CENTER for PRODUCE SAFETY

CPS 2019 RFP
FINAL PROJECT REPORT

Project Title

Sources and prevalence of *Cyclospora cayetanensis* in Southeastern U.S. water sources and growing environments

Project Period

January 1, 2020 – December 31, 2021 (extended to April 30, 2022)

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Objectives

1. Surveillance of Cyclospora cayetanensis *prevalence in irrigation water ponds and on produce (via spent packinghouse water as a proxy).*

2. Surveillance of C. cayetanensis *prevalence in on-farm portable toilets and municipal wastewater influents.*

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FINAL REPORT

Abstract

The increased frequency of produce-associated cyclosporiasis outbreaks highlights *Cyclospora cayetanensis* as an important emerging foodborne pathogen. Most of the produce implicated in cyclosporiasis outbreaks has been grown in areas where the disease is endemic. However, the 2018 and 2020 cyclosporiasis outbreaks associated with produce grown in the United States, including the reported detection *of C. cayetanensis* on US-grown produce in 2018, highlight the need for a better understanding of *C. cayetanensis* prevalence in US agricultural environments and of the risks of oocyst contamination to domestically grown produce. The objectives of this study were to assess *C. cayetanensis* prevalence in irrigation water and harvested produce (via spent packinghouse dump tank water as a proxy) in the Southeastern Coastal Plain growing region in Georgia over a 2-year period. Fecal indicator bacteria, *Escherichia coli*, and the human-specific fecal molecular markers, *Bacteroides* HF183 and crAssphage, were also assessed to evaluate the overall and human-specific fecal contamination, respectively, in the growing environment. Additionally, human sewage samples from municipal wastewater sludge and on-farm portable toilets were analyzed to assess the prevalence of *C. cayetanensis* shedding in the region generally and by the farm worker community, respectively.

Twenty-seven percent of irrigation pond water samples and <1% of dump tank water samples were positive for *C. cayetanensis* by real-time quantitative polymerase chain reaction (qPCR) testing. Additional sequencing-based characterization of a subset of the qPCR positive samples was unable to verify the qPCR results, suggesting false-positive amplification due to cross-reaction. HF183 was detected in 33% of pond water samples and 4% of dump tank water samples. crAssphage was detected in 6% of pond water samples and no dump tank samples. The presence of human-specific fecal markers in the irrigation ponds indicates human sewage in the ponds, likely through groundwater intrusion following rainfall events, as demonstrated in these ponds in a previous CPS-funded project. The *C. cayetanensis* 18S marker was detected in 24% of municipal wastewater sludge samples. Additional molecular characterization of the positive sludge samples confirmed the detection of *C. cayetanensis* in 7 samples and the remaining samples were considered suspected false-positive due to cross-reaction or undetermined.

Data from this study demonstrate the shedding of *C. cayetanensis* oocysts by the growing region community periodically in 2020 and 2021. However, there were no confirmed detections of *C. cayetanensis* in the irrigation ponds or spent dump tank water, despite the detection of human-specific fecal markers throughout the study period. Additional data is needed to assess the utility of the *C. cayetanensis* molecular testing tools for use in environmental matrices.

Background

Produce-associated cyclosporiasis outbreaks have become more frequent in the last decade, but most cases have been linked to produce grown in Central or South America, where the disease is endemic (1–3). Beginning in 1997, *C. cayetanensis* was documented on produce and in wastewater in Peru, on produce in Costa Rica, and in rivers impacted by raw sewage outfalls in Guatemala, providing the first indications that contaminated wastewater may serve as a transmission vehicle of the oocysts to the environment and produce (4–7). A 2013 survey of ready-to-eat produce from Canadian retailers identified *C. cayetanensis* on 1.7% of tested North American–grown produce (US and Canada), suggesting domestic contamination sources (8). In 2018, a multistate leafy greens–associated cyclosporiasis outbreak was linked to domestically grown produce (9). Additionally, during a routine sampling assignment that year, FDA reported

detecting *C. cayetanensis* from domestically grown herbs (10). During a 2020 investigation following an outbreak linked to bagged salad mix, FDA reported detection of *Cyclospora* in a water management canal near the farm (11). Despite this recent implication of domestic produce contamination, little is known regarding the potential *C. cayetanensis* geographic distribution domestically, as well as sources of contamination, extent of environmental contamination, or relative contribution of contamination vehicles to produce (i.e., water, soil, contact).

Currently, there is a lack of knowledge on the presence of *C. cayetanensis* in the environment in the United States, particularly within agricultural regions (*e.g.,* irrigation water, on produce, in wastewater). Potential risk factors for domestic *C. cayetanensis* contamination include a shedding human population and a mode of contamination to the environment. Because *C. cayetanensis* is considered a strictly human pathogen, introduction into the environment must occur from a shedding population. In Georgia, transient farm labor is primarily used only during harvesting periods. Most of the farm workers harvesting produce migrate during harvest seasons from *Cyclospora*-endemic regions, representing a potential shedding population of *C. cayetanensis* oocysts in the domestic growing region. One potential mode of produce contamination in the agricultural environment is runoff of human sewage into irrigation water sources during rain events. In a previous CPS-funded study, we found evidence of human sewage contamination in irrigation ponds in the area that were associated with rainfall events. The goals of this study were to assess *C. cayetanensis* occurrence in irrigation water, harvested produce (via spent packinghouse water as a proxy), on-farm portable toilets, and municipal wastewater influents in the Southeastern Coastal Plain (SECP) growing region in Georgia.

Two Georgia vegetable growers participated in the study. Each grower and their farms represented the standard growing practices in the state and utilized irrigation water sources were representative of the SECP and the various watersheds within it. Each grower produces a variety of fruits and vegetables, including cantaloupe, cabbage, broccoli, greens, peppers, squash, zucchini, eggplant and tomatoes. Most of the irrigation water in this region comes from well-water or surface-water fed holding ponds, which is not unlike irrigation water sources in other agricultural growing regions in the US. Therefore, any risks associated with this type of irrigation water source may be extrapolated to other regions that use surface or surface-stored groundwater for irrigation. Georgia's well-defined seasons provide two vegetable growing seasons, one in the spring and one in the fall, which allowed for data collection during multiple cycles of fallow-growing-harvest seasons. Any risks associated with crops irrigated in a particular way are applicable to those same crops and irrigation methods in other growing regions. As such, the SECP growing region provided an opportunity to conduct a study from which findings and interpretations could be widely applicable to other growing regions where similar produce types are irrigated by well-water fed holding ponds or surface water.

Research Methods and Results

Methods – Objective 1

Sample Collection and Processing

Field sampling was conducted on the two participating grower farms (A and B) from September 2020 through December 2021. Pond water samples were collected from 4 surface-fed holding ponds used for crop irrigation on each farm, for a total of 8 study ponds. In 2020, samples were collected once per month during fallow and growing periods and twice per month during harvest periods. In 2021, the sample frequency was increased during growing periods to twice per month. Fifty liters of water filtered by dead-end ultrafiltration (DEUF) and 250-mL grab samples were collected from each site (12–14). Spent water samples from dump tank flumes were

collected from each grower's packinghouse weekly during harvest periods. For water samples in which the water appeared sufficiently clear to the field team, 20 L was filtered on-site by DEUF. For the majority of samples, the turbidity was too high for DEUF and 20 L was collected and shipped to CDC for concentration. Water temperature, pH, dissolved oxygen, turbidity, oxidation reduction potential, and conductivity were measured using a ProDSS Multiparameter Digital Water Quality Meter (YSI, Yellow Springs, Ohio). Rainfall gauges were in place adjacent to each irrigation pond for the duration of the project. Rainfall data was downloaded from Rain101A Rainfall Data Loggers (Madgetech, Warner, NH) and WatchDog 1120 Data Logging Rain Gauges (Spectrum Technologies, Inc., Aurora, IL). Rainfall data were lost or unable to be collected intermittently due to environmental stress and equipment failures. Because all Grower A ponds were within a 3-mile radius, rainfall data from all 4 of the gauges that were working at various points during the year were merged (or averaged if data from both were available) to create one rainfall dataset for Grower A. Rain gauge malfunctions also occurred at ponds 3 and 4 from Grower B. Therefore, Grower B ponds 3 and 4 rain data were merged (or averaged if data from both were available), as these ponds are within 1 mile of each other. After collection, water samples were stored with cold reusable freezer packs in coolers during sampling and transport. Grab water samples were analyzed the day of collection at the University of Georgia (2021) or Auburn University (2022) laboratory. Ultrafilters were shipped overnight to the Centers for Disease Control and Prevention (CDC) laboratories in Atlanta, GA, for further processing.

Ultrafilters were backflushed as previously described (12,13). Dump tank water samples were concentrated by continuous flow centrifugation (CFC) as previously described (14). Ultrafilter concentrates and CFC eluates were further concentrated by centrifugation at 4000 x *g* for 15 minutes. The resulting pelleted concentrates were stored at -20 °C until nucleic acid extraction. Dump tank concentrates were frozen at -20 °C for at least one month before nucleic acid extraction, to ensure all associated produce was out of market by the time samples were analyzed. Nucleic acid from pond and dump tank water concentrates was extracted using the Qiagen AllPrep® PowerViral® DNA/RNA Kit (Qiagen, Hilden, Germany) with the following modifications. Dithiothreitol (DTT) (Fisher, Fair Lawn, NJ) was used in the place of an equal concentration of β-mercaptoethanol in the initial lysing buffer as recommended by the manufacturer. Bead beating was performed on a FastPrep-24™ Classic bead beating grinder and lysis system (MP biomedicals, Irvine, CA) at a speed of 6 m/s for 60 seconds. The final elution was completed with 100 µL of TE buffer. An extraction blank was included with each batch of extracted samples (n=11). DNA was immediately subjected to molecular testing.

Sample Analysis

Generic *E. coli* and total coliforms were enumerated from 100 mL of the grab samples by the IDEXX Quanti-Tray 2000® method using Colilert-18 media (IDEXX, Westbrook, ME). Real-time quantitative polymerase chain reaction (qPCR) was performed for the detection of *C. cayetanensis*, *Bacteroides* HF183 (HF183) and crAssphage in pond and dump tank water samples (15–17). HF183 and crAssphage are human-specific markers of fecal contamination and were included in the study to evaluate the presence of human fecal contamination to the irrigation ponds and produce (via spent dump tank water). For *C. cayetanensis* qPCR, samples were analyzed in triplicate using 5-µL template in 50-µL reaction volumes. Bovine serum albumin (0.5 mg/mL) and gp32 (25 µg/mL) were added to the reaction mixture to improve amplification efficiency for samples with inhibition. For HF183 and crAssphage qPCR, samples were analyzed in triplicate using 2-µL template in 25-µL reaction volumes. Primer and probe concentrations, internal amplification control quantities, and thermal cycling temperatures was conducted were followed according to the published methods. qPCR amplification was performed in a 7500 Real-Time PCR System with software version 2.3 using 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. Three no-template controls were included with each instrument run.

A sample was considered positive for *C. cayetanensis* and positive for HF183 and crAssphage if amplification was observed for at least 2 of 3 replicates, with each having a crossing threshold (C_q) value <40. *C. cayetanensis*, HF183, and crAssphage were enumerated using standard curves consisting of six 10-fold serial dilutions of a synthetic standard analyzed in triplicate during each instrument run. Gene copy number per reaction was calculated by inputting the average quantification cycle (C_q) of the positive replicates into a pooled standard curve equation. This value was multiplied by the proportions of the original sample that were concentrated, extracted, and tested by qPCR to obtain $\log_{10}$ gene copies detected per 100 mL of original sample. The theoretical detection limit for each sample was calculated in this same manner using a C_q value of 39.99, as it was the highest C_q value possible for a replicate to be scored positive.

After *C. cayetanensis* qPCR, DNA from samples with at least one replicate with a C_q value ≤37 was submitted to the CDC Parasitic Disease Branch for further characterization. Each sample was submitted to eight qPCR assays included in a targeted amplicon sequencing *Cyclospora* typing panel (18). Six assays target markers from the *C. cayetanensis* nuclear genome, and two assays target markers from the mitochondrial genome. The resulting amplicons from each assay were then 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. For samples with *C. cayetanensis* haplotypes, the inclusion criteria for genotyping requires either a) amplification of at least 5 of the 8 markers or b) amplification of 4 markers, including both mitochondrial targets. For samples in which these criteria were met, a pairwise distance matrix was generated and samples were clustered based on genetic relatedness. Samples with haplotypes not matching *C. cayetanensis* were BLAST searched (19) in order to identify matching sequences or sequences from the most closely related organism. A phylogenetic tree was created, including samples that had at least 500 reads mapping to the mitochondrial marker that was most often sequenced; this tree included various taxa that share similarities in this marker. All of the sequences were aligned using Muscle v3.8.425 in geneious (20,21) with default parameters. The resulting alignment was analyzed using the phangorn package in R (22) to generate a neighbor-joining tree with the Jukes-Cantor substitution model, with 100 bootstraps (23).

A workflow algorithm was created to generate a result interpretation for *C. cayetanensis* -positive samples (**Figure 1**). Samples that met the criteria for further characterization and typing were scored based on amplification of the genotyping panel assays and alignment with the resulting amplicons to known *C. cayetanensis* haplotypes. A sample with amplified haplotypes matching *C. cayetanensis* haplotypes was verified as a confirmed detection of *C. cayetanensis*. A sample with haplotypes not matching *C. cayetanensis* haplotypes was scored as a suspected cross-reaction. A sample that did not produce any amplification in the typing assays was scored as undetermined and the qPCR detection may be a result of cross-reaction or detection of *C. cayetanensis* genomic material below the detection limit of the typing methodology. Finally, a sample that was not able to be further characterized due to high C_q value was also scored as undetermined.

Statistical Analysis

All logistic regressions were performed in R version 4.1.1 (24) using lme4 package (25). Figures were created using ggplot2 package (26). Nested mixed effects logistic regression models were run to evaluate the relationship between human fecal marker and *C. cayetanensis* 18S marker presence in pond water. *C. cayetanensis* 18S marker detection was the outcome variable, and

HF183 detection or HF183 concentration were the predictor, with the irrigation pond nested within the grower as a random effect and whether a sample was collected during time periods of the year when domestically acquired cyclosporiasis cases are increased (May through September) as fixed effect covariate (27). A nested mixed effects logistic regression model was run to evaluate the relationship between microbial contamination and rainfall by setting HF183 or *C. cayetanensis* 18S marker detection as the response variable, and cumulative rainfall over the previous 7 days or rainfall incidence in antecedent 48 hours greater than 0.5 inches as the predictor, with the irrigation pond nested within the grower as a random effect.

Results – Objective 1

Irrigation pond sampling occurred over a 16-month period from September 2020 through December 2021 and captured two fall and one spring harvest periods. A total of 27 sampling events allowed for collection of 216 samples from the eight irrigation ponds. One additional pond sample was collected from Grower B during an ad-hoc sampling event, for a total of 217 pond water samples. Measured water quality parameters from the study ponds are presented in **Table 1**. Total coliforms and *E. coli* were routinely detected in the pond water samples throughout the study period (85% prevalence), with median *E. coli* concentrations ranging from 6–63 MPN/100 mL (**Table 2**, **Figure 3**). The *C. cayetanensis* 18S rRNA target was detected in 59 (27%) of the pond water samples (**Table 3**, **Figure 4**). HF183 was detected in 71 (33%) of the pond water samples. crAssphage was detected in 14 (6%) of the pond water samples (**Table 3**, **Figure 4**). The *C. cayetanensis* 18S rRNA target, HF183, and crAssphage were co-detected in 2 samples. The *C. cayetanensis* 18S rRNA target and HF183 were co-detected in 20 samples. HF183 and crAssphage were co-detected in 8 samples.

The presence of the *C. cayetanensis* 18S marker was not significantly associated with HF183 detection [adjusted odds ratio (AOR) = 1.06, 95% confidence interval (95%CI): 0.44–2.36] or levels (AOR = 1.21, 95%CI: 0.70–1.99) in irrigation water. This suggests that human fecal presence or increasing concentrations of human feces in the pond water did not increase the likelihood of detecting the *C. cayetanensis* 18S marker. Rainfall exceeding 0.5 inches in the preceding 48 hours was found to be significantly associated with HF183 detection (AOR = 3.29, 95%CI:1.9–19.25) and the *C. cayetanensis* 18S marker (AOR = 4.03, 95%CI: 1.44–10.87) in irrigation ponds. This means that if it rained greater than 0.5 inch in the previous 48 hours within 3 miles of an irrigation pond, the odds of detecting HF183 and the *C. cayetanensis* 18S marker increased by 3.3 and 4.0-fold, respectively. Microbial contaminants washing into the ponds after rain are likely being detected up by the cayetanensis-specific qPCR assay, but since the human and 18S detections were not associated with one another, the run-off microbial contaminants being detected by the 18S qPCR assay are not of human fecal origin, suggesting likely cross-reaction with a closely related environmental coccidian protozoa. Cumulative rainfall in the previous 7 days was also significantly associated with detection of HF183 (AOR= 1.76, 95%CI: 1.29–2.45) but was not significantly associated with *C. cayetanensis* 18S marker detection (AOR = 1.21, 95%CI: 0.91–1.58) in irrigation water. This means that with every inch increase in the previous 7-day cumulative rainfall, the odds of detecting HF183 in the water increased by 1.75-fold, but this relationship was not seen for the *C. cayetanensis* 18S marker. Human fecal contamination was also associated with increases in rainfall over the previous week, suggesting that human fecal contamination may occur via groundwater intrusion following ground saturation. This route of contamination is less likely for larger parasites such as *Cyclospora*.

A total of 46 dump tank water samples were collected during the study period. Measured water quality parameters from the dump tanks are presented in **Table 4**. Each grower used municipal tap water and chlorinated the dump tank flumes during processing. Chlorine residuals

of the collected water samples could not be measured due to the interfering effects of organic matter. Instead, oxidation-reduction potential (ORP) was measured to assess the oxidizing capacity of the dump tank water at the time of sample collection, and median ORP was 518 mV and 613 mV for Grower A and B, respectively, which is below the industry recommended level of 700 mV for controlling microbial cross-contamination of product within the tanks (28). Culturable total coliforms were routinely detected, and *E. coli* was infrequently detected in the dump tank water samples (**Table 4**, **Figure 5**). HF183 was detected in 2 (4%) dump tank samples and crAssphage was not detected in any dump tank sample (**Table 3**). The *C. cayetanensis* 18S rRNA target was detected in 1 (2%) dump tank sample. However, it should be noted that *C. cayetanensis* oocysts are tolerant to chlorine, so chlorination of dump tank flumes would not be expected to inactivate the oocysts or prevent cross-contamination.

Of pond samples in which the *C. cayetanensis* 18S rRNA target was detected, 45 samples had at least one replicate with a C_q value ≤37 and therefore were submitted for additional characterization and genotyping. Eight additional samples that had amplifications with C_q values >37 were also submitted during the first round of submissions. The single detection in the dump tank water did not meet inclusion criteria, with C_q values >37. For 15 samples submitted for sequencing, no amplification was observed for any of the markers. For 38 samples, only the mitochondrial 16S rRNA gene (MSR) target could be amplified. The mitochondrial haplotype sequences for these water samples did not match any known *C. cayetanensis* haplotypes, but instead only mapped to segments of the mitochondrial target region conserved amongst Apicomplexan protozoa. BLASTN searches revealed that the sequences did not match any known organism, but mitochondrial genome sequences from *Eimeria* and *Isospora* were the closest matches. These results indicate that the DNA amplified by the typing assays did not derive from *C. cayetanensis*, but likely from a closely related organism. The molecular typing results for each sample, along with the result interpretation using the workflow algorithm are presented in **Table 5**. Of the 53 pond samples that were characterized, 38 were determined to be suspected cross-reactions and 15 were undetermined. The remaining 12 qPCR positive samples had C_q values >37, so were not further characterized and therefore were assigned as undetermined for *C. cayetanensis* presence.

Fifty-nine of the 217 irrigation pond water samples were *C. cayetanensis* detections by qPCR, but following sequenced-based typing, no samples were confirmed as *C. cayetanensis*. The sequencing results for these samples suggest at least a portion of the detections were false-positive qPCR results due to cross-reactions with closely related coccidian organisms within the sample, such as *Eimeria* and *Isospora*. *Cyclospora*, *Eimeria,* and *Isospora* belong to the Eimeriidae family and are closely related to one another. There are almost 2,000 known species of *Eimeria* and almost 300 known species of *Isospora*, all of which can infect mammals, birds, reptiles, and fish (29,30). Previous researchers have also detected *C. cayetanensis* in environmental samples by qPCR targeting 18S rRNA that could not be confirmed to be *C. cayetanensis* by subsequent sequencing (31,32).

It remains unknown whether the samples with suspected cross-reactions had qPCR amplification due to closely related Eimeriidae species within the samples outcompeting any *C. cayetanensis* that may have also been present in the sample, or whether *C. cayetanensis* was detected by qPCR but was present at levels too low or in a matrix too complex to be detectable by the subsequent typing assays. There is a scarcity of animal *Eimeria* and *Isospora* gene sequences in NCBI databases, and sequences obtained from amplification of the typing markers were not 100% matches to current organisms in the databases. Amplifications from one of the mitochondrial markers used in the genotyping method were mapped on a neighbor joining tree (Figure 7), demonstrating that the sequences obtained from the pond samples

belonged to coccidia outside the well supported clades occupied by clinical isolated of *C. cayetanensis*. If the criteria outlined in BAM Chapter 19C had been used to determine qPCR detections, which scores any sample with any at least one of three qPCR replicates below a C_q value of 40 as positive, an additional 32 samples would have been detections, exacerbating the risk of false-positive *C. cayetanensis* determinations. Because *Cyclospora* is so closely related to animal coccidian parasites that are naturally present in the environment, more research is needed to understand whether current methodologies are specific enough for research and regulatory purposes. Moreover, additional molecular, microscopic, or culture methods will likely be needed for confirmation of detection of *C. cayetanensis* in environmental samples.

Methods – Objective 2

Sample Collection and Processing

Municipal wastewater sludge was collected twice monthly from the local wastewater treatment plant in Tifton, Georgia from January of 2020 through December 2021. One-liter grab samples were collected from two locations in the processing system, the sludge from the thickener (REC) used for land application and the return activated sludge (RAS) from the aeration basin. From November 2020 until July 2021, only the RAS sample was collected while routine maintenance was performed on the thickener system. 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 hour. After cooling, ~500 mL was concentrated by centrifugation at 4000 x *g* for 15 minutes, and the supernatant was removed from the sludge concentrates. Nucleic acid was extracted using the Qiagen AllPrep® PowerViral® DNA/RNA Kit using the same procedure as for water samples. DNA was stored at -20 °C until molecular testing.

Portable toilet samples were collected every week during harvest seasons, as available. Samples were collected by inserting a SteriWare ViscoThief cream sampler (Sampling Systems, Coleshill, UK). Approximately 200 mL of portable toilet material was drawn into sampler and then ejected 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 4000 x *g* for 15 minutes. The supernatant was removed, and the sample concentrate was stored at 4 °C until further processing. Sample concentrates that exceeded the 10-mL mark of the conical centrifuge tube 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 minute. The eluate was removed 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 4000 x *g* for 15 minutes. The supernatant was removed, and the sample concentrates were stored at -20 °C until further processing. The nucleic acid was extracted from the frozen concentrate using the Qiagen DNeasy® PowerMax® Soil Kit. Finally, the DNA extracts were further concentrated by the ethanol precipitation method described in the PowerMax® Soil Handbook and resuspended in 0.1 mL TE buffer. DNA was immediately subjected to molecular testing for *C. cayetanensis* following the 18S rRNA qPCR method described for water samples.

Sample Analysis

Real-time qPCR was performed for the detection of *C. cayetanensis* in wastewater sludge and portable toilet samples in the same manner as for water samples. *Cyclospora* 18S rRNA qPCR-positive samples were further characterized following the same methods for water samples.

Results – Objective 2

Forty-six RAS sludge and 30 REC sludge samples were collected over the study period. The *C. cayetanensis* 18S rRNA target was detected in 9 (20%) of RAS samples and 9 (30%) of REC samples (**Figure 6**). All qPCR-positive samples were submitted for additional characterization and genotyping, including eight additional RAS samples and three additional REC samples for which only one replicate demonstrated amplification with a C_q value ≤ 37. Of these 26 submissions, no amplification was observed for 18 samples. A summary of the typing results for the nine samples with amplification for at least one typing marker is shown in **Table 6**. One sample amplified 5 of the 8 typing markers, and all amplified haplotypes matched *C. cayetanensis* haplotypes from clinical specimens. This sample met the inclusion criteria for genotyping and it belonged to a genetic cluster that includes clinical specimens from 2018–2021. Six samples produced amplification in 1 to 3 of the typing targets, and all the amplified haplotypes matched *C. cayetanensis* haplotypes from clinical specimens. However, the number of amplifications from each sample were too few to meet the criteria for genotyping. 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 mitochondrial target (**Figure 7**).

Portable toilets were not found every week during the three 2-month harvest seasons covered during this study. A total of 40 portable toilets were collected over the study period (11 from Grower A and 29 from Grower B). Upon initial concentration, 3 portable toilet samples from Grower B did not have any pellet and were therefore discarded, resulting in a total of 37 sample for further analysis. The *C. cayetanensis* 18S rRNA target was not detected by our protocol of detection calling. Therefore, there were no *C. cayetanensis* detections in portable toilet samples. By the protocol outlined in the FDA BAM 19B and 19C methods there was one sample that had 1/3 of the triplicate reactions with a detection. This sample has been submitted for sequencing.

Outcomes and Accomplishments

- This study was the result of a successful partnership between Auburn University and CDC and could not have been accomplished without the trust and collaboration of the two participating growers. This joint effort allowed us to collect samples and information from produce farms to generate meaningful data for the produce industry and other food safety researchers.
- The DEUF method was used successfully in the field by the Auburn field team after training by the CDC team. The continuous flow centrifugation (CFC) method was used successfully for spent dump tank water that was too turbid for ultrafiltration.
- The use of the *C. cayetanensis* typing method developed by CDC's Parasitic Disease Branch aided in determining likely false-positive results of our study samples. The use of the typing method on environmental samples revealed the limitations of a single PCR result. Though the typing method also has limitations for environmental samples, it is an established method that can use to help confirm environmental results.
- The use of the human-specific fecal markers, HF183 and crAssphage, allowed us to assess human sewage contamination of the growing environment and its associated risks even in the absence of *C. cayetanensis* detections. These data demonstrated that meaningful findings and recommendations for prevention of *C. cayetanensis* contamination can generated without the need for detection of the pathogen itself.
- Municipal wastewater surveillance proved to be a valuable way to assess domestic shedding of *C. cayetanensis*. The results of this study verified that wastewater surveillance can be used to determine if, and when, US populations are shedding *C. cayetanensis*.

Summary of Findings and Recommendations

- There were no confirmed detections of *C. cayetanensis* in irrigation pond water or on produce (via spent dump tank water as a proxy). All positive qPCR detections were either undetermined or suspected cross-reactions from closely related coccidia such as *Eimeria* or *Isospora*. Moreover, human fecal contamination in the ponds was not associated with 18S rRNA *C. cayetanensis* qPCR assay detection.
- Human-specific fecal contamination was detected in irrigation pond water, suggesting that produce grown in Southeastern Georgia may be at risk of contamination by human fecal pathogens. Produce growers should be aware of sources of human sewage runoff or septic system leaching in their watersheds, especially after heavy or prolonged rainfall.
- Consistent with the results of previous CPS-funded research, the human fecal marker was more likely to be detected in irrigation ponds after rain events (>0.5 inch in the previous 48 h and previous 7-day total rainfall). Produce growers should continue to use a holistic approach for risk management by monitoring the factors and events that may impact their watersheds, such as heavy rain, and create plans for mitigation procedures or alternative water use practices.
- The results of this study demonstrated that environmental samples should be tested by multiple methods or using multiple detection targets to definitively confirm detection of *C. cayetanensis* and help to avoid false-positive results. The testing methods used should be amenable to environmental samples.
- Often pathogens are present at low concentrations in the environment. There are also often high levels of background organisms, and there are particulates and compounds in environmental samples that may impact detection efficacy. Research is needed to determine the types and prevalence of cross-reactive species in the environment to improve specificity and sensitivity of methods for *Cyclospora* detection in environmental samples.
- *C. cayetanensis* was detected in municipal wastewater influents, demonstrating community shedding of the parasite in Southeastern Georgia. Wastewater surveillance may be useful for understanding domestic shedding of *C. cayetanensis* in the United States.

APPENDICES

Publications and Presentations

Presentations:

Hofstetter, J., *Sources and prevalence of* Cyclospora cayetanensis *in Southeastern U.S. water sources and growing environments*. WDPB All Hands Meeting. March 3, 2020. Atlanta, GA.

Hofstetter, J., Kahler, A., Peterson, A., Richins, Barratt, J., C., Qvarnstrom, Y., Mattioli, M. *Leveraging Partnerships to Evaluate Molecular Methods for* Cyclospora cayetanensis *Detection in Irrigation Water in the United States*. Laboratory Science Symposium. January 26, 2022. Virtual.

Hofstetter, J., Kahler, A., Peterson, A., Richins, T., Jacobson, D., Barratt, J., da Silva A.L.B.R., Rodrigues, C., Qvarnstrom, Y., Mattioli, M. *Evaluation of Prevalence and Methods of Detection of* Cyclospora cayetanensis *in Irrigation Water in the United States*. International Association for Food Protection. August 2022. Pittsburgh, PA.

Kahler, A. *Understanding the Role of Human Waste in* Cyclospora *Contamination of Produce and Strategies to Prevent Contamination in the Field*. CPS Cyclospora Forum II. September 7, 2021. Virtual.

Mattioli, M. *Sources and prevalence of* Cyclospora cayetanensis *in Southeastern US irrigation water sources and growing environments*. Poster. CPS 2020 Symposium. June 23, 2020. Virtual.

Mattioli, M. *Sources and prevalence of* Cyclospora cayetanensis *in Southeastern US irrigation water sources and growing environments*. CPS Cyclospora Forum. June 15, 2021. Virtual.

Budget Summary

At the time of submitting this report, $342,643.30 was sent on the project with a total budget of $339,903. There are no funds remaining to be spent from the project budget, and a total of $2,867.94 was provided in kind from CDC for the project completion.

Contractual	$264,365.84
Supplies and Materials	$76,763.40
Travel	$0
Total	**$341,129.24**

Figures 1–7 and Tables 1–6 (see below)

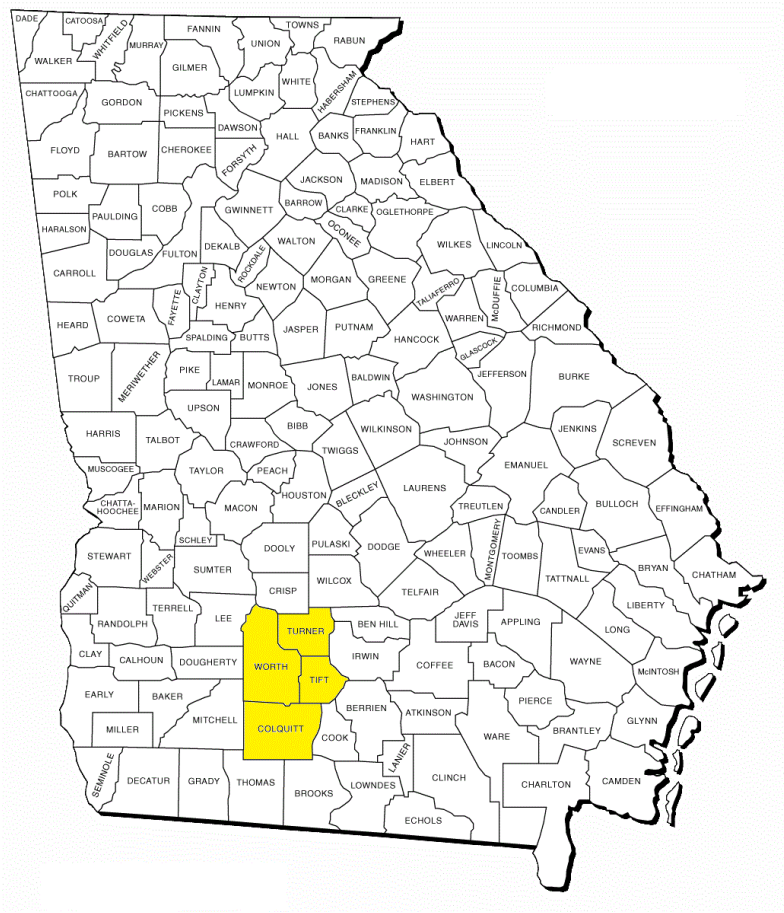


Figure 1. Counties in yellow represent the counties covered by this study.

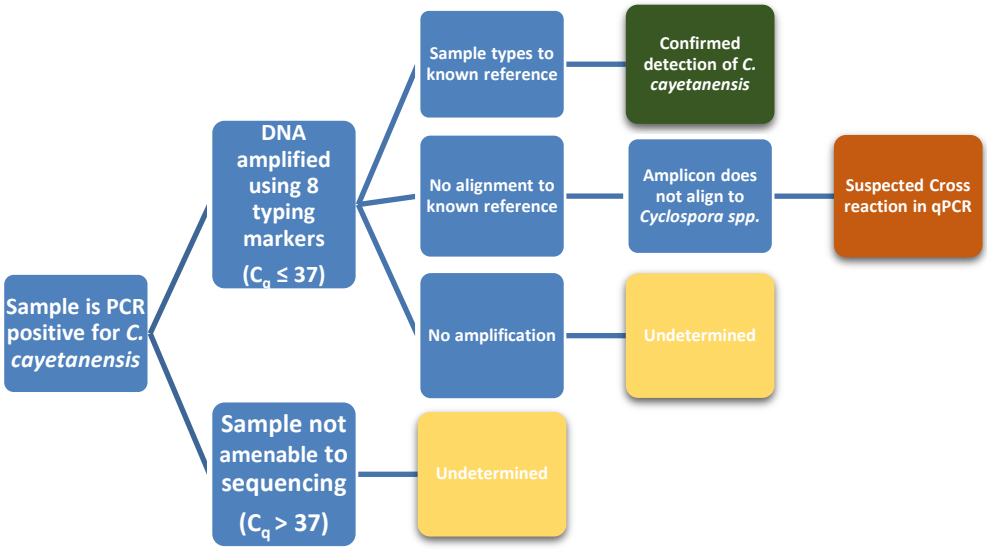


Figure 2. Workflow algorithm for result interpretation of a *C. cayetanensis* qPCR positive result.

Table 1. Median measured water quality parameters (min–max) of the study ponds (N=27).

Grower	Sample Site[a]	Temperature (°C)	Conductivity (µS/cm)	Turbidity (NTU)[a]	Dissolved Oxygen (mg/L)	pH
A	**Pond 1**	21 (13–32)	217 (107–242)	4.3 (0.29–27)	7.9 (3.2–13)	8.0 (7.1–11)
	Pond 2	23 (14–33)	238 (117–324)	8.0 (0.13–270)	8.9 (2.6–13)	8.3 (7.0–10)
	Pond 3	22 (11–31)	211 (102–311)	13 (0.86–420)	4.6 (0.66–15)	7.9 (6.6–9.6)
	Pond 4	24 (15–36)	238 (183–325)	13 (1.7–960)	8.1 (1.5–12)	8.2 (6.9–10)
B	**Pond 1**	21 (14–32)	238 (96–302)	19 (0.16–380)	11 (4.1–17)	9.1 (7.6–11)
	Pond 2[b]	21 (13–30)	118 (16–171)	4.7 (0.15–87)	8.7 (2.5–11)	8.5 (7.5–14)
	Pond 3	21 (11–29)	256 (20–670)	13 (2.3–290)	9.2 (4.2–17)	8.4 (7.0–11)
	Pond 4	21 (14–30)	246 (83–370)	11 (0.29–220)	8.3 (4.0–14)	8.3 (7.4–11)

[a] Maximum turbidity may be artificially high due to water coloration from algal blooms.
[b] N=28 for Pond B-2.

Table 2. The number of detections of total coliforms and *E.* coli (detection rate %) and median concentrations (min–max) for irrigation pond samples, N=27.

Grower	Sample Site[a]	Total Coliforms		*E. coli*	
		n (%)	MPN/100 mL	n (%)	MPN/100 mL
A	**Pond 1**	27 (100)	2420 (127–2420)	25 (93)	7.4 (1–2420)
	Pond 2	27 (100)	2420 (461–2420)	18 (67)	9.8 (1–276)
	Pond 3	27 (100)	2420 (68–2420)	21 (78)	32 (1–1414)
	Pond 4	27 (100)	2420 (1733–2420)	25 (93)	7.5 (1–2420)
B	**Pond 1**	27 (100)	2420 (160–2420)	20 (74)	6.2 (1–2420)
	Pond 2[b]	28 (100)	2420 (48–2420)	26 (93)	13.3 (1–727)
	Pond 3	27 (100)	2420 (152–2420)	23 (85)	13.2 (1–2420)
	Pond 4	27 (100)	2420 (187–2420)	27 (100)	63.3 (2–1986)

[a] Maximum turbidity may be artificially high due to water coloration from algal blooms.
[b] N=28 for Pond B-2.

Table 3. The number of real-time qPCR detections of *C. cayetanensis*, HF183, and crAssphage gene target markers and detection rate (%) for irrigation pond and packinghouse dump tank samples.

Grower	Sample Site[a]	*C. cayetanensis* (%)	HF183 (%)	crAssphage (%)
A	**Pond 1**	4 (15)	12 (44)	3 (11)
	Pond 2	2 (7.4)	7 (26)	1 (3.7)
	Pond 3	17 (63)	14 (52)	6 (22)
	Pond 4	8 (30)	26 (96)	0 (0)
	Grower A Pond Total	31 (29)	59 (55)	10 (37)
	Dump tank	0 (0)	0 (0)	0 (0)
B	**Pond 1**	5 (19)	1 (3.7)	0 (0)
	Pond 2	5 (18)	2 (7.1)	0 (0)
	Pond 3	10 (37)	2 (7.4)	0 (0)
	Pond 4	8 (30)	7 (26)	4 (15)
	Grower B Pond Total	28 (26)	12 (11)	4 (3.7)
	Dump tank	1 (4.3)	2 (8.6)	0 (0)

[a] N=27 for each pond except B-2 (N=28). N=23 for each packinghouse dump tank.

Table 4. Median measured water quality parameters (min–max) and number of detections of total coliforms and *E.* coli (detection rate %) and median concentrations (min–max) of the packinghouse dump tanks (N=23).

Parameter	Grower	
	A	B
Temperature (°C)	15 (12–21)	21 (11–24)
Conductivity (µS/cm)	382 (110–621)	358 (115–680)
Turbidity (NTU)	23 (0.11–310)	23 (1.2–190)
Dissolved Oxygen (mg/L)	9.2 (5.9–11)	8.6 (7.4–11)
pH	8.3 (7.7–10)	8.4 (8.0–9.2)
ORP (mV)	518 (145–695)	613 (248–715)
Total coliforms		
n (%)	18 (78)	21 (91)
MPN/100 mL	2420 (1–2420)	2420 (2–2420)
E. coli		
n (%)	4 (17)	5 (22)
MPN/100 mL	7.2 (1–15)	1 (1–4)

Table 5. Molecular typing results for pond water samples with amplification of the eight typing markers. All mitochondrial amplified markers typed as non-*C. cayetanensis* haplotypes and thus assigned as suspected cross-reaction. Samples with no amplification were assigned undetermined.

Grower	Sample site	No. Samples without Amplification	No. Samples Amplified	Type of amplified markers
A	**Pond 1**	1	2	MSR Mitochondrial
	Pond 2	2	2	MSR Mitochondrial
	Pond 3	4	11	MSR Mitochondrial
	Pond 4	4	2	MSR Mitochondrial
B	**Pond 1**	2	4	MSR Mitochondrial
	Pond 2	0	3	MSR Mitochondrial
	Pond 3	0	9	MSR Mitochondrial
	Pond 4	2	5	MSR Mitochondrial

Table 6. Molecular typing results for sludge samples with amplification of the eight typing markers.

Sludge	Date collected	Number and type of amplified markers in Sample	Marker haplotypes	Genotyping result	Sample result interpretation
REC	8/5/20	1 nuclear	*C. cayetanensis*	NA[a]	Confirmed *C. cayetanensis*
RAS	8/5/20	2 mitochondrial 3 nuclear	*C. cayetanensis*	Clinical *C. cayetanensis*[b]	Confirmed *C. cayetanensis*
RAS	8/18/20	1 nuclear	*C. cayetanensis*	NA	Confirmed *C. cayetanensis*
REC	9/1/20	3 nuclear	*C. cayetanensis*	NA	Confirmed *C. cayetanensis*
RAS	9/1/20	1 nuclear	*C. cayetanensis*	NA	Confirmed *C. cayetanensis*
RAS	9/14/20	1 nuclear	*C. cayetanensis*	NA	Confirmed *C. cayetanensis*
RAS	7/20/21	2 mitochondrial	*C. cayetanensis*	NA	Confirmed *C. cayetanensis*
RAS	8/10/21	1 mitochondrial	Non–*C. cayetanensis*	NA	Suspected cross-reaction

[a] NA: Sample did not meet inclusion criteria for genotyping. See Objective 2 Methods for further detail.
[b] Genetic cluster includes clinical specimens from 2018–2021.

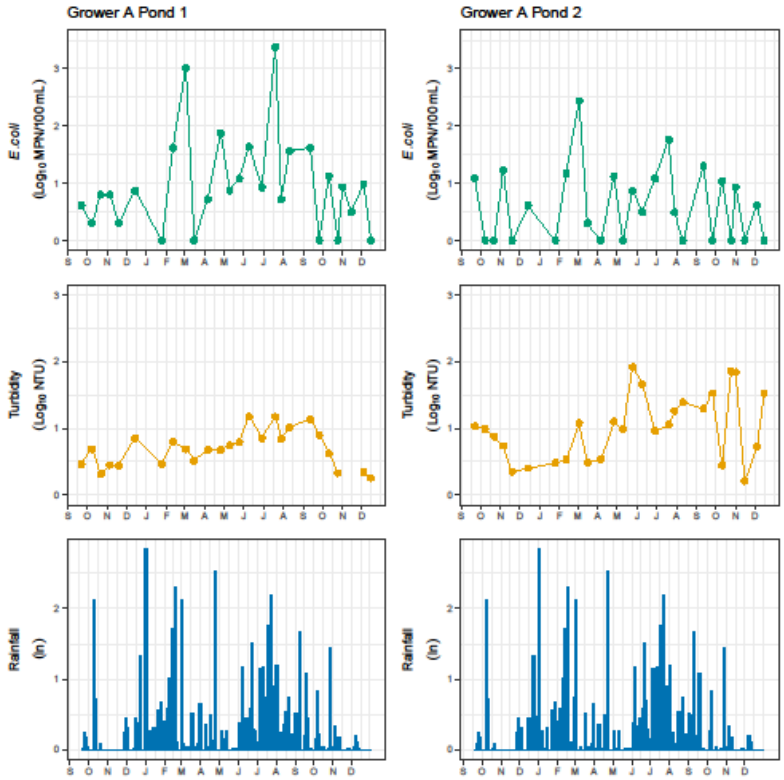


Figure 3: Grower A – Ponds 1 and 2. Log_{10} *E. coli* concentrations (green) and log_{10} turbidity levels (orange) in the irrigation pond samples collected from September 2020 through December 2021. Daily cumulative inches of rainfall from September 2020 through December 2021 are shown in blue.

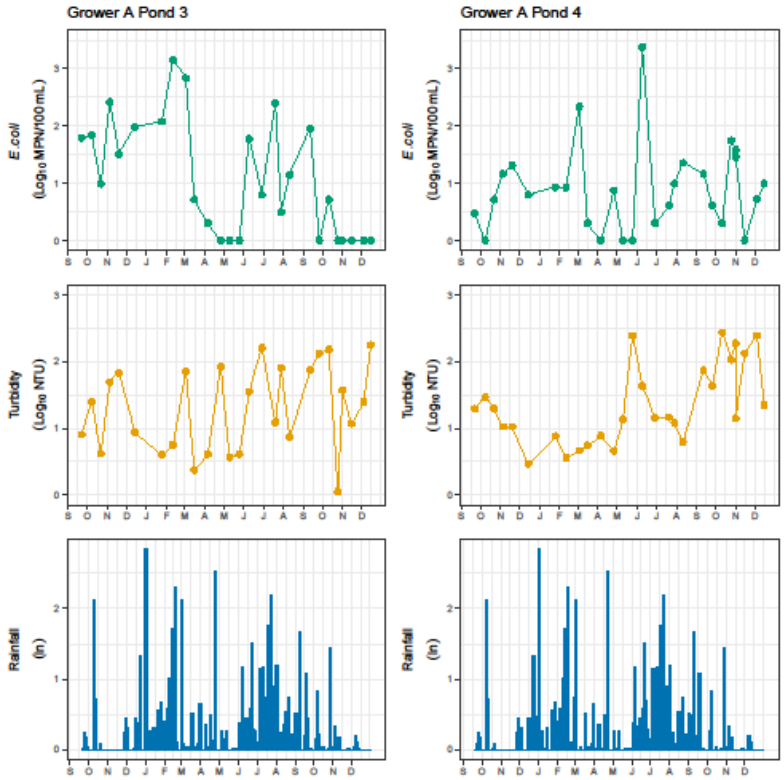


Figure 3: Grower A – Ponds 3 and 4. Log_{10} *E. coli* concentrations (green) and log_{10} turbidity levels (orange) in the irrigation pond samples collected from September 2020 through December 2021. Daily cumulative inches of rainfall from September 2020 through December 2021 are shown in blue.

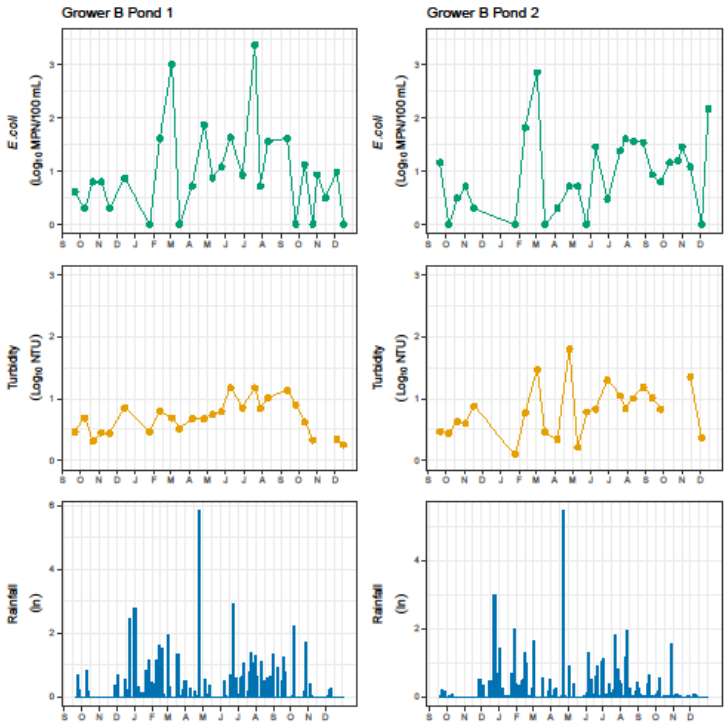
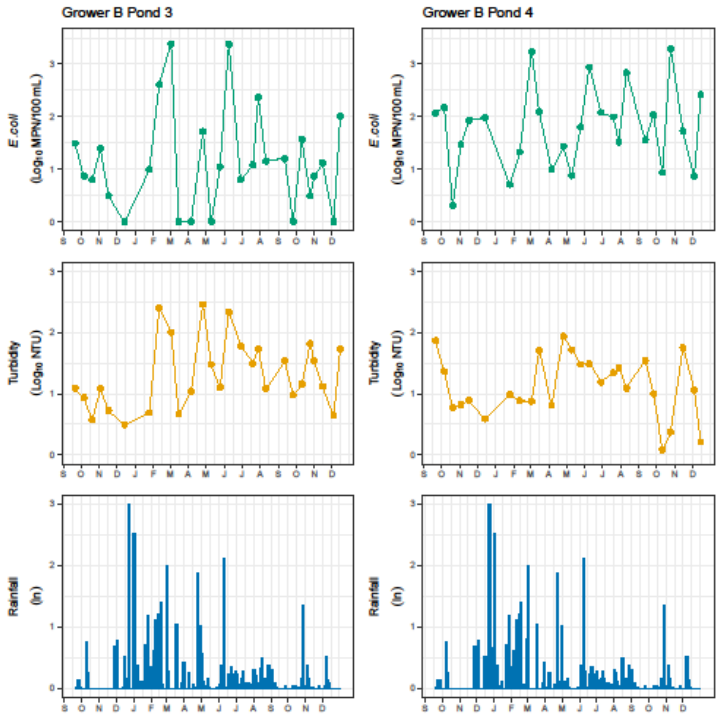


Figure 3: Grower B – Ponds 1 and 2. Log_{10} *E. coli* concentrations (green) and log_{10} turbidity levels (orange) in the irrigation pond samples collected from September 2020 through December 2021. Daily cumulative inches of rainfall from September 2020 through December 2021 are shown in blue.



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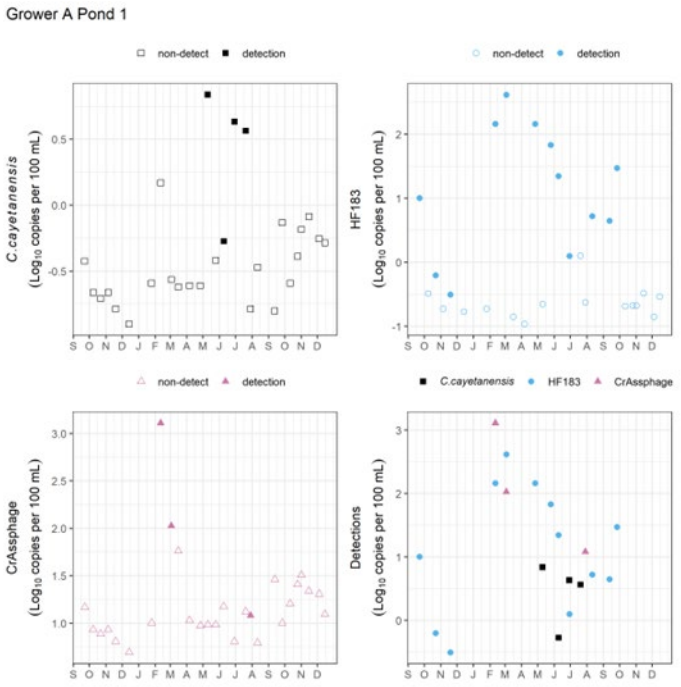


Figure 4: Grower A – Pond 1. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

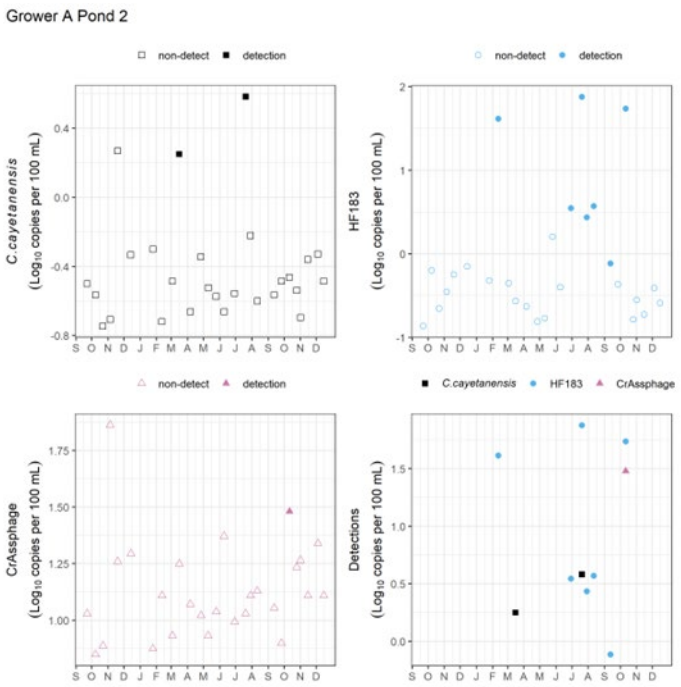
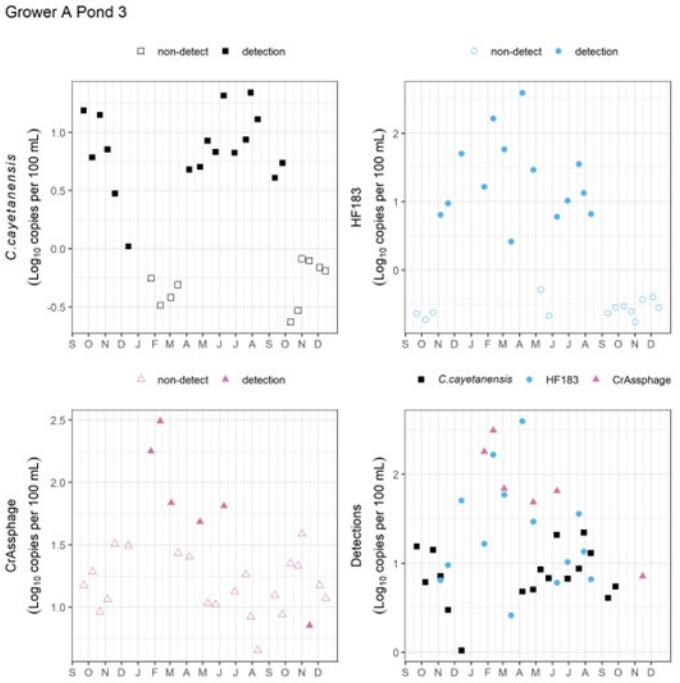


Figure 4: Grower A – Pond 2. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.



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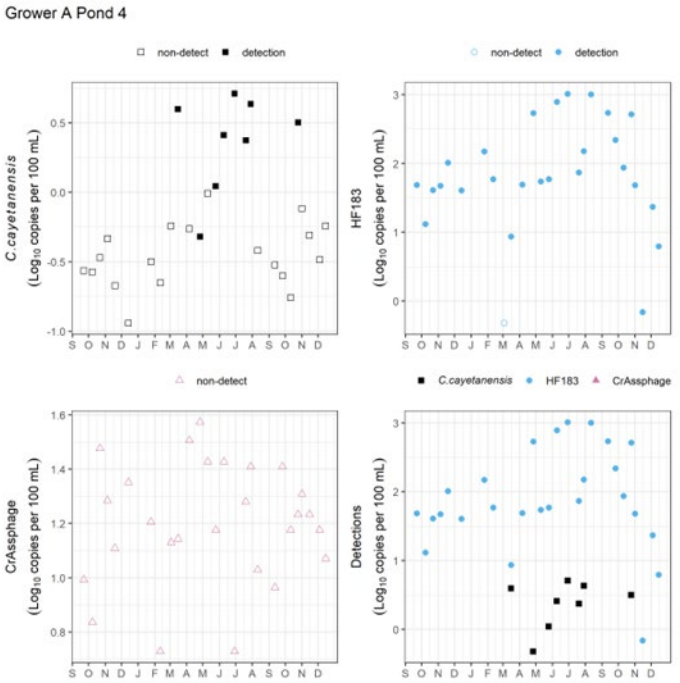


Figure 4: Grower A – Pond 4. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

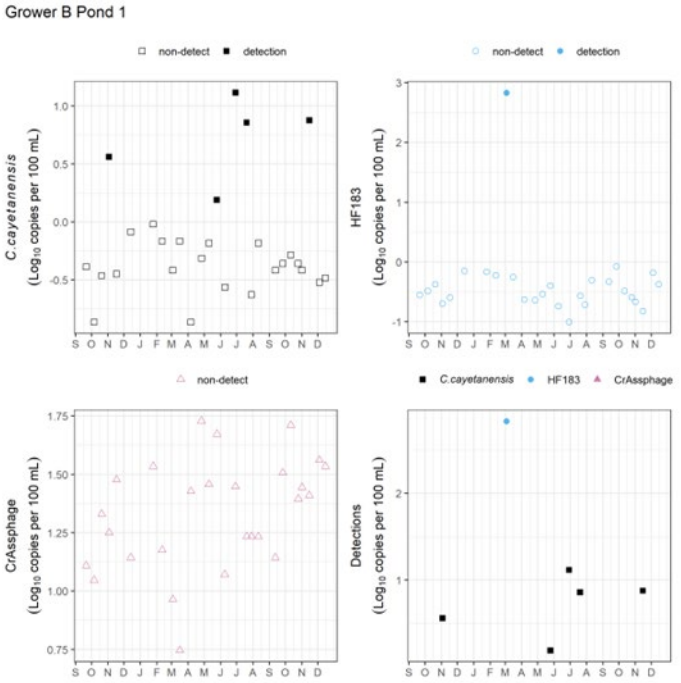


Figure 4: Grower B – Pond 1. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

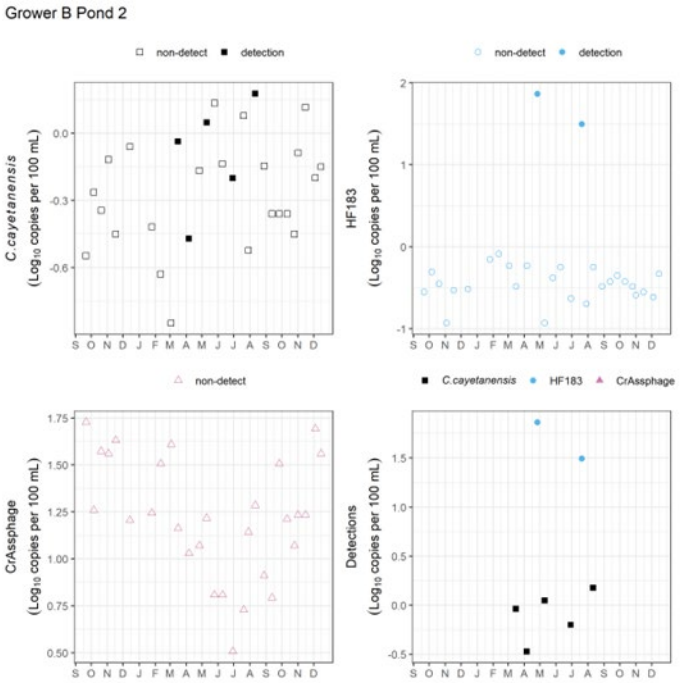


Figure 4: Grower B – Pond 2. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

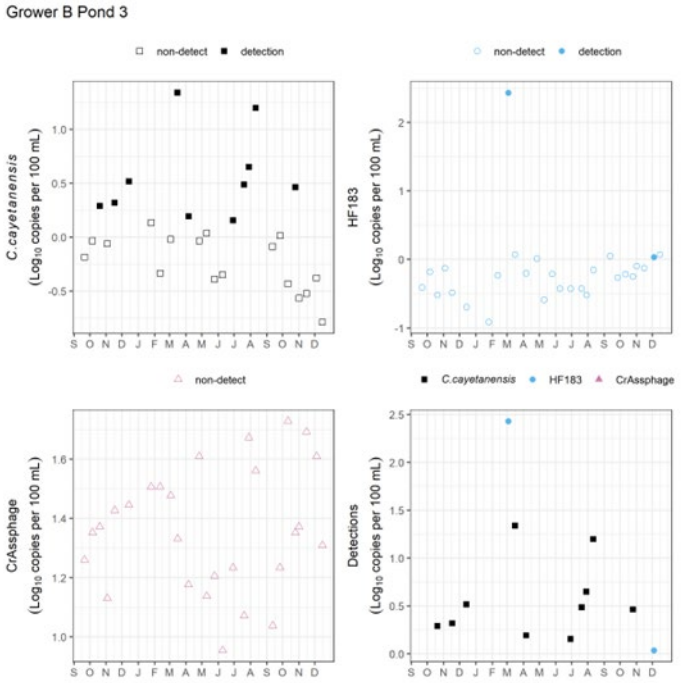


Figure 4: Grower B – Pond 3. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

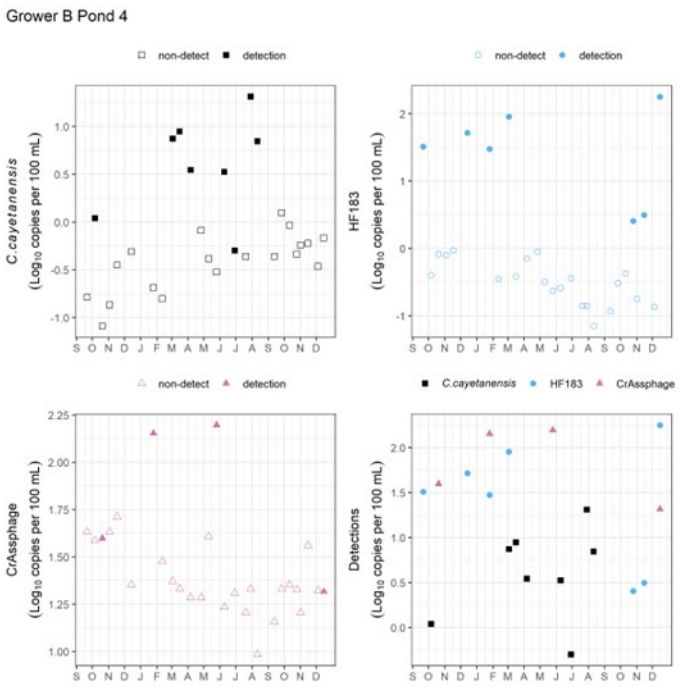


Figure 4: Grower B – Pond 4. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations (black squares), log_{10} HF183 gene target concentrations (blue circles), and log_{10} crAssphage gene target concentrations (pink triangles) in the irrigation pond samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample.

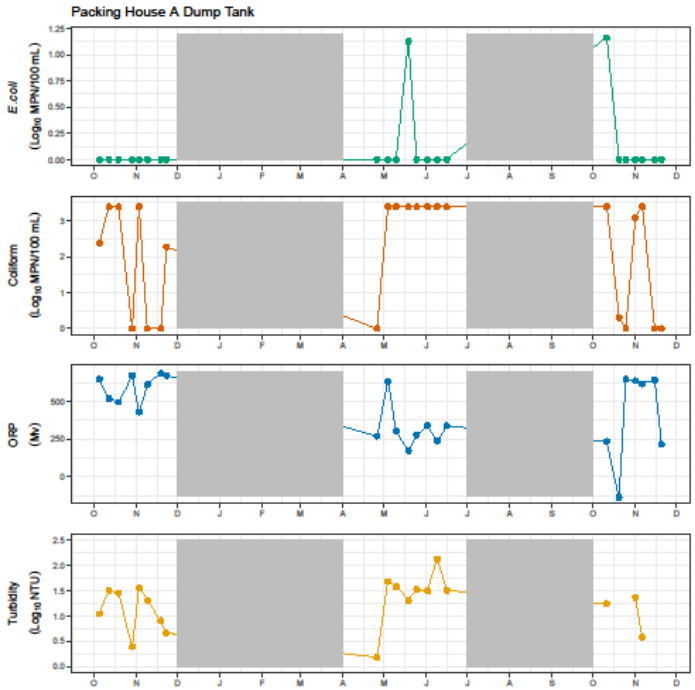
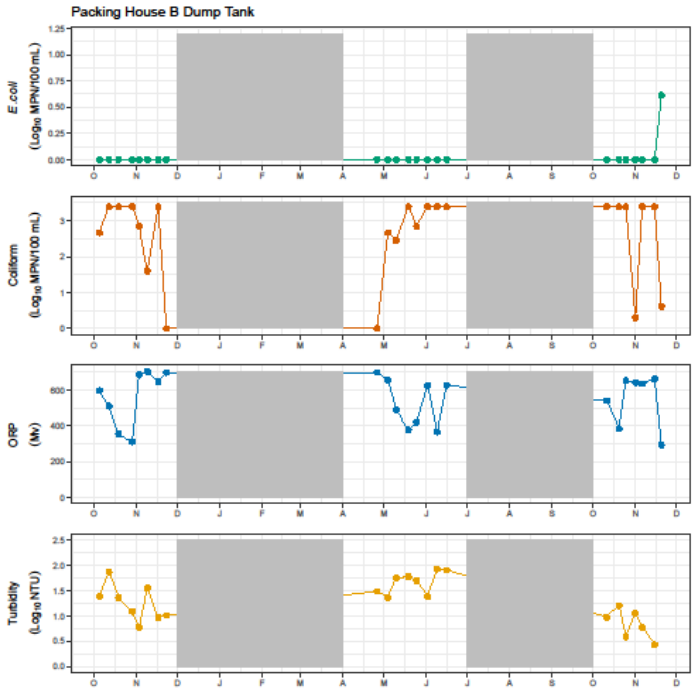


Figure 5: Grower A Dump Tank. Log_{10} *E. coli* (green) and total coliform concentrations (brown), ORP levels (blue), and log_{10} turbidity levels (orange) in the spent dump tank samples collected from October 2020 through December 2021.



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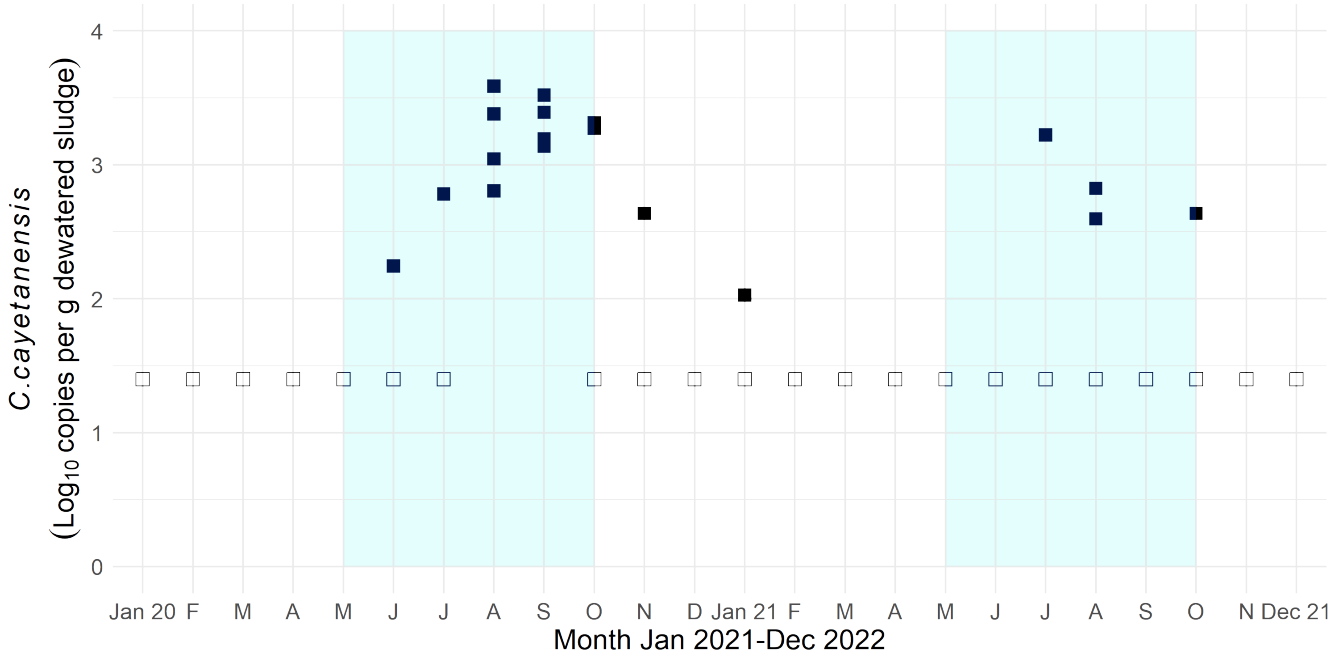

Figure 6. Log_{10} *C. cayetanensis* 18S rRNA gene target concentrations in the municipal sludge samples collected from January 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit. Blue shading depicts the months when domestically acquired cyclosporiasis cases are increased in the US.

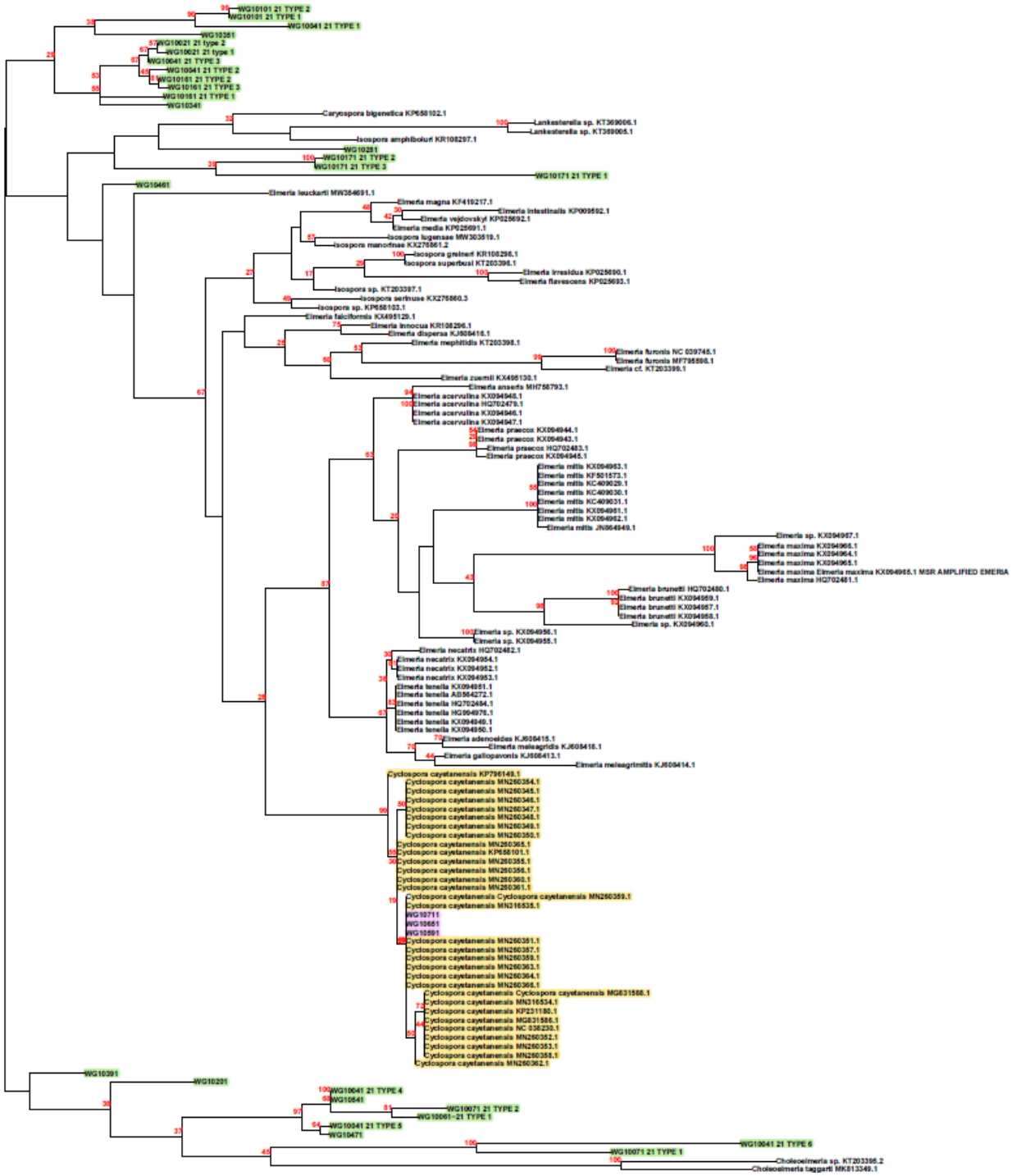

Figure 7. Neighbor joining tree of closely related coccidia species with isolates from irrigation pond samples in green, sludge samples in pink, and clinical *C. cayetanensis* in yellow.

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