CENTER for PRODUCE SAFETY

CPS 2018 RFP
FINAL PROJECT REPORT

Project Title
Cyclospora prevalence in irrigation water in fresh produce growing regions in Arizona

Project Period
January 1, 2019 – December 31, 2019 (extended to March 31, 2020)

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Objective

1. *Determine the prevalence of Cyclospora cayetanensis in irrigation waters in Arizona using the new BAM Chapter 19b method used for produce, with slight modifications. This will allow a determination of any risk from C. cayetanensis and to identify areas of potential risk.*

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research (Rapid Response project)

FINAL REPORT

Abstract

Cyclosporiasis is a gastrointestinal illness caused by the coccidian parasite, *Cyclospora cayetanensis.* Although transmissibility is not entirely known, infection occurs from ingesting fresh produce contaminated by stools infected with *C. cayetanensis* oocysts. *C. cayetanensis* has been implicated in outbreaks in the United States since the early 2000s. In 2019, there were 2,408 laboratory-confirmed cases reported domestically from 37 states, thus making Cyclosporiasis a nationally notifiable disease. This project assessed the prevalence of *C. cayetanensis* in agricultural water in the Yuma Valley fresh produce growing region, by collecting up to 100-liter agricultural water samples monthly for a year; a total of 196 samples were collected. *C. cayetanensis* was collected from Envirochek high volume filters, eluted and concentrated using the EPA method 1623. The newly developed FDA BAM 19b method was used for DNA isolation, purification, and quantitative PCR (qPCR) assays. This study found *C. cayetanensis* in 6/196 water samples from the Yuma Valley growing region, which represents a prevalence of 3%. The six samples positive for *C. cayetanensis* were found during December (2), January (3), and February (1); samples collected in all other months were negative. Four of the six positive samples were from unlined canals (~4%, 4/103) and two samples were from lined canals (~2%, 2/93). There was no statistically significant difference in *C. cayetanensis* gene copies/liter values between lined and unlined canals. These findings suggest that the risk of fresh produce contamination with *C. cayetanensis* from agricultural water in this produce growing region is relatively low, given that no *C. cayetanensis* outbreak has been associated with fresh produce grown in the Yuma Valley and given the volume of raw agricultural commodity irrigated with these agricultural waters. The recommendation is for irrigation districts and growers to continue to implement and practice current water quality safety measures, good agricultural practices (GAPs), and good management practices (GMPs) to continue to reduce the potential contamination of fresh produce with agricultural water potentially contaminated with *C. cayetanensis* oocysts.

Background

Cyclospora cayetanensis is an intestinal coccidian protozoan that has emerged as a major cause of endemic or epidemic diarrheal illness in humans worldwide (Chacín-Bonilla, 2010; Helmy, 2010; Ortega & Sanchez, 2010). *C. cayetanensis* is an obligate intracellular parasite with a complex life cycle not fully understood, requiring a single human host to complete its entire life cycle. The life cycle starts with the ingestion of the transmissible stage, the sporulated oocyst, which excysts (emerges from the cyst) in the gut, releasing infective sporozoites (Chacín-Bonilla, 2010; Helmy, 2010; Ortega & Sanchez, 2010). Oocysts can persist for long periods of time in the environment, maintaining their infectivity even under harsh environmental conditions (Mansfield & Gajadhar, 2004). Oocysts may survive for long periods in external environmental conditions, for up to 2 months at 4°C and 7 days at 37°C (Ortega et al., 1998; Smith et al., 1996). *C. cayetanensis* is highly resistant to not only external environmental conditions, but to many physical and chemical disinfection methods routinely used against bacteria (Chacín-Bonilla, 2010; Galván et al., 2013; Ortega et al., 2010).

The infectious dose of *Cyclospora* oocysts is unknown; however, based on outbreak investigations from other coccidians, it is thought to be relatively low (Dixon et al., 2005; Sterling

& Ortega, 1999), perhaps between 10 and 100 oocysts (Dixon et al., 2005). A draft genome sequence was recently obtained from *C. cayetanensis* oocysts purified from a human stool sample; the genome assembly consists of 865 contigs, with a total length of 44,563,857 bases (Qvarnstrom et al., 2015). These genome sequences will help with the development of subtyping tools to aid in outbreak investigations. Epidemiological studies have shown that *C. cayetanensis* infections are more common during the summer (Chacín-Bonilla, 2010; Helmy, 2010; Ortega & Sanchez, 2010). *C. cayetanensis* oocysts have been found in drinking water (Dowd et al., 2003; Giangaspero et al., 2015a), wastewater (Kitajima et al., 2014), treated wastewater used for irrigation (Giangaspero et al., 2015b), and recreational water in several countries, and are responsible for waterborne outbreaks worldwide (Chacín-Bonilla, 2010; Helmy, 2010; Ortega & Sanchez, 2010). Foodborne outbreaks caused by *C. cayetanensis* associated with fresh produce, including cilantro from Mexico, have recurred annually (2012–2014) and 2015 experienced another ongoing outbreak, with 546 confirmed cyclosporiasis cases from 31 states (Abanyie et al., 2013; CDC 2015; Des Vignes-Kendrick et al., 2013; FDA, 2015; Kozak et al., 2013). *C. cayetanensis* is recognized as an emerging human pathogen, with transmission stages that can be highly resistant to external environmental conditions and to many physical and chemical disinfection methods routinely used as bactericides in drinking water plants, swimming pools, and irrigation systems (Chacín-Bonilla, 2010; Galván et al., 2013; Ortega et al., 2010). Humans and primates are believed to be the only animals infected by the organism (Marangi et al., 2015). A study conducted in Italy reported that *C. cayetanensis* in treated wastewater used for irrigation was transferred to soil and vegetables (Giangaspero et al., 2015b). The findings of this project enable us to have a better understanding of *C. cayetanensis* occurrence and the potential contamination of irrigation waters to help mitigate the microbial health risks of *C. cayetanensis.* A better understanding of its occurrence is critical in mitigating the microbial health risks associated with this microorganism. The research team has been involved in the detection of *Cyclospora* in water since 2003 (Dowd et al., 2003). Most recently, we investigated the occurrence of *C. cayetanensis* in wastewater treatment plants in Arizona (Kitajima et al., 2014). We have been successful in identifying *C. cayetanensis* in wastewater and drinking water (Dowd et al., 2003; Kitajima et al., 2014).

In the United States, outbreaks associated with the *C. cayetanensis* protozoan intestinal parasite have been occurring almost yearly since 2000 (CDC, 2018a). *C. cayetanensis* has been identified as second to *Salmonella* as the most common cause of diarrhea illness cases and outbreaks in the U.S. associated with imported fruits and vegetables (Murphy et al., 2017a). *C. cayetanensis* has been found in drinking water (Dowd et al., 2003; Giangaspero et al., 2015a), wastewater (Kitajima et al., 2014; Lopez et al., 2019) and treated wastewater (Giangaspero et al., 2015b) used for irrigation in several countries where it can be presumably responsible for outbreaks (Chacín-Bonilla et al., 2010; Ortega and Sanchez, 2010; Almeria et al. 2019). In 2018, there were a total of 2,299 laboratory-confirmed cases in 33 states in persons with no history of international travel (CDC, 2018b). Only 761 cases were connected to two outbreaks associated with fresh produce (Casillas et al., 2018). The specific sources of contamination for the remaining 1,538 cases were not determined, even though basil and cilantro were shown to be associated with several clusters (CDC, 2018c). As of November 2019, there had been 2,408 laboratory-confirmed cases of cyclosporiasis in 37 states, including 144 hospitalizations (CDC, 2019a). One multistate outbreak of Cyclospora infections was linked to fresh basil imported from Mexico, with 241 laboratory-confirmed cases of cyclosporiasis in 11 states (exposure occurred in 5 states), including 6 hospitalizations (CDC, 2019b).

This Rapid Response project helped build on our first 2-year Cyclospora project funded by CPS. The previous (2017–2018) Cyclospora project found ~13% (15/119) of water samples tested were positive for *C. cayetanensis*. This project enables us to have a better understanding of *C.*

cayetanensis prevalence and transmission routes in the fresh produce environment and assists in the development of realistic intervention strategies that can impact the fresh produce industry in potentially reducing the contamination of fresh produce with *C. cayetanensis*.

Research Methods and Results

Objective: Determine the prevalence of *Cyclospora cayetanensis* in irrigation waters using the FDA BAM Chapter 19b method, with slight modifications.

The irrigation sampling plan was modified and approved from the original sampling plan due to only five out of the seven irrigation districts agreeing to participate in this 1-year study. Sampling began in February 2019, with 12 samples collected. Beginning in March 2019, five additional samples were added, increasing the monthly total to 17 samples collected for the remaining 11 months (with the exception of three canals not collected due to water not flowing), resulting in a final total of 196 agricultural water samples collected. Data on water quality parameters, such as temperature, pH, electro-conductivity, and turbidity, were also collected. These data were statistically analyzed to determine if they could be correlated with the presence of *C. cayetanensis* in irrigation waters.

Sample collection:
Irrigation water samples were collected using the Gelman Envirochek high-volume (HV) filter (Pall Gelman Sciences, Inc., Ann Arbor, MI) described in the EPA Method 1623 and cited in BAM Chapter 19A, section 6 (Quintero-Betancourt et al., 2003). The majority of agricultural water volume samples ranged from 30 to 100 liters, depending on the turbidity of the water. Filters were placed in sterile plastic bags and stored on ice in a cooler and transported to the Lopez Lab at the UA, where they were stored at 4°C and processed within 48–72 hours of collection. Water quality parameters of temperature, pH, electro-conductivity, and turbidity were taken and recorded on chain-of-custody forms for each sample.

Filter elution and concentration:
C. cayetanensis was eluted and concentrated from the 100-liter irrigation/watershed samples from the Gelman Envirochek high-volume (HV) filters. Briefly, each filter was filled with 125 mL of parasite buffer (EPA Method 1623) and placed on the laboratory shaker (Pall Gelman Sciences) at the 12 o'clock position and shaken for 5 min at 800 oscillations. The parasite buffer was decanted into a 250-mL sterile Nalgene bottle and an additional 125 mL of parasite buffer was added to the filter and shaken at the 4 o'clock and 8 o'clock positions for 5 min each at 800 oscillations. The remaining parasite buffer was decanted into the 250-mL bottle and centrifuged at 4,000 ×*g* for 45 min. The supernatant was discarded and the pellet was resuspended with phosphate-buffered saline (PBS) and transferred to a conical tube. Final elution and concentrated sample volumes ranged from 2 to 30 mL and were stored at -20°C until DNA isolation.

The methods used from this point forward were previously described (Murphy et al., 2017, 2018) and are part of the FDA BAM 19b method.

DNA isolation:
C. cayetanensis DNA was isolated using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA), with slight modifications, and performed in a class II Biosafety cabinet (ThermoFisher Scientific). Briefly, samples were thawed and vortexed thoroughly before taking 800 µL of sample and adding it to the Lysing Matrix E tube (containing beads); water was added to the negative DNA extraction/process control. Then 122 µL of MT buffer and 350 µL of sodium

phosphate buffer were added to tubes and transferred to the FastPrep-24 bead beater instrument (MP Biomedicals) and homogenized at a setting of 6.5 m/s (approx. 4,000 rpm) for 60 s twice, followed by incubation on ice for 3 min. Samples were then centrifuged at 14,000 ×*g* for 15 min. The supernatants were transferred to clean 2-mL tubes, and 250 µL of protein precipitation solution was added; tubes were gently mixed by inverting by hand 10 times. Tubes were then centrifuged at 14,000 ×*g* for 5 min, and the supernatants were transferred to clean 15-mL conical tubes containing 1.0 mL of resuspended binding matrix. Tubes were once again gently mixed by hand for 2 min, and the silica matrix was allowed to settle for 3 min. Tubes were then centrifuged at 1,000 ×*g* for 1 min in a swinging bucket rotor, followed by the removal of 1.4 mL (700 µL x 2) of supernatant from each tube. The matrix was resuspended in the remaining supernatant and 700 µL was transferred to a SPIN filter in a catch tube, which was centrifuged at 14,000 ×*g* for 1 min and then decanted. This step was repeated with any remaining resuspended binding matrix. Then 500 µL of prepared SWES-M was added to each filter, gently resuspended by pipetting up and down, and then followed by centrifugation at 14,000 ×*g* for 1 min, then emptied the catch tube and replaced. Centrifuged again at 14,000 ×*g* for 2 min to dry the matrix, discarded the catch tube and replaced. Filters were air dried for 5 min at room temperature, and 75 µL DES was added to the matrix in the spin filter. The binding matrix was resuspended by gently stirring with a small pipet tip, and tubes were incubated at 55ºC in a heat block for 5 min. Tubes were centrifuged at 14,000 ×*g* for 1 min to recover the eluted DNA and then the SPIN filter was discarded. DNA purification protocol was then followed.

DNA Purification:
C. cayetanensis DNA purification was performed with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Briefly, approximately 375 µL of Buffer PB was added to the DNA sample tube and mixed. Then the DNA/Buffer PB mix was added to QIAquick column and centrifuged at 14,000 ×*g* for 30 to 60 s to bind DNA. Flow-through was discarded and the column was placed back on same tube followed by adding 750 µL of Buffer PE to QIAquick column, centrifuged again as described above. The column was centrifuged once more for 1 min and the filter columns were placed in clean 1.5-mL microcentrifuge tubes. DNA was eluted by adding 30 µL of Buffer EB, letting it stand for 1 min, and then centrifuging at 14,000 ×*g* for 30 to 60 s. Columns were discarded, and DNA tubes were stored at 4 or -20ºC until ready for qPCR.

Quantitative PCR (qPCR)/Real-time PCR (RT-PCR):
The TaqMan qPCR assay was performed as a duplex assay targeting both *C. cayetanensis* 18S rRNA gene and an exogenous Internal Amplification Control (IAC). A synthetic DNA double-stranded 998bp gBlock gene fragment that corresponds to the 18S rRNA was used as a positive control and as standards to quantify the gene copies/well. Sample DNA was analyzed in duplicate, both the undiluted sample and at a 1/10 dilution. The duplex qPCR assay was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, CA). Reactions were performed using 2.0 µL of template in a final reaction volume of 20 µL. The application protocol consisted of an initial step of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 67°C for 30 s. Analysis was performed using the CFX Manager Software (Bio-Rad). A quantification cycle (Cq) ≤38 was used to determine positive samples. In the case of only one well resulting in a Cq value, the average was not taken and that single value was used to determine whether positive or negative.

Statistical Analysis:
A two-sample t-test at $P \leq 0.05$ was used to determine statistical difference between gene copies/liter of *C. cayetanensis* in lined and unlined canals and for the comparison of physicochemical properties among canals positive, negative, and overall.

Outcomes and Accomplishments

The objective of the project was achieved in that 196 water samples were collected over the course of one year from five irrigation districts across the Yuma Valley. The samples taken during the 12-month study provide a reasonable representation of the prevalence of *C. cayetanensis* in agricultural water throughout the produce growing region. The newly developed FDA BAM 19b molecular method for the detection of *C. cayetanensis* was utilized and shown to be effective.

Summary of Findings and Recommendations

This project showed a 3% prevalence of *C. cayetanensis* overall in the sampled canal water of the Yuma Valley growing region, with six positive samples out of the 196 collected (Table 1). The six samples positive for *C. cayetanensis* were found during February and December 2019, and January 2020; samples collected in all other months were negative. Four of the six positive samples were collected from unlined canals (~4%, 4/103) and the other two samples were from lined canals (~2%, 2/93) (Table 1). The values for gene copies/well ranged from less than 1 to 1.68×10^0. There was no statistically significant difference between lined and unlined canals for *C. cayetanensis* gene copies/liter (Table 1). The value of gene copies/liter is an estimation of what could be found per liter of agricultural water since only less than 1 mL was used for DNA extraction from sample volumes ranging from 5–15 mL.

Determining the concentration of *C. cayetanensis* oocysts from agricultural water still presents challenges due to the high levels of sediment and organic material found in samples when collecting large volumes of up to 100 liters of agricultural water. For canal water positive for *C. cayetanensis*, there was a statistically significant difference between unlined and lined canals for total dissolved solids (TDS), and statistical differences between positive overall and lined canals for conductivity, salinity and TDS (Table 2). In addition, there was a significant difference between positive and negative canals for air, water, salinity, TDS and pH (data not shown).

These findings show an overall lower prevalence from the previous (2017–2018) Cyclospora project, where ~13% (15/119) samples were positive for *C. cayetanensis*. The three-year average from the combined studies shows a ~7% (21/315) prevalence of *C. cayetanensis*. These findings suggest that the risk of fresh produce contamination by *C. cayetanensis* from agricultural water in this produce growing region seems to be relatively low, given that no *C. cayetanensis* outbreak has been associated with fresh produce grown in the Yuma Valley and given the volume of raw agricultural commodity irrigated with these agricultural waters.

The recommendation is for irrigation districts and growers to continue to implement and practice current water quality safety measures, good agricultural practices (GAPs) and good management practices (GMPs) to continue to reduce the potential contamination of fresh produce with agricultural water contaminated with *C. cayetanensis* oocysts. Since we know humans infected with *C. cayetanensis* can transmit the oocysts into the environment, it is recommended that agricultural workers continue to practice hygiene and sanitation practices to minimize the potential of *C. cayetanensis* oocysts contamination of fresh produce. Stakeholders should continue to monitor human wastewater and nearby encampments, and both wild and domesticated animal intrusion into agricultural water, which can act as physical transporters of *C. cayetanensis* oocysts after coming across human waste or garbage contaminated with human feces.

APPENDICES

Publications and Presentations
No publications have been completed; however the research team anticipates that one paper will be submitted.

Budget Summary
A total of $167,632.77 was awarded to this project and all funds have been spent pending final reconciliation. Project personnel and travel expenses were adjusted to cover operational expenses.

Suggestions to CPS
None

Tables and Figures
(see Tables 1–2 below)

Table 1. *C. cayetanensis* detected in agricultural water from unlined and lined canals

Canal	Average Cq	Avg. Gene Copies/Well	Avg. Gene Copies of Cyclospora/Liter
Overall *n=196*			
Mean ± SD	36.86 ± 0.82	$5.37 \times 10^{-1} \pm 0.57$	$1.01 \times 10^{4} \pm 1.07 \times 10^{4}$
Range		$6.08 \times 10^{-2} – 1.68 \times 10^{0}$	$1.14 \times 10^{3} – 3.15 \times 10^{4}$
% Positive			3% (6/196)
Unlined[a] *n=103*			
Mean ± SD	36.96 ± 0.60	$3.71 \times 10^{-1} \pm 0.28$	$6.95 \times 10^{3} \pm 5.27 \times 10^{3}$
Range		$1.21 \times 10^{-1} – 8.43 \times 10^{-1}$	$2.26 \times 10^{3} – 1.58 \times 10^{4}$
% Positive			~ 4% (4/103)[b]
Lined[a] *n=93*			
Mean ± SD	36.76 ±1.14	$8.70 \times 10^{-1} \pm 0.81$	$1.63 \times 10^{4} \pm 1.52 \times 10^{4}$
Range		$6.08 \times 10^{-2} – 1.68 \times 10^{0}$	$1.14 \times 10^{3} – 3.15 \times 10^{4}$
% Positive			~ 2% (2/93)[c]

[a] There was no statistically significant difference between gene copies/liter for unlined and lined canals (with $P \leq 0.05$).
[b] Two of the four positive samples had average gene copies/well from both wells; the remaining two samples only had gene copies in one well.
[c] Both positive samples only had gene copies in one well.

Table 2. Environmental parameters for agricultural canal water positive for *C. cayetanensis*

Canal	Air temp (°C)	Water temp (°C)	Relative humidity (%)	Conductivity (uS/cm)	Salinity (ppt)	TDS (ppt)	pH	Turbidity (NTU)
Overall *n=196*								
Arithmetic mean	20.49[a]	14.47[a]	37.27[a]	1098.00[a]	613.00[a]	549.18[a]	8.02[a]	2.22[a]
Unlined *n=103*								
Arithmetic mean	18.85[a]	14.15[a]	40.27[a]	1158.75[ab]	563.75[ab]	823.25[a]	8.11[a]	2.50[a]
Lined *n=93*								
Arithmetic mean	23.77[a]	15.10[a]	32.77[a]	1445.00[b]	711.50[b]	1.03[b]	7.82[a]	1.67[a]

[ab] Within each column, different letters indicate a statistically significant difference between the physicochemical parameter values for the three sample types ($P < 0.05$). (If the letters are the same there is no statistical difference.)

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