis* in agricultural water samples, which could be very useful to identify environmental sources of contamination.

KEYWORDS agricultural water, *Cyclospora cayetanensis*, dead-end ultrafiltration, agricultural water

According to recent estimates, parasitic protozoan outbreaks are one of the leading causes of the 1.7 billion cases of diarrhea every year (1). Poor water, sanitation, and hygiene have a major impact on undernutrition and on several neglected tropical diseases (2). Most infections and deaths caused by these pathogens affect people in

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developing countries. Nevertheless, significant morbidity associated with protozoan parasite infections can also be seen in developed countries (3).

Intestinal protozoan parasites can be transmitted through food, water, and fecal-oral routes. Protozoan parasites such as *Cryptosporidium* spp., *Giardia duodenalis*, microsporidia, *Isospora* spp., *Entamoeba histolytica*, *Toxoplasma gondii*, *Balantidium coli*, *Sarcocystis* spp., *Naegleria* spp., *Acanthamoeba* spp., *Blastocystis hominis*, and *Cyclospora cayetanensis* are among those that can be acquired through exposure to contaminated water (1, 4, 5).

Among these foodborne and waterborne protozoa, *C. cayetanensis* is a coccidian parasite that causes cyclosporiasis, an intestinal illness characterized by watery diarrhea, nausea, stomach cramps, loss of appetite, and weight loss. Cyclosporiasis is normally self-limiting, although the course of the infection can be more severe in immunosuppressed patients (6, 7). Humans may become infected with *C. cayetanensis* by consuming fresh produce or water contaminated with sporulated oocysts of the parasite (8, 9).

In recent years, outbreaks and sporadic cases of cyclosporiasis associated with the consumption of fresh produce were reported in different countries, including Canada (10, 11), Poland (12), England, Scotland, and Wales (10), as well as Latin American countries (6). In the United States, foodborne outbreaks of cyclosporiasis have been reported annually since the mid-1990s with a continuous increase of reported cases since 2013. For instance, in 2013, a total of 631 cases with 49 hospitalizations were reported from 25 states to the Centers for Disease Control and Prevention (CDC) (13). In 2018 and 2019, the number of cases, hospitalizations, and the number of states reporting cyclosporiasis cases increased significantly. In 2018, a total of 2,299 domestically acquired cases with 160 hospitalizations were reported to the CDC from 33 states (14), and 511 of the 2,299 cases were epidemiologically linked to salads sold in a quick-service restaurant chain (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/b-071318/index.html). In 2019, a total of 2,408 cases were reported from 37 states with 144 hospitalizations (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2019/a-050119/index.html). Advances in laboratory detection of *C. cayetanensis* were of crucial importance during the investigations that took place in 2018. By using a novel laboratory method validated for the detection of *C. cayetanensis* in produce, the U.S. Food and Drug Administration (FDA) was able to detect the parasite's DNA in one of the unopened packages of the bagged salad mix sold by the implicated restaurant chain. The results of the testing conducted by the FDA supported the epidemiologic evidence that the salad mix was the source of the outbreak (https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-fdas-ongoing-efforts-prevent-foodborne-outbreaks).

Foodborne cyclosporiasis is becoming a significant public health concern in food production, and agricultural water could play a role as a vehicle for contamination of fresh produce (6, 15). The potential use of contaminated water for agricultural purposes poses a serious threat to millions of people worldwide (4). Although no estimates are available for the economic and public health impacts of cyclosporiasis in the United States and abroad, the burden of cyclosporiasis should not be underestimated. The absence of practical tools to detect *C. cayetanensis* in potential sources of contamination in the environment was considered a major challenge when investigating the cyclosporiasis outbreaks that took place in Texas in 2013 (13). This limitation creates a challenge for regulatory agencies, and, to close this gap, new methods for detection of *C. cayetanensis* in different matrices, including water, must be developed and validated.

For detection of *C. cayetanensis* in water, the only method recommended by the FDA was the EPA 1623 method (16), published in the FDA's Bacteriological Analytical Manual (BAM) in chapter 19A (17). However, this method was originally designed and validated to detect *Cryptosporidium* sp. and *Giardia* sp. in drinking water and not in agricultural water. Because of these limitations, we decided to evaluate an alternative method using hollow-fiber ultrafilters. This filtration system has proven to be cost-effective and capable of recovering bacteria, viruses, and parasites from large-volume

TABLE 1 Comparison of qPCR IAC detection results for purified and nonpurified samples

Sample[b]	C_T value from IAC[a]	
	Before purification	After purification
W461	43.81	28.66
W462	44.02	28.99
W463	31.09	28.1
W471	32.79	25.9
W472	38.07	25.33
W473	29.37	24.21

[a]Runs were set up according to settings established in BAM, chapter 19B (23).
[b]Samples used for this comparison were not seeded with *C. cayetanensis*.

water samples (18). Using hollow fibers in a dead-end ultrafiltration (DEUF) configuration would be useful for recovering multiple microbe classes (19).

In this study, a method to detect *C. cayetanensis* in agricultural water by combining the DEUF method for recovery of oocysts with a sensitive and specific PCR method targeting the *C. cayetanensis* 18S rRNA gene was developed and evaluated. A single-laboratory validation study (SLV) was conducted to assess the performance of the ultrafiltration combined with a robust DNA extraction and quantitative PCR (qPCR) methods.

RESULTS AND DISCUSSION

Optimization of the DNA extraction and qPCR methods for the detection of *C. cayetanensis* in agricultural water. An efficient protocol for the detection of *C. cayetanensis* in agricultural water was developed and evaluated in this study. This method combines the high performance of DEUF with robust molecular detection using a previously developed TaqMan qPCR targeting the *C. cayetanensis* 18S rRNA. Inhibitors of PCR are often present in agricultural water and can impair detection using molecular-based methods (20–22). Due to the inhibitory potential of this matrix, the DNA extraction and qPCR steps from the FDA BAM chapter 19B method (23) were modified for optimal performance with agricultural water.

The addition of a DNA purification step after the DNA extraction improved results based on a notable shift to lower internal amplification control (IAC) cycle threshold (C_T) values in the qPCR. A comparison of results for purified and nonpurified DNA samples is presented in Table 1. Purification lowered C_T values by many cycles in most of the samples. Further purification of DNA samples using the QIAquick PCR purification kit was shown to be an essential step in this method as demonstrated by a notable decrease in IAC C_T values. Furthermore, the optimization eliminated the need for more than 40 cycles and the C_T cutoff for qPCR used in the FDA BAM chapter 19B method. This allowed the detection method to be faster and more straightforward.

Detection of *C. cayetanensis* in agricultural water samples seeded with different numbers of oocysts. All 10-liter agricultural water samples seeded with 12, 25, 100, and 200 oocysts produced 100% positive replicates when tested with the qPCR. In addition, all unseeded samples were negative. Spiking with 3 oocysts was also tested and produced no positive results (data not shown). Detection rates for water samples seeded with 6 oocysts were 66.6% (8 of 12 filters). The evaluation conducted in this study followed internationally recognized guidelines for method validation (24, 25) and demonstrated that the method described can detect as few as 6 *C. cayetanensis* oocysts in 10 liters of agricultural water. The method relies on the DEUF filtration system and molecular detection according to the FDA's BAM, chapter 19B (23). The detection limit established in this study can be applied only to agricultural water and should be further evaluated for other water sources, which were not part of this SLV. The number of positive qPCR replicates and the C_T values for each seeding level are shown in Table 2. Table 3 shows a summary of the results and the detection rates at each seeding level.

Detection of *C. cayetanensis* in environmental water samples. Water samples filtered from the Chesapeake and Ohio Canal (C&O Canal) and Potomac River were

TABLE 2 qPCR detection results for agricultural water samples seeded with *Cyclospora cayetanensis* oocysts

No. of seeded oocysts[a]	qPCR 18S target C_T values[b,d]	No. of qPCR-positive replicates[b]	IAC target C_T value (mean ± SD)[c]
200	32.4 ± 0	3	29.2 ± 0.1
200	32.3 ± 0.5	3	28.1 ± 0.3
200	32.5 ± 0.1	3	27.8 ± 0.5
200	32.8 ± 0.2	3	28.4 ± 0.5
200	31.7 ± 0.2	3	27.7 ± 0.5
200	31.6 ± 0.2	3	27 ± 0.4
100	33.6 ± 0.2	3	32.1 ± 0.3
100	33.2 ± 0.1	3	32.3 ± 1.2
100	31.3 ± 0.1	3	28.1 ± 0.4
25	35.3 ± 0.3	3	26.9 ± 0.1
25	36.1 ± 0.7	3	26.1 ± 0.4
25	35.8 ± 0.3	3	26.4 ± 0.1
25	34.9 ± 0.4	3	28 ± 0.4
25	36.2 ± 0.2	3	28.9 ± 0.8
25	35.5 ± 0.7	3	26.6 ± 0.3
12	36.2 ± 0.3	3	29.1 ± 0.5
12	36.8 ± 0.6	3	29 ± 0.7
12	37.7 ± 2.7	3	29.2 ± 0.8
6	38.1 ± 0.4	3	30.4 ± 1.2
6	38.4 ± 0.6	2	28.7 ± 0.4
6	38.8	1	32.7 ± 1.5
6	36.8	1	28.7 ± 0.5
6	38.2 ± 0.8	2	34.3 ± 1.6
6	Undetermined[e]	0	31.3 ± 0.9
6	38.9	1	29.2 ± 0.2
6	Undetermined	0	29.4 ± 0.4
6	38.6	1	28.2 ± 0.3
6	38.1 ± 0.3	2	26.4 ± 0.4
6	Undetermined	0	24 ± 0.3
6	Undetermined	0	24.6 ± 1.6
0	Undetermined	0	27.7 ± 0.8
0	Undetermined	0	29.2 ± 0.6
0	Undetermined	0	27.1 ± 0.6
0	Undetermined	0	29.4 ± 0.3
0	Undetermined	0	27 ± 0.1
0	Undetermined	0	27.6 ± 0.3
0	Undetermined	0	26.7 ± 0.4
0	Undetermined	0	26 ± 0.1
0	Undetermined	0	27.4 ± 0.5

[a]Oocysts were seeded in 10-liter agricultural water samples as described in Materials and Methods.
[b]Number of qPCR positives out of the 3 replicates tested.
[c]Mean (±SD) C_T value for the *C. cayetanensis* 18S rRNA target reaction based on replicates producing a positive result ($C_T \leq 40.0$). No SD is shown when only one replicate produced a positive result.
[d]Data represent mean values from positive replicates.
[e]Undetermined, no C_T in qPCR.

analyzed to verify the usefulness of this method for the analysis of surface water. A total of 6 samples were collected at different sites, and 50% (3/6) were identified as positive for *C. cayetanensis*. Table 4 shows a summary of the results obtained with these samples. DNA samples derived from positive stool samples were used as positive controls for the molecular detection steps.

The results obtained with water samples from the C&O Canal are preliminary and do not allow conclusions to be drawn regarding the dispersion of *C. cayetanensis* in water sources. However, the results concur with other studies which detected *C. cayetanensis* in environmental samples, including the influent and effluent of wastewater treatment plants (WWTP), public drinking water supplies, and surface water (26–29). Further surveillance studies, using a more systematic approach and validated methods on a larger number of samples, must be conducted to provide more consistent data related to the dispersion of *C. cayetanensis* in the environment.

In this study, a qPCR was used targeting the *C. cayetanensis* 18S rRNA, a conserved gene within the coccidian group. The 18S rRNA gene was previously estimated to be

TABLE 3 Summary of detection results using DEUF and qPCR for agricultural water samples seeded with *C. cayetanensis* oocysts or unseeded

Matrix	Seeding level[a]	No. of samples analyzed	No. of oocysts/liter	qPCR	
				No. of positive samples	% positive
Irrigation water (10 liters)	0	12	0	0	0
	6	12	0.6	8	66.6
	12	3	1.2	3	100
	25	6	2.5	6	100
	100	3	10	3	100
	200	6	20	6	100

[a]Oocysts were seeded in 10-liter agricultural water samples as described in Materials and Methods.

18 copies per genome (30). Considering the biology of the parasite, most oocysts in the environment should be sporulated and each contain 4 genomes; therefore, the number of targets available should allow sensitive detection. Included in the specificity evaluation were microorganisms phylogenetically related to *C. cayetanensis*, which are dispersed in the environment, and microorganisms not closely related to *C. cayetanensis* but which have caused significant food safety impact. There was no cross-amplification using this DNA panel, but it is impossible to rule out potential cross-amplification of related microorganisms, which might be found in environmental samples, and for which full-length 18S rRNA sequencing data are not available. Additional work to develop molecular methods based on different regions of the genome, including organellar genomes, is underway. One of these tools, designed to amplify a DNA fragment of the mitochondrial genome, was used to confirm the presence of *C. cayetanensis* in the C&O Canal samples, and it should strengthen the analysis of environmental samples (M. Durigan and A. J. da Silva, unpublished data).

Dead-end ultrafiltration has shown great performance in the detection of other organisms like bacteriophage, *Enterococcus faecalis*, *Clostridium perfringens* spores, and *Cryptosporidium parvum* oocysts (19). The method described herein allows the analysis of water with high turbidity (18), which represents a major advantage over other methods designed to recover and detect parasites in water, such as the EPA 1623 method (16). This study demonstrated that the method was highly effective for analysis of samples contaminated with low levels of the parasite, with detection of as few as 6 oocysts of *C. cayetanensis* in 10 liters of agricultural water. *C. cayetanensis* was also detected in surface water samples from multiple locations of the C&O Canal in Maryland, United States, demonstrating that the method can be applied in the field with real environmental samples of various turbidity.

TABLE 4 qPCR results for detection of *C. cayetanensis* in DNA extracted from environmental samples processed by the DEUF method and qPCR

Sample	Origin[a]	Turbidity (NTU)	qPCR result	qPCR C_T[c]
W33	C&O Canal (Great Falls, MD)	10.2	Positive	35.7
W37	C&O Canal (Great Falls, MD)	17.3	Negative	Undetermined
W40	C&O Canal (Great Falls, MD)	14.7	Negative	Undetermined
W41	C&O Canal (Great Falls, MD)	14.9	Negative	Undetermined
W42	C&O Canal (Great Falls, MD)	14.6	Positive	36.6
W43	C&O Canal (Great Falls, MD)	16.5	Positive	33.9 ± 0.4
Stool 28 control[b,d]	USA	NA	Positive	29 ± 0.2
Stool 19 control[b,d]	USA	NA	Positive	27.1 ± 0.3
Oocysts control[b,e]	Purified oocysts (Indonesia)	NA	Positive	31 ± 0.3

[a]A through F indicate different locations of the C&O Canal.
[b]DNA extracted from clinical samples positive for *C. cayetanensis* and purified oocysts were included for comparison.
[c]No SD is shown when only one replicate produced a positive result.
[d]Stools 19 and 28 represent DNA from clinical samples from two individuals positive for *C. cayetanensis* by microscopic and molecular analysis.
[e]Oocysts control represents DNA from oocysts purified from stool from an Indonesian patient.

In conclusion, this laboratory evaluation demonstrates that the robust DEUF method combined with a well-designed molecular approach allows the detection of low levels of *C. cayetanensis*, which may be dispersed in water. The approach was also successfully used to collect, recover, concentrate, and analyze surface water in the field. This method will be a useful tool to detect and further characterize *C. cayetanensis* from a variety of environmental sources such as well water, surface water, reclaimed water, and effluent water, among others. Based on the data generated in this single laboratory validation study, the FDA conducted a collaborative study to validate this method for regulatory testing (unpublished data). This method is currently available through the FDA's Bacteriological Analytical Manual (BAM) (17). The FDA's BAM presents the agency's preferred laboratory procedures for microbiological analyses of foods and cosmetics. Additionally, there is a need for new *C. cayetanensis* detection markers based on other genomic regions and subtyping markers. Advances in these areas will allow the development of specific and sensitive tools to better understand how contaminated water can affect the food chain and potentially cause waterborne protozoan outbreaks.

MATERIALS AND METHODS

Study design. This study was conducted following the FDA Food Program's Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds (https://www.fda.gov/media/83812/download), which aligns well with those from the AOAC International and the ISO 16140-2:2016 standard (24, 25).

***C. cayetanensis* oocysts.** The oocysts used in the experiments designed to evaluate this new method were purified from individual human stool samples and stored in 2.5% potassium dichromate as described elsewhere (23). The study was approved by the institutional review board of the FDA (protocol number 15-039F). The oocysts were enumerated using a hemocytometer on an Olympus BX51 microscope (Optical Elements Corporation, Dulles, VA, USA). Oocysts were diluted in 0.85% NaCl to contain 10 oocysts/μl and 0.6 oocysts/μl for seeding experiments in water.

Water collection, storage, and seeding of oocysts. All irrigation water samples were collected from one irrigation pond located in Upper Marlboro, Prince George's County, Maryland (38°51′41.7″N, 76°46′28.0″W). The water was collected using a Honda WX10TA water pump into 20-liter carboys that were autoclaved prior to use. Each 20-liter carboy used in these experiments was sterilized before the experiments using the following procedure. First, 20 ml of 0.65% bleach solution was sprayed on each of the carboys, which were then left for approximately 30 min at room temperature. The carboys were then rinsed three times with deionized (DI) water and autoclaved for 45 min at 121°C. Collected water was stored for no more than 2 weeks at 4°C until used in experiments. The turbidity of all the water samples was measured with a model TL2300 tungsten lamp turbidimeter (Hach Company). Ten-liter samples of the collected water were separated into independent containers and kept in the lab at room temperature for approximately 45 min prior to seeding. The turbidity range of the agriculture water samples was 2.36 to 10.7 nephelometric turbidity units (NTU).

The study was designed as required by FDA validation guidelines in which the low-level seeding was a level giving 50% ± 25% positive results. *C. cayetanensis* oocysts were seeded in 10-liter agricultural water samples in the following manner. First, a stock oocyst preparation was serially diluted to obtain seeding preparations containing 0.6, 1.2, 2.5, 10, and 20 oocysts/μl. Unseeded water samples from the same source were also included in the experiments. Then, 10 μl of each preparation above was inoculated into individual tubes containing approximately 50 ml irrigation water taken from the carboys containing 10-liter samples to be seeded. The 50-ml preparations containing oocysts were then mixed back into the carboys containing the 10-liter samples. Each carboy containing oocysts was then swirled to evenly distribute oocysts in the sample prior to filtration. Three replicates of each sample at each of the five seeding levels were tested.

Surface water samples were also collected from the Potomac River and the C&O Canal at Lock 22 (39°03′13.1″N, 77°17′20.0″W). The turbidity range of the surface water samples was 10.2 to 17.3 NTU. These surface water samples were processed and tested for the presence of *C. cayetanensis* to demonstrate the usefulness of this method for the analysis of water samples.

DEUF procedure. The filtration was performed using a Geopump peristaltic pump with EZ-Load II pump head model 91352123 (Geotech) and silicone tubing L/S 36 (Cole Parmer). New tubing was used for each experiment. The DEUF setup was performed as described elsewhere (19) with minor modifications. The hollow-fiber ultrafilter Rexeed-25S (Asahi Kasei Medical Co.) was set up with the input blue port on the top and connected to the pump with L/S 36 tubing, which was clamped to prevent leakage. The water was pumped from the input tubing to the filter and exited the ultrafilter through the permeate red side port. This port was connected with L/S 36 tubing to a flow meter CLX-15D-C (Clark Solutions) using an autoclavable DIN adapter model MPC855 NS.375 (Molded Products), and filtrate was collected in a second "permeate" carboy. After filtration, the permeate port was closed with an end port cap, model MPC-40 (Molded Products), to avoid leakage. All tubing connectors and clamps were sanitized with 0.65% bleach, washed with DI water, and then autoclaved as described earlier.

For the backflush, the filtration setup was adjusted with the input and output ports inverted. New L/S 36 tubing was connected to the red side port as an input port. Five hundred milliliters of a solution containing 0.5% Tween 80, 0.01% sodium polyphosphate (Sigma-Aldrich; catalog no. 305553), and 0.001% Y-30 antifoam emulsion (Sigma-Aldrich; catalog no A5758) were pumped through the permeate port to backflush the content retained in the filters. The pump rate used to backflush the content through the blue end port was approximately 600 ml/min; the final backflush sample, volumes of approximately 600 ml, were collected in1-liter beakers. To concentrate, the backflush eluate for each sample was split into equal parts in four 175-ml conical bottles and centrifuged at 4,000 $\times$ *g* for 45 min. The supernatant was then removed by vacuum aspiration, and 30 ml of residual supernatant was left per bottle. The pellets from each bottle were resuspended and transferred to eight (two per bottle) 15-ml conical tubes, which were then centrifuged at 4,000 $\times$ *g* for 45 min. The supernatant from this step was aspirated, and all pellets from the same sample were combined in a new 15-ml conical tube and centrifuged once more at 4,000 $\times$ *g* for 45 min. All but approximately 300 μl of the supernatant was aspirated, and this final resuspended pellet was then stored at 4°C for 24 h or promptly subjected to DNA extraction.

DNA extraction and quantitative PCR. DNA extraction was performed using the FastDNA spin kit for soil in conjunction with a FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA) following the procedure described in the FDA's BAM, chapter 19B (23) with some optimization for this type of matrix as follows: (i) MT buffer and 830 μl sodium phosphate buffer were added to the 15-ml conical tubes containing the final sample pellet for DNA extraction, and the mixture was mixed and transferred to the lysing matrix tube; (ii) if the total volume to be transferred to the lysing matrix tube was too large, the mixture was split into two tubes; and (iii) if a sample was split into two lysing matrix tubes, the lysate from the first tube was transferred to one spin filter and centrifuged, and then the lysate from the second tube was added to the same spin filter, which was centrifuged again.

After the DNA extraction procedure, the DNA extracts were purified further using the QIAquick PCR purification kit as described in the kit's protocol. Final elution was performed with 30 μl of elution buffer (10 mM Tris-HCl, pH 8.5) to improve DNA recovery as recommended. The DNA samples were stored at 4°C for up to 5 days or at −20 or −80°C for longer-term storage.

To detect *C. cayetanensis* DNA in the samples, a TaqMan PCR assay (qPCR) targeting a region of the 18S rRNA gene (31, 32) was used. The optimized format of this reaction was a duplex TaqMan PCR (qPCR) that included a noncompetitive exogenous internal amplification control (IAC) to monitor reaction failure or lack of efficiency due to PCR inhibition (31).

A commercial synthetic gene fragment (gBlock; Integrated DNA Technologies, Coralville, CA) was used as positive control following the same protocol described previously (31). This control was prepared in a solution containing Tris-EDTA buffer with 5 μg/ml herring sperm DNA (Thermo Fisher Scientific, Waltham, MA) as a carrier. The synthetic control was serially diluted to contain from 5 $\times$ 10^4 to 0.5 copies per μL. For all qPCRs, 2 μl of the appropriate positive-control dilutions were used as the template to achieve the desired concentration. A few modifications from the qPCR conditions described previously (31) were implemented as follows: (i) each DNA sample was analyzed in triplicate both undiluted and at a 1/10 dilution instead of a one-fourth dilution; (ii) the qPCRs were run for 40 cycles instead of 45; and (iii) The cycle threshold (C_T) was set manually to 0.03 instead of 0.02.

Each run consisted of study samples, a no-template control (NTC), a DNA extraction control sample, and positive controls from 10 to 10^3 copies of the synthetic DNA positive control. All reactions were run in triplicate on an Applied Biosystems 7500 Fast real-time PCR system (Thermo Fisher Scientific, Waltham, MA) in fast mode using the QuantiFast Multiplex PCR +R kit (Qiagen). Analysis was performed using the Applied Biosystems 7500 software v.2.3.

The specificity of the qPCR was tested by analysis of a panel consisting of DNA samples from foodborne bacterial and parasitic pathogens in addition to *in silico* testing using sequences available in GenBank. This panel included DNA from the following microorganisms: *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cyclospora cayetanensis*, *Cyclospora* sp. (from simians), *Eimeria acervulina*, *Eimeria tenella*, *Eimeria maxima*, *Entamoeba histolytica*, *Giardia duodenalis*, *Blastocystis hominis*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Salmonella* sp., *Escherichia coli*, and *Trypanosoma cruzi*.

Interpretation of qPCR results. DNA samples were considered positive by qPCR when at least one reaction replicate produced a C_T value of less than or equal to 40.0 for the *C. cayetanensis* 18S rRNA target. If a sample *C. cayetanensis* 18S (Ccay 18S) target reaction produced all replicates with undetermined C_T values and all replicates of the IAC target reaction produced a positive result crossing the threshold, the sample was considered negative. If one or more replicates of the NTC sample, or the DNA extraction control sample Ccay18S target reactions, produced a positive result crossing the threshold, or if one or more replicates of the positive-control sample Ccay18S target reaction was undetermined, the experimental run was considered invalid. If a DNA sample Ccay18S target produced all replicates with undetermined C_T values and one or more of the undiluted DNA sample IAC target reactions was undetermined, the result was considered inconclusive.

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M.D. participated in the experiments' design, collected the environmental samples, and performed the experiments. H.R.M. participated in the experiments' design. A.J.D.S. coordinated the study, participated in the experiments' design, and supervised the experiments. All the authors cowrote the manuscript and approved the final manuscript.

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