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Sources and Prevalence of *Cyclospora cayetanensis* in Southeastern U.S. Growing Environments

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Abstract

Recent cyclosporiasis outbreaks associated with fresh produce grown in the United States highlight the need to better understand *Cyclospora cayetanensis* prevalence in U.S. agricultural environments. In this study, *C. cayetanensis* occurrence was assessed in municipal wastewater sludge, on-farm portable toilets, irrigation pond water, and spent packing house dump tank water in a Southeastern Georgia growing region over two years. Detection of the *C. cayetanensis* 18S rRNA qPCR gene target in pond samples was 0%, 28%, and 42% ($N = 217$) depending on the detection definition used, and ≤1% in dump tank samples ($N = 46$). However, no qPCR detections were confirmed by sequencing, suggesting false detection occurred due to cross-reactions. *C. cayetanensis* qPCR detections were confirmed in 9% of wastewater sludge samples ($N = 76$). The human-specific fecal markers HF183 and crAssphage were detected in 33% and 6% of pond samples, respectively, and 4% and 0% of dump tank samples, respectively. Despite community *Cyclospora* shedding and evidence of human fecal contamination in irrigation water, there was

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

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no correlation between *C. cayetanensis* and HF183 qPCR detections, further supporting that 18S gene target qPCR amplifications were due to cross–reactions. When evaluating *C. cayetanensis* qPCR environmental detection data, the impact of assay specificity and detection criteria should be considered. Moreover, additional sequence-based testing may be needed to appropriately interpret *Cyclospora* qPCR environmental data.

Keywords

Cyclospora ; Foodborne pathogens; Food safety; Irrigation water; Produce; Wastewater

In recent years, produce-associated cyclosporiasis outbreaks have caused concern in the United States (U.S.) public health and food safety communities. Historically, most cyclosporiasis outbreaks have been attributed to produce grown in Central or South America, where the disease is endemic (Abanyie et al., 2015; Buss et al., 2016; Katz et al., 1999). However, in 2013, a survey of ready-to-eat produce from Canadian retailers reported the identification of *Cyclospora cayetanensis*, on 1.7% of tested North American-grown produce (U.S. and Canada), suggesting the existence of domestic contamination sources (Dixon et al., 2013). In 2018, two multistate cyclosporiasis outbreaks occurred in which U.S.-grown produce was the suspected source (U.S. Food and Drug Administration, 2020b, 2022). During routine sampling in 2018, the U.S. Food and Drug Administration (FDA) reported the detection of *C. cayetanensis* from U.S.-grown herbs (U.S. Food and Drug Administration, 2020c). Subsequently, the FDA reported the detection of *Cyclospora* in a canal near the implicated farm during a 2020 investigation following an outbreak associated with the consumption of bagged salad mix (U.S. Food and Drug Administration, 2020d). Despite these recent implications of domestic produce contamination, more environmental data are needed to determine whether *C. cayetanensis* is present in U.S. growing environments, potential sources in these environments, and potential contamination pathways to produce.

As an obligate intracellular parasite of humans, the sole sources of *C. cayetanensis* contamination to the environment are human feces or sewage (Ortega & Sanchez, 2010). In resource-limited settings, surface water sources may become contaminated with *C. cayetanensis* due to insufficient wastewater sewerage and treatment or the presence of untreated sewage outfalls. Wastewater sewerage and treatment systems in the U.S. are designed to remove parasites and prevent the release of untreated wastewater into the environment. However, a significant portion of U.S., wastewater systems are aging and have compromised infrastructures. These include systems that are over capacity, damaged, or utilize combined sewer overflows that can release untreated wastewater into the environment (McGinnis et al., 2018). Failure of on-site septic systems may also provide a route of insufficiently treated wastewater to the environment, particularly after rainfall events (Lusk et al., 2017; Murphy et al., 2020). Once in the environment, *C. cayetanensis* requires an estimated seven to 15 days to sporulate (Ortega & Sanchez, 2010). Environmental water sources that become contaminated with wastewater can serve as an environmental reservoir for *C. cayetanensis* oocysts, and given enough time in the environment for oocyst

sporulation, contaminated water sources could also serve as effective vehicles for produce contamination (Bern et al., 1999; Sturbaum et al., 1998).

Surveillance to assess the prevalence, sources, and transport of *C. cayetanensis* in the environment poses distinct challenges. Surveillance testing may require expensive and time-consuming sample collection and testing methods to detect low concentrations of oocysts. Additionally, the complexities of environmental matrices and background microbial flora may adversely affect the sensitivity and specificity of molecular testing methods. Finally, the criteria used to determine the limit of detection of a quantitative real-time PCR (qPCR) assay (i.e., quantification cycle (C_q) threshold) is not standardized, which can result in discrepant interpretation of environmental contamination data and related food safety risk estimates (Klymus et al., 2020; Stokdyk et al., 2016).

The objectives of this study were to assess potential sources of *C. cayetanensis* and its occurrence within farm environments in a southeastern Georgia growing region. This region was selected because it represents a large vegetable production area in the Southeastern Coastal Plain, where most growers utilize surface-fed holding ponds for irrigation and employ seasonal farm workers from *Cyclospora* endemic countries. *C. cayetanensis* shedding within the community was evaluated by analyzing municipal wastewater biosolids in the growing region and on-farm portable toilets during harvest periods. *C. cayetanensis* occurrence in water and on produce was assessed by analyzing irrigation pond water and spent packing house dump tank water, which served as a proxy to assess contamination of washed produce. Water samples were also analyzed for total coliforms and generic *Escherichia coli* and the human-specific fecal markers *Bacteroides* HF183 (HF183) and crAssphage to evaluate overall and human-specific fecal contamination, respectively, as previous research documented human fecal contamination in ponds in the study area (Hill et al., 2017). Finally, this study evaluated whether further sequence-based approaches can be used to confirm environmental *C. cayetanensis* detections by qPCR and how these additional sequence data and qPCR detection criteria impact the understanding of *C. cayetanensis* environmental occurrence and implications for food safety.

Materials and methods

Water sample collection and processing

Water samples were collected from irrigation ponds and postharvest packing house dump tanks of two produce farms in southeastern Georgia between September 2020 and December 2021. Irrigation water sampling sites consisted of four surface-fed holding ponds used for crop irrigation using drip systems on each grower's farm. Pond water samples were collected once a month during fallow periods and twice per month during harvest periods. In 2020, pond water samples were collected once a month during growing periods, but were collected twice a month in 2021. During harvest periods, spent water from one dump tank from each grower's packing house was collected weekly. A total of 217 pond water samples and 46 dump tank water samples were collected during the study period.

Pond water samples were collected on – site by filtering 50 L of water using dead-end ultrafiltration (DEUF) (Kahler et al., 2021; Mull & Hill, 2012; Smith & Hill, 2009).

Twenty liters of dump tank water was collected in 20-L cubitainers. Harvested produce was submerged in municipal water containing chlorine within the dump tank flume to cool the produce and remove debris. Dump tank water was sampled during produce washing before a water change to collect microorganisms that had been rinsed from the produce into the water. A 250-mL grab sample was collected alongside both pond and dump tank large-volume samples for quantification of total coliforms and *E. coli*. At the time of sampling, water temperature, pH, dissolved oxygen, turbidity, and conductivity were measured using a YSI Inc. ProDSS Multiparameter Digital Water Quality Meter (Yellow Springs, OH). Each grower used municipal tap water for dump tanks and an automated fertilizer dosing system for chlorinating the water during processing. Oxidation-reduction potential (ORP) was measured using the YSI meter to assess the oxidizing capacity of the dump tank water at the time of sample collection. Four replicate measurements were taken for each parameter to ensure accurate measurement in heterogeneous water matrices. Water samples were stored in insulated coolers with ice packs. Sodium thiosulfate was added to the dump tank (5 g) and grab water (2.5 g) samples immediately after collection to neutralize any chlorine residual. Grab water samples were analyzed for total coliforms and *E. coli* on the day of collection at the University of Georgia (2021) or Auburn University (2022) laboratories. Ultrafilters and dump tank samples were shipped overnight to the Centers for Disease Control and Prevention (CDC) laboratories in Atlanta, GA, for further processing.

At CDC, ultrafilters were backflushed by pumping 500 mL of a 0.5% Tween 80/0.01% sodium polyphosphate/0.001% Antifoam Y-30 solution through the dialysate port and recovering captured microbes from the blood port (Kahler et al., 2021; Smith & Hill, 2009). Due to their high organic load, dump tank water samples were concentrated by continuous flow centrifugation (CFC) as previously described (Kahler et al., 2021). Briefly, samples were pumped through a CFC Express unit at 750 mL/min using CFC 210 disposable bowls (Scientific Methods Inc., Granger, IN). The resulting concentrates were decanted, and the bowls were rinsed two times with 5 mL of a 0.5% Tween 80/0.01% sodium polyphosphate/0.001% Antifoam Y-30 solution. Samples were further concentrated by centrifugation at 4,000*g* for 15 min, the supernatants were removed by pipetting, and the pellets were resuspended with PBS. The resuspended concentrates were stored at −20 °C until nucleic acid extraction.

Wastewater biosolid and portable toilet sample collection and processing

Municipal wastewater biosolids (sludge) were collected twice a month from the local wastewater treatment plant in Tifton, Georgia, from January 2020 through December 2021. This facility serves the county in which the two study farms were located and is located 10–25 miles from the irrigation ponds, depending on the pond. One liter grab samples were collected from two locations in the processing system, the sludge from the gravitational thickener (REC) used for land application and the return-activated sludge (RAS) from the aeration basin. Only the RAS sample was collected from November 2020 until July 2021 while routine maintenance was performed on the thickener system. Forty-six RAS sludge and 30 REC sludge samples were collected in total. Sludge samples were placed in an insulated cooler with cold reusable freezer packs and shipped overnight to CDC in Atlanta, GA, for further processing. Samples were frozen at −20 °C upon arrival until further

processing. Thawed sludge samples were heat inactivated in a 60 °C water bath for 1 h. After cooling, approximately 500 mL was concentrated by centrifugation at 4,000*g* for 15 min, and the supernatants were removed by pipetting. The sludge concentrates were stored at −20 °C until nucleic acid extraction.

Portable toilet samples were collected every week during harvest seasons, as available, for a total of 37 samples. Samples were collected by inserting a SteriWare ViscoThief cream sampler into the toilet seat opening (Sampling Systems, Ltd., UK). Approximately 200 mL of portable toilet material was drawn into the sampler and then transferred into a sterile 250-mL Nalgene bottle. Samples were placed in an insulated cooler with cold reusable freezer packs and shipped overnight to CDC for further processing. At CDC, samples were concentrated by centrifugation at 4,000*g* for 15 min. The supernatant was removed by pipetting, and the sample concentrate was stored at 4 °C until further processing. Sample concentrates that exceeded a volume of 10 mL were placed in a filtered stomacher bag. Parasites were then eluted off the concentrate in the stomacher bag by adding 100 mL of 0.1% Alconox and stomaching at 230 rpm for 1 min using a Stomacher® 400 Circulator Lab Blender (Seward Ltd., UK). The eluate was removed from the bag, and the stomacher bag was rinsed manually with an additional 100 mL of 0.1% Alconox. The sample eluates were combined and further concentrated by centrifugation at 4,000*g* for 15 min. The supernatant was removed, and the sample concentrates were stored at −20 °C until nucleic acid extraction.

Nucleic acid extraction

Nucleic acid from pond and dump tank water concentrates was extracted using the Qiagen AllPrep® PowerViral® DNA/RNA Kit (Germantown, MD) with the following modifications. Dithiothreitol (Thermo Fisher Scientific, Waltham, MA) at a concentration of 0.01 μM was used in place of β-mercaptoethanol in the initial lysing buffer, which acts to prevent RNase activity for total nucleic acid extractions. Bead beating was performed on a FastPrep-24™ Classic bead beating grinder and lysis system (MP Biomedicals, Santa Ana, CaA) at 6 m/s for 60 s. The final elution was completed with 100 μL of Tris-EDTA (TE) buffer. Nucleic acid was extracted from the municipal wastewater sludge concentrates using the Qiagen AllPrep® PowerViral® DNA/RNA Kit following the same procedure as water samples. DNA was extracted from the frozen portable toilet sample concentrates using the Qiagen DNeasy® PowerMax® Soil Kit and the extracts were further concentrated by ethanol precipitation and resuspension in 0.1 mL TE buffer as described in the PowerMax® Soil Kit Handbook. An extraction blank was included with each batch of extracted samples ($N = 11$). Nucleic acid that was not immediately subjected to molecular testing was stored at −20 °C.

qPCR

Quantitative real-time PCR (qPCR) was performed for the detection of a *C. cayetanensis* 18S rRNA gene target for all samples (Qvarnstrom et al., 2018). Samples were analyzed in triplicate using a 5 μL template in 50-μL reaction volumes. Bovine serum albumin (BSA, Millipore Sigma, Burlington, MA) and T4 Gene 32 protein (gp32, New England Biolabs, Ipswich, MA) were added to the reaction mixture at concentrations of 0.5 mg/mL and 25 μg/mL, respectively, to improve amplification efficiency and reduce inhibition. Primer

and probe concentrations were 0.5 μM and 0.1 μM, respectively. An internal amplification control (IAC) consisting of a synthetic 200-bp ultramer DNA sequence was included in the *C. cayetanensis* assay at a concentration of 500 copies per reaction (U.S. Food and Drug Administration, 2017). IAC primer and probe concentrations were 0.5 μM and 0.1 μM, respectively.

qPCR was performed for the detection of *Bacteroides* HF183 (HF183) and crAssphage (056 assay) in pond and dump tank water samples (Stachler et al., 2017; U.S. Environmental Protection Agency, 2019). Samples were analyzed in triplicate using a 2 μL template in 25-μL reaction volumes. BSA was added to the reaction mixtures at a concentration of 0.2 mg/mL. Primer and probe concentrations for the assays were 1 μM and 0.08 μM, respectively. An internal amplification control (IAC) consisting of a linearized synthetic plasmid was included in the HF183 assay at a concentration of 100 copies per reaction to evaluate assay interference. IAC primer and probe concentrations were 1 μM and 0.08 μM, respectively.

qPCR amplification was performed in a 7500 Real-Time PCR System with software version 2.3 using TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, Waltham, MA). Forty-five cycles of annealing and extension were performed for all assays to visualize amplification curves for samples with late-stage amplification (C_q 35–40). For *C. cayetanensis*, the annealing and extension temperature was 67 °C, and for HF183 and crAssphage, the annealing and extension temperature was 60 °C. Three no-template controls with nuclease-free water as template were included with each instrument run.

All gene targets were enumerated using a standard curve consisting of at least six, 10-fold serial dilutions of a synthetic standard (Integrated DNA Technologies, Coralville, IA) analyzed in triplicate during each instrument run (Table S1). Gene copy number per reaction was calculated by inputting the average quantification cycle (C_q) of the detected replicates into a pooled standard curve equation. This value was multiplied by the proportion of the original sample that was concentrated, extracted, and tested by qPCR to obtain $\log_{10}$ gene copies detected per 100 mL of the original sample. A sample was considered to have a detection for HF183 or crAssphage if amplification was observed for at least 2 of 3 replicates, each having a C_q value less than 40.

Three definitions were used for determining qPCR detection of the *C. cayetanensis* 18S rRNA gene target in a sample. For Definition 1, the target was considered detected in a sample if amplification was observed for at least 2 of 3 qPCR replicates each having a C_q value less than 40 (Mattioli et al., 2021; Rao et al., 2022). Definition 2 corresponds to the criteria outlined in FDA's Bacteriological Analytical Manual for the detection of *C. cayetanensis* in agricultural water, in which the target was considered detected in a sample if amplification was observed for at least one qPCR replicate having a C_q value less than 40 (U.S. Food and Drug Administration, 2020a). Definition 3 is a proposed approach for standardization of detection limits for environmental qPCR assays, in which the detection limit for an assay is the lowest concentration of a standard that produces amplification in at least 95% of qPCR replicates (Klymus et al., 2020). For the *C. cayetanensis* 18S rRNA gene target assay, the qPCR detection limit was 35 gene target copies per reaction ($C_q \leq 33$).

Confirmation of C. cayetanensis qPCR detections

A workflow algorithm was used to confirm qPCR detections of the *C. cayetanensis* 18S rRNA gene target in irrigation water or sludge (Fig. 1). Samples that met the criteria for sequence characterization ($C_q < 37$) were subjected to testing using a *C. cayetanensis* PCR genotyping panel, consisting of six assays targeting regions from the *C. cayetanensis* nuclear genome and two assays targeting regions from the mitochondrial genome (Nascimento et al., 2020). Amplicons from all eight assays were deep sequenced on the Illumina MiSeq (Illumina, San Diego, CA) to identify haplotypes. The haplotypes were compared against known clinical *C. cayetanensis* haplotype sequences in a reference database compiled from U.S. *Cyclospora* outbreak strains, and a sample with amplified haplotypes matching *C. cayetanensis* haplotypes was considered a confirmed detection of *C. cayetanensis* (Barratt et al., 2021). For samples with confirmed *C. cayetanensis* haplotypes, genotyping could be attempted for samples with either (a) amplification of at least 5 of the eight markers or (b) amplification of 4 markers, if at least 3 of those 4 markers were any combination of the following: MSR, Mt-Junction, HC360i2, and HC378 (Barratt et al., 2021). A pairwise distance matrix was generated for samples in which these criteria were met, and samples were clustered based on genetic relatedness as previously described (Barratt et al., 2021).

A sample with haplotypes possessing low homology to known *C. cayetanensis* haplotypes was considered a false detection caused by suspected cross-reaction in the qPCR assay. Amplicon sequences were BLAST searched against the NCBI nucleotide database to identify closely related organisms (percent identity > 95%) that may have been present in the sample, leading to cross-reaction with the qPCR assay (Camacho et al., 2009). A phylogenetic tree was created that included a subset of samples in which the mitochondrial MSR marker amplified (Barratt et al., 2021; Nascimento et al., 2020). For comparison, mitochondrial DNA sequences homologous to the MSR locus were obtained from GenBank for several other coccidian parasites; these sequences were also included in our phylogenetic analysis. Sequences were aligned using MUSCLE v3.8.425 in Geneious Prime (version 2022.1.1) with default parameters (https://www.geneious.com) (Edgar, 2004; Kearse et al., 2012). The resulting alignment was analyzed using the phangorn package in R (version 4.0.4) to generate a neighbor-joining tree using the Jukes-Cantor substitution model, with 1,000 bootstraps (Saitou & Nei, 1987; Schliep, 2011).

Samples that did not produce any PCR amplification by the typing assays were considered unconfirmed detections, meaning the qPCR detection may have been the result of a cross-reaction or detection of *C. cayetanensis* genomic material that was below the detection limit of the typing assay. Finally, samples that were not able to be further characterized due to high qPCR C_q values ($C_q \geq 37$) were also considered unconfirmed detections.

Fecal indicator bacteria quantification

Total coliforms and *E. coli* were enumerated from 100 mL of the grab samples within 6 h of sample collection by the IDEXX Quanti-Tray 2000® method using Colilert-18 media (IDEXX, Westbrook, ME) (American Public Health Association, 2017).

Statistical analysis

Statistical analyses were performed in R version 4.2.2 (R Core Team, 2017). Figures were created using the ggplot2 package (Wickham, 2016). Cochran-Mantel-Haenszel Chi-Squared test was used to evaluate the association between qPCR detections of the *C. cayetanensis* 18S rRNA gene target and the human fecal indicator HF183 while controlling for pond using the mantelhaen.test function (R Core Team, 2017). P values ≤0.05 were considered statistically significant.

Results

Physical, chemical, and fecal indicator characteristics of water

Measured water quality parameters from the study ponds and dump tanks are presented in Table S2. Total coliforms and *E. coli* were routinely cultured in the pond water samples throughout the study period (100%; 85%, N = 217), with median *E. coli* concentrations ranging from 6 to 63 MPN/100 mL (Table 1). While total coliforms were also routinely cultured from dump tank water (84%, N = 46), *E. coli* was rarely detected in the samples (20%). Time series values of total coliform and *E. coli* concentrations are presented in the research project report (Mattioli et al., 2022). HF183 and crAssphage were detected in 71 (33%) and 14 (6%) of the pond water samples, respectively (Table 1 and Figs. S1–S8). HF183 was detected in 2 (4%) dump tank samples, and crAssphage was not detected in any dump tank sample.

C. cayetanensis qPCR detections

The *C. cayetanensis* 18S rRNA gene target was detected by qPCR in 59 (27%) pond water samples using Definition 1 detection criteria, in 91 (42%) pond water samples using Definition 2, and in none of the water samples using Definition 3 (Table 2 and Figs. S1–S8). The *C. cayetanensis* gene target was detected by qPCR in 1 (2%) dump tank sample using Definitions 1 and 2. There was no association between incidence of detection of HF183 and incidence of *C. cayetanensis* qPCR detection using either Definition 1 or Definition 2 in water {Definition 1: $\chi^2(1)$ = [0.006], 95% CI [0.39, 2.37], p = 0.94; Definition 2: $\chi^2(1)$ = [0.04], 95% CI [0.39, 1.86], p = 0.84; Definition 3 had no *C. cayetanensis* detections}. The *C. cayetanensis* gene target was detected by qPCR in 9 (20%) RAS samples using Definition 1, in 16 (35%) samples using Definition 2, and in 2 (4%) samples using Definition 3 (Fig. 2). Finally, the *C. cayetanensis* gene target was detected in 9 (30%) REC samples using Definition 1, 12 (40%) samples using Definition 2, and 1 (3%) sample using Definition 3. There were no *C. cayetanensis* qPCR detections in portable toilet samples.

Confirmation of C. cayetanensis qPCR detections

Of pond samples in which the *C. cayetanensis* 18S rRNA gene target was detected by qPCR, 45 (76%) samples had at least one replicate with a C_q value <37 and were further tested using the *C. cayetanensis* genotyping panel PCR assays (Nascimento et al., 2020). To evaluate the C_q value cut-off for sequencing confirmation, eight additional samples with qPCR C_q values ≥37 were also tested (N = 53 total water samples tested by PCR panel). The single qPCR detection in the dump tank water did not meet inclusion criteria, with all

replicate C_q values >37. All sludge samples with detections by qPCR were tested by the PCR genotyping panel, including five additional RAS samples and three additional REC samples for which only one replicate amplified at a C_q value <37 (N = 26 total sludge samples tested by PCR panel).

No amplification was observed for any genotyping marker for 15 pond water samples, and only the mitochondrial 16S rRNA gene (MSR) target amplified for 38 irrigation pond water samples. The MSR haplotype sequences for these pond water samples did not match any known *C. cayetanensis* haplotypes but were closely related to segments of the mitochondrial target region conserved amongst other Apicomplexan protozoa. MSR amplicon sequences were analyzed on a neighbor-joining tree, demonstrating that the sequences from the irrigation pond samples belonged to coccidia outside the clades occupied by clinically isolated *C. cayetanensis* sequences (Fig. S9). BLASTN searches revealed that the MSR amplicon sequences did not match any known organism, but mitochondrial genome sequences from *Eimeria, Isospora, and Caryospora* were the closest matches. Therefore, these 38 samples were determined to be false detections caused by suspected cross-reactions from closely related coccidian parasites in the irrigation pond water. Of the remaining samples, 15 were considered unconfirmed detections for *C. cayetanensis* due to the lack of amplification of any of the genotyping markers and 12 were considered unconfirmed detections as all three qPCR replicates had $C_q \geq 37$.

Of the 26 sludge samples tested by the *C. cayetanensis* genotyping panel, no amplification was observed for 18 samples. One sample amplified five of the 8 typing markers and matched *C. cayetanensis* haplotypes from clinical specimens from 2018 to 2021; the MSR locus obtained from this sample is included in our phylogeny (Table S3 and Fig. S9). Six additional samples produced amplification in one to three of the typing panel targets, and all the amplified haplotypes matched *C. cayetanensis* haplotypes from clinical specimens. However, the number of markers amplified from each of these six samples was too few to meet the criteria for clustering via the previously described genotyping procedure (Nascimento et al., 2020). One sample had only one mitochondrial target amplify, and the haplotype sequence did not match any *C. cayetanensis* haplotypes but did match a conserved segment of the Apicomplexan mitochondrial target and thus was considered a suspected cross-reaction.

Discussion

There were no confirmed detections of *C. cayetanensis* in irrigation pond water or in spent dump tank water. While up of 42% of the irrigation pond water samples analyzed during this study detected the *C. cayetanensis* 18S rRNA gene target by qPCR (Definition 2 criteria), the *C. cayetanensis* genotyping assay panel indicated that at least some of the qPCR detections were likely cross-reactions with closely related coccidian organisms, such as *Eimeria, Isospora*, and *Caryospora*. Cross-reaction in the *C. cayetanensis* qPCR assay caused by these other Eimeriidae is plausible, as there are almost 2,000 known species of *Eimeria* and almost 300 known species of *Isospora*, which can infect mammals, birds, reptiles, and fish that could be present in or near the irrigation ponds in the study area (Lindsay et al., 1997; López-Osorio et al., 2020). Previous studies using the 18S rRNA

gene target for environmental samples have also detected *C. cayetanensis* gene targets by qPCR that could not be confirmed as *C. cayetanensis* by subsequent sequencing (Ben Ayed et al., 2012; Fan et al., 2021; Kniel, 2022). Consequently, appropriate caution should be exercised when assessing reports of *C. cayetanensis* detection in environmental samples and on produce in the absence of confirmatory Supporting data.

While there is no perfectly established correlation between human pathogens and their indicators, the lack of association between detections of human fecal markers and the *C. cayetanensis* 18S rRNA gene target provided evidence for the conclusion that the *C. cayetanensis* qPCR detections in pond water were likely false detections. However, HF183 and crAssphage presence in the irrigation ponds did demonstrate environmental contamination from human sewage sources. These findings highlight the risks of human pathogen contamination in this growing region, even in the absence of confirmed *C. cayetanensis* detections. Another potential route of pathogen contamination, particularly for human pathogens, is from septic systems. Smaller pathogens such as bacteria and viruses may contaminate the environment via groundwater intrusion originating from septic system leach fields (Lusk et al., 2017; Sowah et al., 2017). Large protozoan parasites such as *Cyclospora* are more likely to be retained in the leach field due to the filtration effects of the soil core. Defects in septic system design, poor maintenance, septic density in the area, the proximity of septic systems to surface waters, or various geological, chemical, and biological properties of the soil and aquifer may all increase the likelihood *C. cayetanensis* contamination to the environment (Bitton & Harvey, 1992; Lusk et al., 2017).

For environmental surveillance, the methods and criteria used for pathogen detection and confirmation are critically important for result interpretation. Using a high limit of detection for a qPCR assay will reduce the number of false detections but may consequently result in false negatives if the assay has either a low sensitivity or the pathogens are present at low concentrations. However, using a low limit of detection for a qPCR assay can increase the chance of false detections, especially if there are closely related organisms co-occurring in the environment (Wilcox et al., 2013). These concepts are illustrated by the *C. cayetanensis* 18S rRNA gene target qPCR detection rates obtained in this study. While Definition 1 and 2 criteria resulted in numerous false detections of *C. cayetanensis* due to suspected cross-reactions from closely related organisms in irrigation pond samples, the results obtained with the more stringent Definition 3 criteria aligned better with the sequence-confirmed results. But Definition 3 also resulted in false negatives in sludge, only detecting 3 of the 7 sequence-confirmed samples. For *C. cayetanensis*, creating a qPCR assay that is 100% specific and sensitive in environmental samples may not be possible. There are proposed statistical approaches to estimate the probability that a qPCR amplification is a true detection of a pathogen, which take into account the sensitivity and specificity of the assay (Wang et al., 2010). However, this methodology requires a precise understanding of the assay specificity in environmental samples, which is not well characterized for the *C. cayetanensis* 18S rRNA gene target assay to date. Thus, for pathogens such as *C. cayetanensis*, confirmatory steps or a multiple-tool testing panel such as some combination of molecular (qPCR and sequencing), microscopic, or statistical methodologies will likely be needed for confirmation of detection in environmental samples (Chen et al., 2021).

Municipal wastewater biosolid surveillance proved to be a valuable way to assess U.S. community shedding of *C. cayetanensis* in the Southeastern U.S. produce growing region. The seasonal pattern of the confirmed *C. cayetanensis* detections in sludge, which all occurred from July to September of 2020 and 2021, coincided with the time period of most produce-related U.S. cyclosporiasis outbreaks (Hadjilouka & Tsaltas, 2020). However, the drivers of the relationship between *Cyclospora* infections and season are unknown, even in endemic areas, therefore, we cannot ascertain whether the population shedding *C. cayetanensis* during this period was due to an increase in U.S.-acquired outbreak-associated cases exposed in the U.S. or due to a transient population shedding *C. cayetanensis* acquired elsewhere (Bern et al., 2002; Ortega, 2009). The sludge samples also proved valuable in that they demonstrated the effectiveness of the *C. cayetanensis* confirmation algorithm and sequence typing methods.

This study was subject to several limitations. The performance of the *C. cayetanensis* sequencing panel assays has not been evaluated for environmental samples and the genotyping assay is not used for diagnostic confirmation in clinical samples. Therefore, it is not known whether the false detections in the irrigation pond samples exhibited qPCR amplification due solely to cross-reaction from closely related Eimeriidae species within the samples, or whether *C. cayetanensis* was detected by qPCR but was present at levels too low to be detectable by the subsequent typing assays. Additionally, it was not possible to determine or predict which cross-reacting species of Eimeriidae were detected due to the absence of Eimeriidae gene sequences in the NCBI nucleotide database producing an identical match to the sequences we detected. Finally, the centrifugation methods used were not optimal for viral detection, which may have resulted in underestimating crAssphage occurrence in irrigation pond and dump tank water samples (Ikner et al., 2012). However, results from both HF183 and crAssphage demonstrated contamination from human sewage sources in the irrigation ponds and dump tanks.

The results of this study provide data that are critically needed to determine whether *C. cayetanensis* is present in U.S. growing environments. There were no confirmed detections of *C. cayetanensis* in irrigation pond water or on produce (via spent dump tank water as a proxy) at these Southeastern Georgia farms. However, human-specific fecal contamination was detected in the irrigation pond water and spent dump tank water, highlighting the risk of produce contamination by sewage-impacted irrigation water sources in this area. Molecular sequencing tools previously used only for clinical samples showed that the *C. cayetanensis* 18S rRNA gene target qPCR detections in water samples were false detections from suspected cross-reactions from closely related Eimeriidae parasites. This study demonstrated that when evaluating *C. cayetanensis* qPCR detections from environmental samples, the impact of assay specificity and detection criteria should be considered. Thus, testing environmental samples by multiple methods (i.e., addition of HF183 or crAssphage to evaluate human fecal contamination) or using sequencing-based confirmation assays may be needed to appropriately interpret *Cyclospora* qPCR environmental data and avoid false detections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abanyie F, Harvey RR, Harris JR, Wiegand RE, Gaul L, Desvignes-Kendrick M, Irvin K, Williams I, Hall RL, Herwaldt B, Gray EB, Qvarnstrom Y, Wise ME, Cantu V, Cantey PT, Bosch S, Da Silva AJ, Fields A, Bishop H, … Multistate Cyclosporiasis Outbreak Investigation Team (2015). 2013 multistate outbreaks of *Cyclospora cayetanensis* infections associated with fresh produce: Focus on the Texas investigations. Epidemiology and Infection, 143(16), 3451–3458. 10.1017/S0950268815000370. [PubMed: 25865140]

American Public Health Association (2017). 9223 enzyme substrate coliform test. American Public Health Association. 10.2105/SMWW.2882.194.

Barratt J, Houghton K, Richins T, Straily A, Threlkel R, Bera B, Kenneally J, Clemons B, Madison-Antenucci S, Cebelinski E, Whitney BM, Kreil KR, Cama V, Arrowood MJ, & Qvarnstrom Y (2021). Investigation of US *Cyclospora cayetanensis* outbreaks in 2019 and evaluation of an improved *Cyclospora* genotyping system against 2019 cyclosporiasis outbreak clusters. Epidemiology and Infection, 149, e214. 10.1017/S0950268821002090. [PubMed: 34511150]

Ben Ayed L, Yang W, Widmer G, Cama V, Ortega Y, & Xiao L (2012). Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis, Enterocytozoon bieneusi, Cyclospora cayetanensis* and *Eimeria* spp.. Journal of Water and Health, 10(3), 431–444. 10.2166/wh.2012.204. [PubMed: 22960487]

Bern C, Hernandez B, Lopez MB, Arrowood MJ, de Mejia MA, de Merida AM, Hightower AW, Venczel L, Herwaldt BL, & Klein RE (1999). Epidemiologic studies of *Cyclospora cayetanensis* in Guatemala. Emerging Infectious Diseases, 5(6), 766–774. 10.3201/eid0506.990604. [PubMed: 10603209]

Bern C, Ortega Y, Checkley W, Roberts JM, Lescano AG, Cabrera L, Verastegui M, Black RE, Sterling C, & Gilman RH (2002). Epidemiologic differences between cyclosporiasis and cryptosporidiosis in Peruvian children. Emerging Infectious Diseases, 8(6), 581–585. 10.3201/eid0806.01-0331. [PubMed: 12023913]

Bitton G, & Harvey RW (1992). Transport of pathogens through soils and aquifers. In Environmental microbiology (pp. 103–124). Wiley.

Buss BF, Joshi MV, O'Keefe AL, Allensworth CD, Garvey A, Obbink K, Mandernach S, & Safranek TJ (2016). Regional investigation of a cyclosporiasis outbreak linked to imported romaine lettuce – Nebraska and Iowa, June–August 2013. Epidemiology and Infection, 144(9), 1807–1817. 10.1017/S0950268815002484. [PubMed: 26489789]

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, & Madden TL (2009). BLAST+: architecture and applications. BMC Bioinformatics, 10(1), 421. 10.1186/1471-2105-10-421. [PubMed: 20003500]

Chen H, Liu C, Li Y, & Teng Y (2021). Integrating metagenomic and Bayesian analyses to evaluate the performance and confidence of crAssphage as an indicator for tracking human sewage contamination in China. Environmental Science & Technology, 55(8), 4992–5000. 10.1021/acs.est.1c00071. [PubMed: 33715349]

Dixon B, Parrington L, Cook A, Pollari F, & Farber J (2013). Detection of *Cyclospora, Cryptosporidium*, and *Giardia* in ready-to-eat packaged leafy greens in Ontario, Canada. Journal of Food Protection, 76(2), 307–313. 10.4315/0362-028X.JFP-12-282. [PubMed: 23433379]

Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5), 1792–1797. 10.1093/nar/gkh340. [PubMed: 15034147]

Fan Y, Wang X, Yang R, Zhao W, Li N, Guo Y, Xiao L, & Feng Y (2021). Molecular characterization of the waterborne pathogens *Cryptosporidium* spp., *Giardia duodenalis, Enterocytozoon bieneusi, Cyclospora cayetanensis* and *Eimeria* spp. In wastewater and sewage in Guangzhou, China. Parasites & Vectors, 14, 66. 10.1186/s13071-020-04566-5. [PubMed: 33472683]

Hadjilouka A, & Tsaltas D (2020). Cyclospora cayetanensis—Major outbreaks from ready to eat fresh fruits and vegetables. Foods, 9(11), 1703. 10.3390/foods9111703. [PubMed: 33233660]

Hill V, Vellidis G, & Levy K (2017). Improved sampling and analytical methods for testing agricultural water for pathogens, surrogates, and source tracking indicators. Center for Produce Safety [Final report].

Ikner LA, Gerba CP, & Bright KR (2012). Concentration and recovery of viruses from water: A comprehensive review. Food and Environmental Virology, 4(2), 41–67. 10.1007/s12560-012-9080-2. [PubMed: 23412811]

Kahler AM, Mattioli MC, da Silva AJ, & Hill V (2021). Detection of *Cyclospora cayetanensis* in produce irrigation and wash water using large-volume sampling techniques. Food and Waterborne Parasitology, 22, e00110. [PubMed: 33681488]

Katz D, Kumar S, Lowdermilk M, & Hopkins R (1999). Cyclosporiasis associated with imported raspberries, Florida, 1996. Public Health Reports, 1, 427–438. 10.1093/phr/114.5.427.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, & Drummond A (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647–1649. 10.1093/bioinformatics/bts199. [PubMed: 22543367]

Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, & Richter CA (2020). Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA, 2(3), 271–282. 10.1002/edn3.29.

Kniel K. (2022). Analysis of the presence of Cyclospora in waters of the mid-Atlantic states and evaluation of removal and inactivation by filtration. Center for Produce Safety [Final report].

Lindsay DS, Dubey JP, & Blagburn BL (1997). Biology of Isospora spp. From humans, nonhuman primates, and domestic animals. Clinical Microbiology Reviews, 10(1), 19–34. 10.1128/cmr.10.1.19. [PubMed: 8993857]

López-Osorio S, Chaparro-Gutiérrez JJ, & Gómez-Osorio LM (2020). Overview of poultry *Eimeria* life cycle and host-parasite interactions. Frontiers in Veterinary Science, 7, 384. 10.3389/fvets.2020.00384. [PubMed: 32714951]

Lusk MG, Toor GS, Yang Y-Y, Mechtensimer S, De M, & Obreza TA (2017). A review of the fate and transport of nitrogen, phosphorus, pathogens, and trace organic chemicals in septic systems. Critical Reviews in Environmental Science and Technology, 47(7), 455–541. 10.1080/10643389.2017.1327787.

Mattioli MC, Benedict KM, Murphy J, Kahler A, Kline KE, Longenberger A, Mitchell PK, Watkins S, Berger P, Shanks OC, Barrett CE, Barclay L, Hall AJ, Hill V, & Weltman A (2021). Identifying septic pollution exposure routes during a waterborne norovirus outbreak—a new application for human-associated microbial source tracking qPCR. Journal of Microbiological Methods, 180, 106091. 10.1016/j.mimet.2020.106091. [PubMed: 33137355]

Mattioli MC, da Silva ALBR, Kahler AM, & Arrowood MJ (2022). Sources and prevalence of Cyclospora cayetanensis in Southeastern US water sources and growing environments [Final report]. Center for Produce Safety.

McGinnis S, Spencer S, Firnstahl A, Stokdyk J, Borchardt M, McCarthy DT, & Murphy HM (2018). Human *Bacteroides* and total coliforms as indicators of recent combined sewer overflows

and rain events in urban creeks. Science of the Total Environment, 630, 967–976. 10.1016/j.scitotenv.2018.02.108. [PubMed: 29554782]

Mull B, & Hill VR (2012). Recovery of diverse microbes in high turbidity surface water samples using dead-end ultrafiltration. Journal of Microbiological Methods, 91(3), 429–433. 10.1016/j.mimet.2012.10.001. [PubMed: 23064261]

Murphy HM, McGinnis S, Blunt R, Stokdyk J, Wu J, Cagle A, Denno DM, Spencer S, Firnstahl A, & Borchardt MA (2020). Septic systems and rainfall influence human fecal marker and indicator organism occurrence in private wells in Southeastern Pennsylvania. Environmental Science and Technology, 54(6), 3159–3168. 10.1021/acs.est.9b05405. [PubMed: 32073835]

Nascimento FS, Barratt J, Houghton K, Plucinski M, Kelley J, Casillas S, Bennett CC, Snider C, Tuladhar R, Zhang J, Clemons B, Madison-Antenucci S, Russell A, Cebelinski E, Haan J, Robinson T, Arrowood MJ, Talundzic E, Bradbury RS, & Qvarnstrom Y (2020). Evaluation of an ensemble-based distance statistic for clustering MLST datasets using epidemiologically defined clusters of cyclosporiasis. Epidemiology and Infection, 148, e172. 10.1017/S0950268820001697. [PubMed: 32741426]

Ortega YR (2009). Environmental factors influencing the survival of *Cyclospora cayetanensis*. In Giardia and Cryptosporidium: from molecules to diseases (pp. 248–254).

Ortega YR, & Sanchez R (2010). Update on *Cyclospora cayetanensis*, a food-borne and waterborne parasite. Clinical Microbiology Reviews, 23(1), 218–234. 10.1128/CMR.00026-09. [PubMed: 20065331]

Qvarnstrom Y, Benedict T, Marcet PL, Wiegand RE, Herwaldt BL, & da Silva AJ (2018). Molecular detection of *Cyclospora cayetanensis* in human stool specimens using UNEX-based DNA extraction and real-time PCR. Parasitology, 145(7), 865–870. 10.1017/S0031182017001925. [PubMed: 29113617]

R Core Team (2017). R: a language and environment for statistical computing. R Foundation for Statistical Computing.

Rao G, Kahler A, Voth-Gaeddert LE, Cranford H, Libbey S, Galloway R, Molinari N-A, Ellis EM, Yoder JS, Mattioli MC, & Ellis BR (2022). Microbial characterization, factors contributing to contamination, and household use of cistern water, U.S. Virgin Islands. ACS ES&T Water, 2(12), 2634–2644. 10.1021/acsestwater.2c00389. [PubMed: 36530952]

Saitou N, & Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4(4), 406–425. 10.1093/oxfordjournals.molbev.a040454. [PubMed: 3447015]

Schliep KP (2011). phangorn: phylogenetic analysis in R. Bioinformatics, 27(4), 592–593. 10.1093/bioinformatics/btq706. [PubMed: 21169378]

Smith CM, & Hill VR (2009). Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water. Applied and Environmental Microbiology, 75(16), 5284–5289. 10.1128/AEM.00456-09. [PubMed: 19561183]

Sowah RA, Habteselassie MY, Radcliffe DE, Bauske E, & Risse M (2017). Isolating the impact of septic systems on fecal pollution in streams of suburban watersheds in Georgia, United States. Water Research, 108, 330–338. 10.1016/j.watres.2016.11.007. [PubMed: 27847149]

Stachler E, Kelty C, Sivaganesan M, Li X, Bibby K, & Shanks OC (2017). Quantitative crAssphage PCR assays for human fecal pollution measurement. Environmental Science & Technology, 51(16), 9146–9154. 10.1021/acs.est.7b02703. [PubMed: 28700235]

Stokdyk JP, Firnstahl AD, Spencer SK, Burch TR, & Borchardt MA (2016). Determining the 95% limit of detection for waterborne pathogen analyses from primary concentration to qPCR. Water Research, 96, 105–113. 10.1016/j.watres.2016.03.026. [PubMed: 27023926]

Sturbaum GD, Ortega YR, Gilman RH, Sterling CR, Cabrera L, & Klein DA (1998). Detection of *Cyclospora cayetanensis* in wastewater. Applied and Environmental Microbiology, 64(6), 2284–2286. 10.1128/aem.64.6.2284-2286.1998. [PubMed: 9603852]

U.S. Environmental Protection Agency. (2019). Method 1696: characterization of human fecal pollution in water by HF183/BacR287 TaqMan® quantitative polymerase chain reaction (qPCR) Assay. Office of Water.

U.S. Food and Drug Administration (2017). BAM Chapter 19b: molecular detection of *Cyclospora cayetanensis* in fresh produce using real-time PCR. Bacteriological analytical manual. FDA.

U.S. Food and Drug Administration (2020a). BAM 19c: dead-end ultrafiltration for the detection of Cyclospora cayetanensis from agricultural water. Bacteriological analytical manual. .

U.S. Food and Drug Administration (2020b). Del Monte Fresh Produce N.A., Inc. voluntarily recalls limited quantity of vegetable trays in a multistate outbreak of Cyclospora illnesses in select retailers in Illinois, Indiana, Iowa, Michigan Minnesota, and Wisconsin, because of possible health risk. U.S. Food and Drug Administration, FDA.

U.S. Food and Drug Administration (2020c). FDA sampling assignment update identifies Cyclospora in herbs. U.S. Food and Drug Administration, FDA.

U.S. Food and Drug Administration (2020d). Outbreak investigation of Cyclospora: bagged salads (June 2020). U.S. Food and Drug Administration, FDA.

U.S. Food and Drug Administration (2022). FDA investigated a multistate outbreak of Cyclospora illnesses linked to fresh express salad mix served at McDonald's ends. FDA.

Wang D, Silkie SS, Nelson KL, & Wuertz S (2010). Estimating true human and animal host source contribution in quantitative microbial source tracking using the Monte Carlo method. Water Research, 44(16), 4760–4775. 10.1016/j.watres.2010.07.076. [PubMed: 20822794]

Wickham H. (2016). ggplot2: elegant graphics for data analysis. New York: Springer-Verlag.

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, & Schwartz MK (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. PLOS ONE, 8(3)e59520. 10.1371/journal.pone.0059520. [PubMed: 23555689]

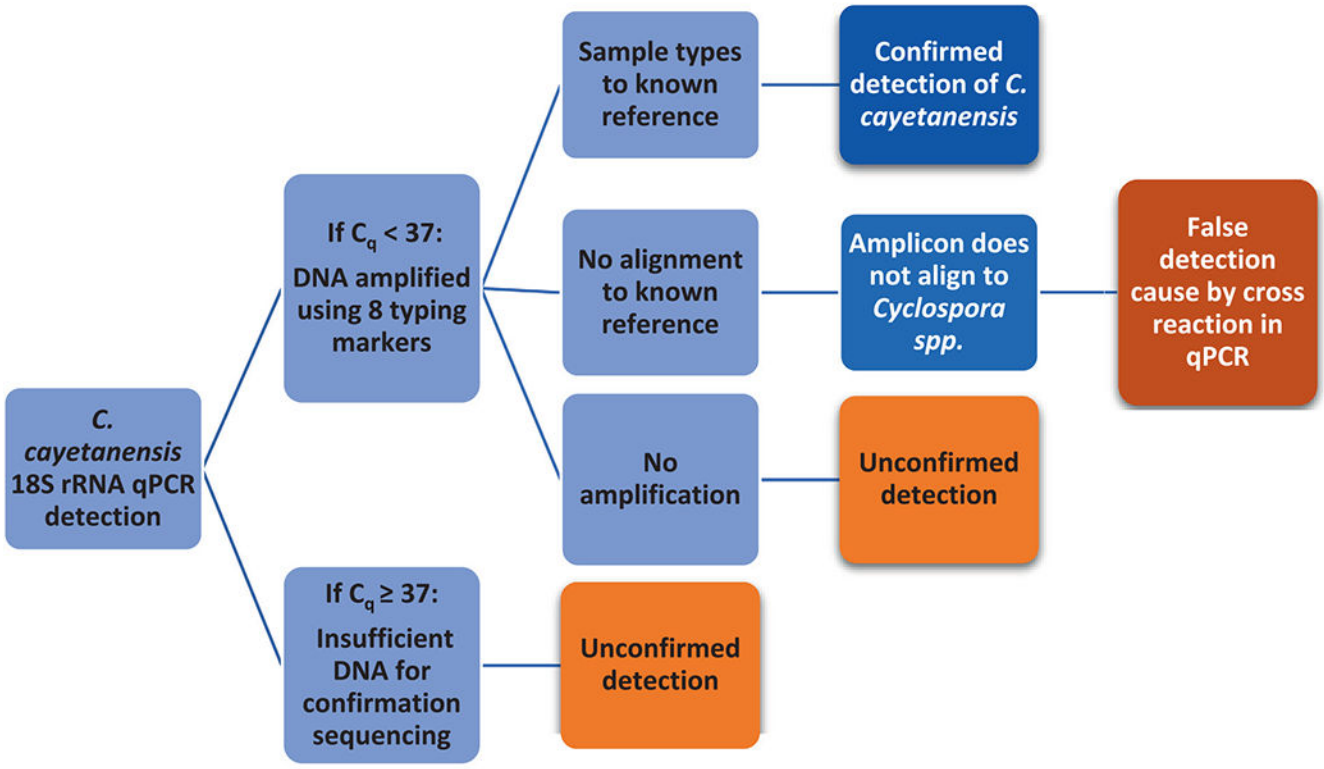

Figure 1.
Workflow algorithm for confirmation of a *C. cayetanensis* 18S rRNA gene target detection by qPCR.

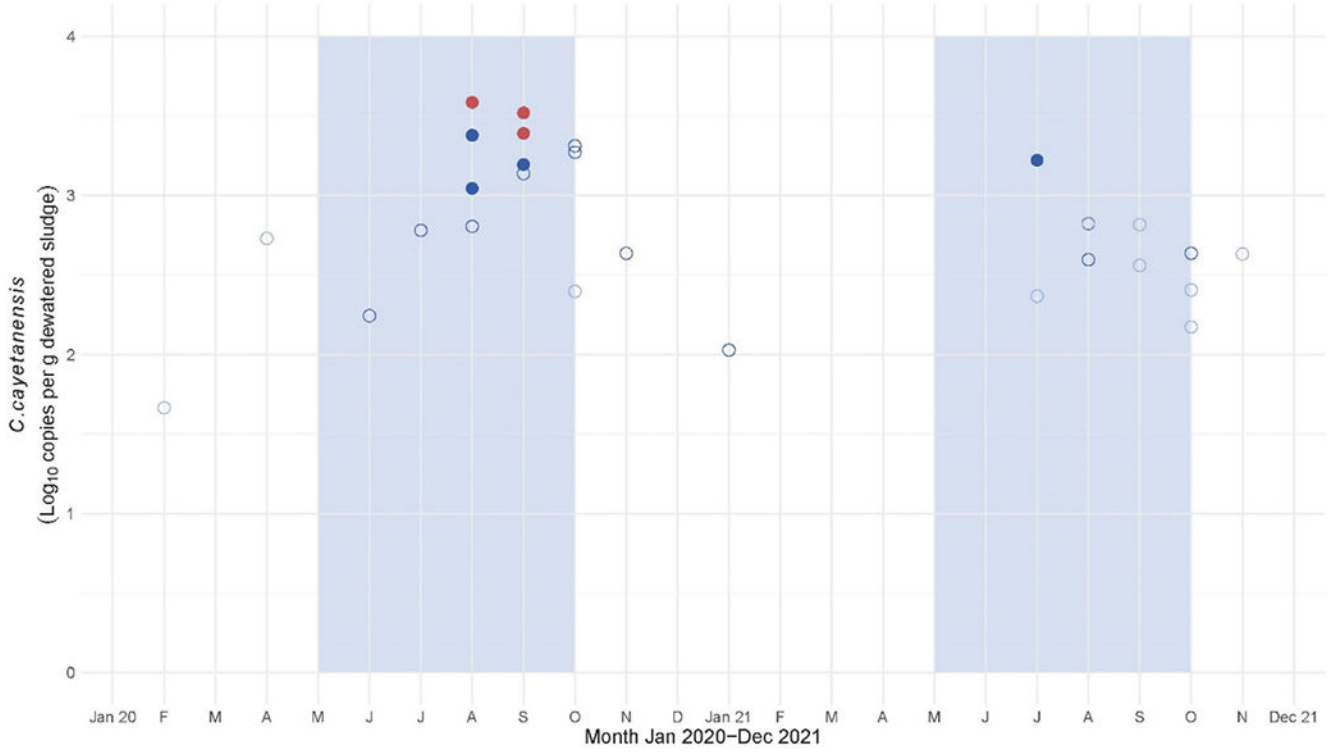

Figure 2.

Concentrations of the *C. cayetanensis* 18S rRNA gene target in municipal sludge samples (either REC or RAS) collected from January 2020 through December 2021. Samples with sequence-confirmed detections are plotted as solid circles and detections by qPCR only are plotted as open circles. Red circles represent qPCR detections by Definitions 1, 2, and 3. Dark blue circles represent qPCR detections by Definitions 1 and 2. Light blue circles represent qPCR detections by Definition 2 only. Definition 1: at least duplicate amplification with $C_q < 40$; Definition 2: at least one replicate amplification with $C_q < 40$; Definition 3: 35 gene target copies per reaction ($C_q \leq 33$). Blue shading depicts the months when domestically acquired cyclosporiasis cases are higher in the U.S. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

The number of detections, detection rates (%), and median concentrations (range) of total coliforms, *E. coli*, HF183, and crAssphage for irrigation pond ($N = 27$) and packing house dump tank ($N = 23$) samples

Grower	Location	Total coliforms		*E. coli*		HF183	crAssphage
		n (%)	MPN/100 mL	*n* (%)	MPN/100 mL	*n* (%)	*n* (%)
A	Pond 1	27 (100)	2,420 (127–2,420)	25 (93)	7.4 (1–2,420)	12 (44)	3 (11)
A	Pond 2	27 (100)	2,420 (461–2,420)	18 (67)	9.8 (1–276)	7 (26)	1 (3.7)
A	Pond 3	27 (100)	2,420 (68–2,420)	21 (78)	32 (1–1,414)	14 (52)	6 (22)
A	Pond 4	27 (100)	2,420 (1,733–2,420)	25 (93)	7.5 (1–2,420)	26 (96)	0 (0)
A	Packing house	18 (78)	2,420 (1–2,420)	4 (17)	7.2 (1–15)	0 (0)	0 (0)
B	Pond 1	27 (100)	2,420 (160–2,420)	20 (74)	6.2 (1–2,420)	1 (3.7)	0 (0)
B	Pond 2[a]	28 (100)	2,420 (48–2,420)	26 (93)	13.3 (1–727)	2 (7.1)	0 (0)
B	Pond 3	27 (100)	2,420 (152–2,420)	23 (85)	13.2 (1–2,420)	2 (7.4)	0 (0)
B	Pond 4	27 (100)	2,420 (187–2,420)	27 (100)	63.3 (2–1,986)	7 (26)	4 (15)
B	Packing house	21 (91)	2,420 (2–2,420)	5 (22)	1 (1–4)	2 (8.6)	0 (0)

[a] $N = 28$ for Pond B-2.

Table 2

The number of qPCR detections of the *C. cayetanensis* 18S rRNA gene target and detection rates (%) for irrigation pond samples ($N = 27$ per pond) and packing house dump tank samples ($N = 23$)

Grower	Location	Definition 1[a]	Definition 2[b]	Definition 3[c]
A	Pond 1	4 (15)	12 (44)	0 (0)
A	Pond 2	2 (7.4)	7 (26)	0 (0)
A	Pond 3	17 (63)	20 (74)	0 (0)
A	Pond 4	8 (30)	9 (33)	0 (0)
A	Packing house	0 (0)	0 (0)	0 (0)
B	Pond 1	5 (19)	10 (37)	0 (0)
B	Pond 2[d]	5 (18)	10 (36)	0 (0)
B	Pond 3	10 (37)	12 (44)	0 (0)
B	Pond 4	8 (30)	11 (41)	0 (0)
B	Packing house	1 (4.3)	1 (4.3)	0 (0)

[a] Target was considered detected by qPCR if two replicates had $C_q < 40$.

[b] Target was considered detected by qPCR if any replicate had $C_q < 40$.

[c] Target was considered detected by qPCR if any replicate had $C_q < 33$.

[d] $N = 28$ for Pond B-2.