CENTER for PRODUCE SAFETY

CPS 2019 RFP
FINAL PROJECT REPORT

Project Title
The prevalence of *Cyclospora* in water and produce

Project Period
January 1, 2020 – December 31, 2021 (extended to March 31, 2022)

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Objectives

1. *Compare three molecular tools for the detection of Cyclospora in water.*
2. *Examine the presence of Cyclospora oocysts in waters in Florida and California.*
3. *Perform a systematic survey of fresh produce in Florida based on the current knowledge of the distribution of this parasite in the state.*

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FINAL REPORT

Abstract
In the US, gastrointestinal infections caused by *Cyclospora cayetanensis* have been reported in travelers and non-travelers. Most of the outbreaks that occurred in the US were associated with products imported from agricultural regions endemic to *Cyclospora*. However, in recent years, outbreaks have also been associated with products produced and processed domestically. Florida and California are two states where there is great production of fresh vegetable products. The objective of this study was to determine the prevalence of *Cyclospora* in surface water in these two states. Water samples from the Salinas River in California and from canal waters in Florida were collected over the two years of the study. Ten-liter samples of water were examined for *Cyclospora.* Likewise, vegetables purchased from Florida markets were examined for the presence of *Cyclospora.* Our results indicate that presumptive *Cyclospora* are present in the environment in both states. Presumptive positives were most frequently identified during the months of April to August.

Background
Cyclospora has been associated with illness outbreaks in the US since the 1990s. Reports of cyclosporiasis in travelers were frequent. In individuals with no travel history, most of the cases with cyclosporiasis were associated with consumption of fresh vegetables imported from regions endemic to *Cyclospora* (Ortega and Sanchez, 2010). However, in recent years, outbreaks of cyclosporiasis have also been associated with produce produced and processed in the US.

In 2018, two important U.S. cyclosporiasis outbreaks implicated different food items and sources of the products. In the first outbreak, 250 people got sick after ingesting pre-packaged vegetable trays containing broccoli, carrots, cauliflower, and dill dip that were prepared commercially in the Midwest. Epidemiological data or traceback studies were not able to determine which item was contaminated, nor the source or potential point of contamination for any item that comprised the vegetable trays (CDC, 2018). The second outbreak implicated a variety of salads sold by a large fast-food restaurant and produced by a Fresh Express processor in Illinois using products that were presumably grown in the US. Analysis of a package of lettuce and carrots was confirmed positive for *Cyclospora*. In total, 511 cases of cyclosporiasis resulted from this outbreak. An FDA sampling of a lettuce field in California tested positive and in 2020, the US had another outbreak implicating salad mixes of which cabbage has been grown in Florida (FDA, 2020).

Because surveys of environmental and produce samples also gave positive results, it was imperative to determine the prevalence of *Cyclospora* in environmental samples. These findings persuaded our team to learn more about the distribution of *Cyclospora* in the US. It was important to determine if *Cyclospora* had seasonal distribution in Florida and California, as was previously observed in endemic areas.

Research Methods and Results
Frequent communications were established with collaborators from academia and the fresh produce industry to identify relevant points where the collection of water samples should be done. Once the points of interest were determined, we proceeded to search for public and accessible areas. Water was collected from 14 sampling sites in each of the states (**Figure 1**).

Water samples were collected for two years in Florida and California. These samples were collected monthly during the months of April to August (**Figure 2**). Each water sample consisted

of 10 liters of water collected *in situ* using hollowfiber filters. Data on the temperature, turbidity, and chemical composition (nitrites, nitrates, total chlorine, KH ppm, ammonia, and pH) of each water sample were collected. The filters were transported to the parasitology laboratory at the University of Georgia. Once the information of each sample was entered, the filters were processed, and the filtrate was eluted, centrifuged, and concentrated (EPA, 1999).

Lettuce, cilantro, parsley, and basil were obtained from seven markets in Florida. The vegetables were transported to the parasitology laboratory at the University of Georgia. Samples (100 grams) of the vegetables were placed in stomacher bags. Samples were washed with elution buffer and concentrated by centrifugation. Samples were run in duplicate for nPCR (Li et al., 2007) and in triplicate for qPCR (FDA BAM19b, Murphy et al., 2017).

Presumptive *Cyclospora* samples were detected in water samples in California and Florida (**Figure 3**). In California, the months with the highest number of presumptive-positive samples were December (3.99%), April (1.17%) and June (0.7%); the distribution by collection points was between 1–4 presumptive positives per collection point, with the highest being 0.94%. In Florida, the highest number of presumptive positives was detected in the months of November and August (1.89%) followed by the month of March (1.35%).

Presumptive *Cyclospora* samples were detected in vegetables purchased in Florida. Of the products collected (n=767), 21 were presumptive positives. The largest number of presumptive positives was detected in vegetables purchased at farmers markets (1.69%). The distribution by product indicated that cilantro contained the most presumptive positives (10/767; 1.3%), followed by lettuce (0.65%), basil (0.52%), and parsley (0.26%).

The nPCR amplifications were sequenced. The sequences were compared in GenBank. Samples with undetermined profiles are being further examined by next generation DNA sequencing.

Outcomes and Accomplishments

- Presumptive *Cyclospora* positives samples were identified in vegetables and surface waters in Florida and California.
- Interaction of the research team with the fresh produce industry increased in both states. We had the opportunity to meet with food safety workers in these communities to discuss potential sources of *Cyclospora* contamination in water and agricultural fields.
- By talking with farmers and processors we were able to provide more information about *Cyclospora* and have a better understanding of the challenges the fresh produce industry is facing.
- An opportunity was created to work with the Florida Department of Health to determine the temporal-spatial presence of *Cyclospora* in Florida.
- A collection of environmental samples will be used in later studies to generate diagnostic tests.
- Our graduate and undergraduate students had the opportunity to participate in this large and highly relevant study for the fresh produce industry.
- Two manuscripts are being prepared using the results of this study and a third will be prepared in collaboration with the Florida Department of Health.
- Multiple oral and poster presentations have resulted from this study.

Summary of Findings and Recommendations

- Presumptive *Cyclospora* samples were identified in Florida and California.
- More than one diagnostic test is needed to confirm results.
- Sequencing can provide information on the presence of *Cyclospora* in food and environmental samples.
- The study led to a number of recommendations to CPS:
    - Offer informative seminars on *Cyclospora* to companies (small and large) that produce fresh vegetables in the US and other countries, particularly those that export to the US. It is recommended that these seminars be offered during the months of January to March of each year.
    - Trainings must be promoted and directed to personnel of farms and processing plants.
    - Support research studies to identify methods of inactivation and control and encourage work that is carried out mainly with *Cyclospora*.

APPENDICES

Publications and Presentations

Presentations, oral:

1. The prevalence of Cyclospora in fresh produce and water. Cyclospora forum, CPS Research Symposium webinar. June 15, 2021.
2. Ortega, Y. Comparison of Methods/Techniques that are Currently Available and Used to Identify Presumptive Positive Samples: Harmonization of Detection to Confirm Positives Samples. Cyclospora Forum II. September 8, 2021.

Presentations, posters:

3. Atis L, J DeMent, MP Torres, and Y Ortega. Occurrence of *Cyclospora cayetanensis* in Florida, 2014–2018. 2019 IAFP Annual Meeting, Louisville, KY, July 21–24.
4. Studebaker, A, M Aaron, R Raad, L Atis, and YR Ortega. Presumptive *Cyclospora* findings in surface waters. 2022 IAFP Annual Meeting, Pittsburgh, PA, July 31–August 3.

Budget Summary

This project was awarded $213,861 in research funds, and the majority of funds were spent.

Figures 1–3 (see below)

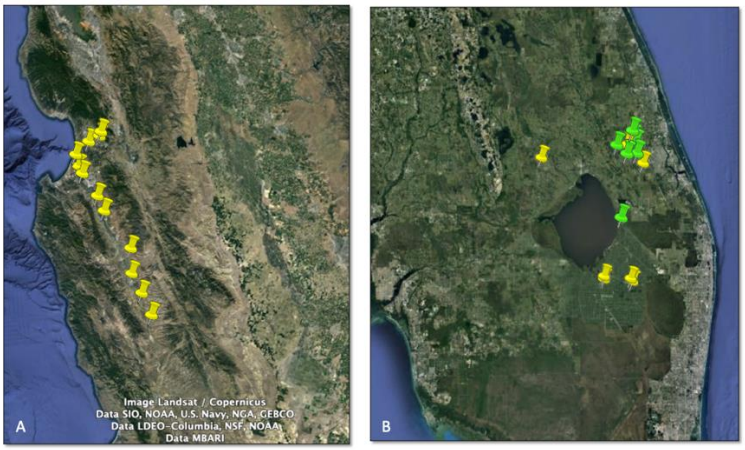

Figure 1. Selected sites where water samples were collected. A) California B) Florida

Figure 2. Water availability throughout the year. A, C) Salinas River; B, D) Florida canal.

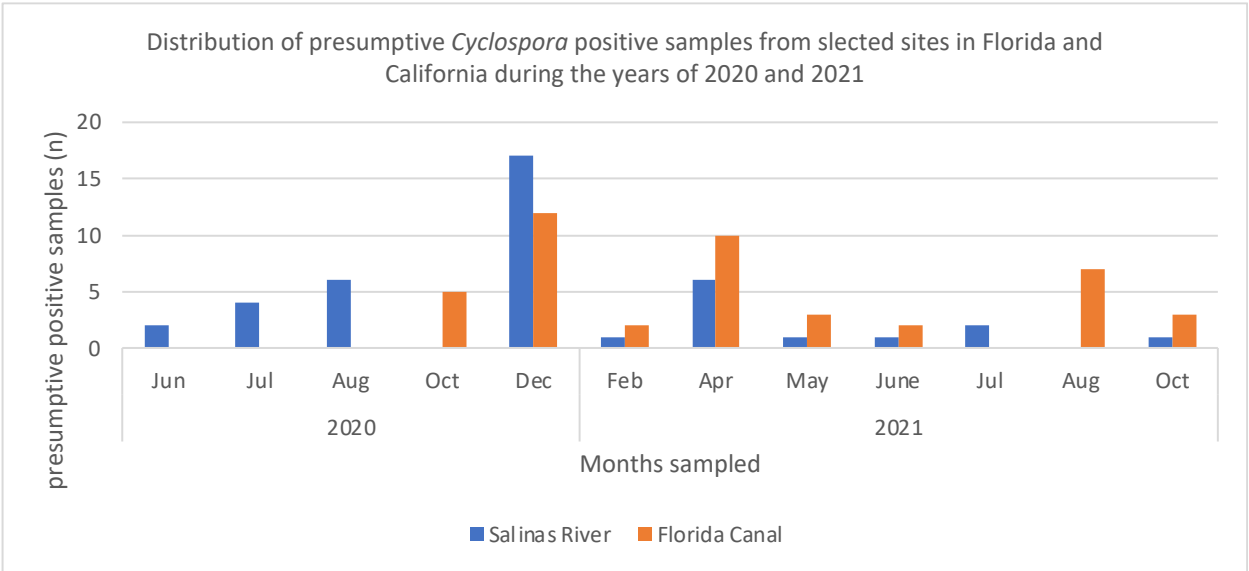

Figure 3. Distribution of presumptive *Cyclospora*-positive water samples collected in California (Salinas River) and Florida canals.

References cited

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Li G, Xiao S, Zhou R, Li W, Wadeh H, 2007. Molecular characterization of Cyclospora-like organism from dairy cattle. Parasitol Res 100: 955-61.

Murphy HR, Lee S, da Silva AJ. 2017. Evaluation of an improved U.S. Food and Drug Administration Method for the detection of Cyclospora cayetanensis in produce using Real- Time PCR. J Food Prot 80:1133-1144.

Ortega YR, Sanchez R. 2010. Update on Cyclospora cayetanensis, a food-borne and waterborne parasite. Clin Microbiol Rev 23:218-234.

ADDENDUM – Summary of additional project findings based on further analysis of samples (provided November 2022)

A two-year survey of surface waters in California and Florida was conducted during 2020-2021; 426 water samples were collected from California and 370 from Florida. Using the 18S RNA gene quantitative real-time PCR (qPCR) described in the BAM 19c, 27 and 56 presumptive positives were identified in Florida and California, respectively. Using the 18S RNA gene nested PCR (nPCR), 44 and 27 presumptive positive samples were identified in Florida and California respectively. Products from the nPCR of presumptive positives were sequenced, and *Cyclospora cayetanensis* was not detected in any of the presumptive positive samples.

Amplified products from California identified *Eimeria* sp.*, Eimeria sciurorum*, *E. myoxi*, *E. uptoni*, *E. arizonensis*, *E. rioarribaensis*, *E. gruis*, *E. paludosa*, *Isospora* sp.*,* and *Gousia carpelli.* Presumptive positive samples from Florida amplified as *E. melogale, I. gekkonis, I. butcherae,* and *Caryospora* sp*.* During the survey, animal feces found nearby the water sampling sites in California were also examined, and *E. rioarribensis*, *E. cantolensis*, *E. melogade*, *E. reichenowi*, *E. grus*, *E. yeidovskyi*, *E. stiedai*, *Isospora*, and *Caryospora* were detected. In addition, the presumptive positive samples by qPCR (BAM 19c) were examined using two other targets: CYC-13 and CYC-21. *Cyclospora* was not detected in these samples.

In conclusion, results from a 2-year survey study of surface waters in Florida and California revealed that although some samples from Florida and California were presumptive positive by qPCR and nPCR, none of them could be confirmed as *Cyclospora cayetanensis*. Two additional PCR assays were performed and none of the samples amplified as *C. cayetanensis*.

Also in 2020-2021, fresh produce from markets in Florida was surveyed. Of 767 vegetable samples collected in Florida, 21 were presumptive positive by nPCR and 2 by qPCR. Additional testing of these presumptive positive vegetable samples is ongoing using the CYC-13 and CYC-21 PCR tests.

Additional or alternative assays are needed to confirm the presence of *Cyclospora cayetanensis* from environmental samples.