CENTER for PRODUCE SAFETY

CPS 2016 RFP
FINAL PROJECT REPORT

Project Title
Cyclospora: Potential reservoirs and occurrence in irrigation waters

Project Period
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Objectives

1. *Determine the occurrence of Cyclospora cayetanensis in irrigation waters in Arizona and Texas. (This will allow a determination of any risk from C. cayetanensis and to identify areas of potential risk.)*
2. *Determine the occurrence of C. cayetanensis in raw sewage and treated wastewater effluents in produce-growing areas such as Yuma, AZ, and El Paso, TX. (This data will allow an assessment of the incidence of C. cayetanensis infection among these communities. Treated wastewater effluents are also sometimes released into watersheds and could potentially impact irrigation waters.)*

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FINAL REPORT

Abstract
Cyclospora cayetanensis has been implicated in outbreaks in the United States associated with produce imported from Mexico since the mid ’90s and more recently has been associated with produce grown in the United States. This study examined the occurrence of *C. cayetanensis* in irrigation waters, raw sewage, and treated wastewater from the produce producing areas of Yuma, Arizona (AZ) and the Upper Rio Grande Valley, Texas/New Mexico (TX/NM), including El Paso, Texas (TX). *Cyclospora cayetanensis* was concentrated from 100-liter samples of irrigation/watershed water and treated wastewater (effluent) by using Gelman Envirochek high-volume (HV) filters, and from 1-liter raw sewage (influent) samples by filtration. A TaqMan assay probe targeting the internal transcriber 2 gene was developed and tested for detection of *C. cayetanensis* in environmental samples using quantitative and droplet digital PCR. A total of 181 irrigation water samples were collected over the 2-year period from the two growing regions. For the AZ irrigation water samples, 15/119 (~13%) tested presumptive positive for *C. cayetanensis*, ranging from 8.6 to 1.28E+03 gene copies/liter. For the TX/NM irrigation water samples, 3/62 (~5%) tested presumptive positive, ranging from 3.81E+02 to 4.17E+03 gene copies/liter. A total of 243 wastewater treatment plant (WWTP) samples were collected over the 2-year period from six treatment plants (three each in AZ and TX). For the AZ WWTP samples, 76/165 (~46%) tested presumptive positive for *C. cayetanensis*, ranging from 1.47E+00 to 7.93E+06 gene copies/liter; ~55% (46/83) of the 1-liter influent samples were presumptive positive whereas ~37% (30/82) of the 100-liter effluent samples were presumptive positive. For the TX WWTP samples, 28/78 (~36%) tested presumptive positive for *C. cayetanensis*, ranging from 1.37E+02 to 2.80E+06 gene copies/liter; ~46% (18/39) of the 1-liter influent samples were presumptive positive whereas ~26% (10/39) of the 100-liter effluent samples were presumptive positive.

This study showed that even though *C. cayetanensis* is presumptively present in irrigation waters in Arizona and Texas the risk from *C. cayetanensis* seems to be relatively low, given that no outbreaks have been linked to fresh produce grown in these two regions. Secondary confirmation by sequencing or alternative testing is required to confirm presumptive positive samples. These findings do not determine the viability or the infectivity of the presumptive positive samples. The study also showed that *C. cayetanensis* is presumptively present in influent (raw sewage) and effluent (treated wastewater) in the produce producing areas of Yuma, AZ, and the Upper Rio Grande, TX. These findings indicate that there is a presumptive incidence of *C. cayetanensis* infection among both the Arizona and Texas communities. However, no determination was made of the potential source of contamination of *C. cayetanensis* in irrigation waters. Although effluent is released into watersheds, no connection was made to impacting irrigation waters in these growing regions. These findings also suggest that persons involved in the production and harvesting of fresh produce and who live in these communities might pose a potential risk through some cross-contamination pathway.

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* oocycts 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, 2013, 2015; 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).

Research Methods and Results

Objective 1: *Determine the occurrence of C. cayetanensis in irrigation waters in AZ and TX.*

Irrigation water samples were collected from canals with various characteristics (e.g., main canals and lateral canals, cement-lined and non-lined canals, urban and rural canals) in the Yuma Valley in Arizona and Upper Rio Grande Valley in Texas/New Mexico over a 2-year period. The growing season for leafy greens in the Yuma region takes place from approximately October through early April each year, and in the Upper Rio Grande Valley the primary growing season is during the summer. Agriculture in the El Paso region is based along the Rio Grande River. Agricultural land runs from the east of El Paso along the Rio Grande up to the town of Fabens and west to New Mexico to the town of Truth or Consequences. This area utilizes the

Upper Rio Grande Valley irrigation canal system. The normal growing season is from March to October, but chronic drought has reduced the irrigation season from May to August. The majority of the crops grown in this region are pecans, onions, and peppers. After the growing season, most of the available water is from irrigation return flows and effluents from wastewater treatment plants. The water flow in the Rio Grande River is controlled by the Caballo Dam and the American Dam.

The irrigation sampling plans for Arizona and Texas were modified and approved from the original plan. The modified sampling plan in Yuma AZ for 100-liter irrigation canal samples was as follows: (4) 100-liter samples/month x 12 months = 48 samples/year x 2 years = 96 samples. The modified sampling plan in Upper Rio Grande Valley TX/NM was as follows: (8) 100-liter samples/month x 6 months (irrigation season) = 48 samples/year x 2 years = 96 samples. The combined total of samples was 192 100-liter irrigation canal samples over a 2-year period. In addition, a 1-liter grab sample was collected monthly from each of the irrigation sampling sites over the 2-year period for the detection of *Escherichia coli* from each sampling site, for a total of 192 1-liter samples (96 from each region) over the project period.

C. cayetanensis was concentrated from the 100-liter irrigation/watershed samples using the Gelman Envirochek high-volume (HV) filter (Pall Gelman Sciences, Inc., Ann Arbor, MI) (Quintero-Betancourt et al., 2003). Filters were placed in sterile plastic bags, shipped overnight on ice to the Water & Energy Sustainable Technology (WEST) Center where samples were processed within 24-48 hours of collection. Molecular work was conducted in the PI's laboratory. Briefly, a 200-µl portion of 1.5-ml concentrated irrigation/watershed samples (obtained after concentration and elution from HV filter) was used for the nucleic acid extraction, as described previously (Kitajima et al., 2014). The samples were subjected to ten cycles of freeze-thaw, followed by nucleic acid extraction and purification using the QIAmp DNA mini kit (Shields et al., 2013). A TaqMan assay probe targeting the internal transcriber 2 gene was developed and tested for detection of *C. cayetanensis* in environmental samples using quantitative and droplet digital PCR.

All 1-liter irrigation/watershed grab samples were collected in sterile plastic bottles, stored on ice, and transported to the respective research team labs in Yuma or El Paso and then processed for coliforms and *Escherichia coli* within 24 hours of collection using the Colilert quantitative MPN method (IDEXX Laboratories, Inc., Westbrook, ME). Data was also collected on water quality parameters such as temperature, pH, electro-conductivity, and turbidity. In addition, the sources of irrigation water, the irrigation methods used (e.g., flood, drip), the last occurrence of a rainfall event, and the types of crops irrigated in the study areas were also noted. These data were statistically analyzed to determine if they can be correlated with the presence of *C. cayetanensis* in irrigation waters.

Objective 1 Results:
A total of 181 irrigation water samples were collected over the 2-year project from the two growing regions in Arizona and Texas (representing ~94% of the original sampling plan for 192). Due to the subcontract PI leaving the project mid-way during 2018, samples were no longer collected in the Upper Rio Grande Valley Texas/New Mexico. However, additional irrigation water samples (40) were collected in AZ during October–December during year 2, to make up for not sampling in TX/NM.

A total of 119 samples (79 original + 40 additional) were collected in AZ, with 15/119 (~13%) testing presumptive positive for *C. cayetanensis* and ranging in concentration from 8.6 to 1.28E+03 gene copies/liter (**Table 1**). A total of 62 samples were collected in TX/NM, with 3/62 (~5%) testing presumptive positive for *C. cayetanensis* and ranging in concentration from 3.81E+02 to 4.17E+03 gene copies/liter (**Table 2**). The cycle threshold (C_T) values obtained

from PCR assay runs for the presumptive positive samples ranged from 30.58 to 37.12 based on our limit of detection.

Coliform data from the AZ samples showed an increase during the months of April to November for both years. During year 1, coliform levels were particularly high for all four sites during June and July (>2419.6 MPN/100 mL). *Escherichia coli* data from the AZ samples also showed an increase during April to August, but levels were still below the acceptable limit of 410 MPN/100 mL; only one sample in year 1 and two samples in year 2 were above the acceptable limit. Coliform data from the TX/NM samples showed levels >2419.6 MPN/100 mL during the growing season of March through September. *E. coli* data from the TX/NM samples showed that sample site A was above the acceptable limit once in each year, and two other samples also were above the limit in year 1.

Objective 2: *Determine the occurrence of C. cayetanensis in raw sewage and treated wastewater in produce producing areas such as Yuma, AZ, and El Paso, TX.*

To determine the incidence of *C. cayetanensis* in influent (raw sewage) and effluent (treated wastewater), 1-liter raw sewage grab samples and 100-liter effluent samples were collected monthly over a 2-year period from three wastewater treatment plants (WWTPs) located near agricultural fields in both Yuma, AZ, and El Paso, TX, for a total of 144 samples collected from each region (n = 288). *C. cayetanensis* was concentrated from the 100-liter effluent samples using the Gelman Envirochek HV filter as described in Obj. 1. In addition, for the detection of *Escherichia coli* from influent (raw sewage) and effluent (treated wastewater), a 1-liter grab sample was also collected monthly over the 2-year period, for a total of 144 samples from each region (n = 288).

Influent samples were collected in sterile plastic bottles, shipped overnight on ice to the WEST Center where samples were processed within 24–48 hours of collection. *C. cayetanensis* was concentrated from the influent samples by filtration. Briefly, the influent was passed through a 90-mm diameter nitrocellulose filter (with a 0.45-µm pore size) placed in a glass filter holder, and the oocysts were retained on the filters by size exclusion (Kitajima et al., 2014). The oocysts were then recovered from the filter by elution and concentrated by centrifugal ultrafiltration to a final volume of 1.5 ml. The additional 1-liter influent and effluent grab samples for the detection of *E. coli* were collected in sterile plastic bottles, stored on ice, and transported to the respective research team labs in Yuma or El Paso and then processed for coliforms and *E. coli* within 24 hours of collection as described in Obj. 1.

Processing methods at each WWTP also were identified and then compared to the different WWTPs in Arizona and Texas. *Escherichia coli* concentrations in the samples were determined by the Colilert quantitative MPN method. These data were statistically analyzed to determine any correlation with the presence of *C. cayetanensis* in irrigation waters.

Molecular work was conducted in PI's laboratory. Briefly, a 200-µl portion of the 1.5-ml concentrated treated wastewater samples (obtained after filtration and elution) were used for the nucleic acid extraction, as described previously (Kitajima et al., 2014). The samples were subjected to ten cycles of freeze-thaw, followed by nucleic acid extraction and purification using the QIAmp DNA mini kit (Shields et al., 2013). A TaqMan assay probe targeting the internal transcriber 2 gene, as described in Obj. 1, was used for detection of *C. cayetanensis* using quantitative and droplet digital PCR.

Objective 2 Results:
A total of 243 WWTP samples were collected over the 2-year project from the six WWTPs, three each in Arizona and Texas (representing ~84% of the original sampling plan for 288). The

WWTP monthly sampling plan was only slightly modified when the subcontract PI left the project in 2018 and samples were no longer collected in TX; additional samples (36) were collected in AZ during October–December to make up the difference.

A total of 165 samples (129 original + 36 additional) were collected in AZ, with 76/165 (~46%) testing presumptive positive for *C. cayetanensis* and ranging in concentration from 1.47E+00 to 7.93E+06 gene copies/liter (**Table 1**). Among these WWTP samples, ~55% (46/83) of the 1-liter influent samples were presumptive positive whereas ~37% (30/82) of the 100-liter effluent samples were presumptive positive.

A total of 78 samples were collected in TX, with 28/78 (~36%) testing presumptive positive for *C. cayetanensis* and ranging in concentration from 1.37E+02 to 2.80E+06 gene copies/liter (**Table 2**). Among these WWTP samples, ~46% (18/39) of the 1-liter influent samples were presumptive positive whereas ~26% (10/39) of the 100-liter effluent samples were presumptive positive. The C_T values obtained for the presumptive positive samples ranged from 26.15 to 37.05. Coliform and *E. coli* data obtained for both the AZ and TX samples were as expected, especially the high levels observed for the influent samples (Table 1 and 2, respectively).

Outcomes and Accomplishments

The objectives of the project were achieved even though delays were experienced and modifications to the sampling plans had to be made. Irrigation water samples were collected (94% of original number planned) and WWTP samples were collected (84% of original number planned) even though the subcontract PI (University of Texas El Paso Regional Campus) left the project in mid-2018 and additional samples had to be collected in Arizona. A major accomplishment of the project is that a TaqMan assay probe targeting the internal transcriber 2 gene (ITS-2) was developed and tested for detection of *C. cayetanensis* in environmental samples using quantitative and droplet digital PCR. A manuscript is being finalized for publication.

Summary of Findings and Recommendations

This study showed that even though *Cyclospora cayetanensis* is presumptively present in irrigation waters in Arizona and Texas the risk from *C. cayetanensis* seems to be relatively low, given that no outbreaks have been linked to fresh produce grown in these two regions. These findings do not determine the viability or the infectivity of the presumptive positive samples. No relationship was observed between coliform and *E. coli* data in connection with *C. cayetanensis* presumptive positive samples. No seasonality pattern was noticed with *C. cayetanensis* for the presumptive positive samples; however, a trend was observed with the increase of coliform levels in irrigation water samples during the summer months. The study also showed that *C. cayetanensis* is presumptively present in influent (raw sewage) and effluent (treated wastewater) in the produce producing areas of Yuma, AZ, and the Upper Rio Grande Valley, TX. These findings indicate that there is a presumptive incidence of *C. cayetanensis* infection among both the Arizona and Texas communities. However, no determination was made of the potential source of contamination of *C. cayetanensis* in irrigation waters. Although effluent is released into watersheds no connection was made to impacting irrigation waters in these growing regions. These findings also suggest that persons involved in the production and harvesting of fresh produce and who live in these communities might pose a potential risk through some cross-contamination pathway.

The project findings lead to three recommendations. First, further research should be conducted to determine the similarity between the *C. cayetanensis*–presumptive positive irrigation water

samples and WWTP samples. Second, for persons in the community who are involved in the production and harvesting of fresh produce, precautionary measures should be considered to reduce the potential for cross-contamination of fresh produce by infected persons. And third, effluent (treated wastewater) released into watersheds that flow to fresh produce growing regions in Mexico should be investigated to determine the risk of *C. cayetanensis*.

APPENDICES

Publications and Presentations

No publications have been submitted but one draft manuscript is currently being finalized.

The PI presented interim results at the annual CPS Research Symposium in 2017 and 2018, and will present final results in June 2019. Student poster presentations also have been given.

Budget Summary

Total funds awarded were $310,980. The grant funds provided were appropriate to complete the 2-year project. Due to the subcontract PI leaving the project, the remaining subcontract balance was approved for transfer to the PI's account to complete the additional irrigation and WWTP sampling and processing. These funds included the purchasing of additional supplies, travel, wages and benefits. Co-PI accounts were completely spent for collecting and processing samples, which included personnel and operations. Total expenditures were approximately as follows: $175,796 for personnel, $77,805 for operations including travel and supplies, $40,420 for subcontract PI, $3,200 for other direct costs, and $10,188 for indirect costs.

Tables and Figures

See below for Tables 1–2

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Table 1. Arizona overall findings in Canal, Effluent, and Influent samples

	E. coli (MPN/100 ml)	Total Coliforms (MPN/100 ml)	Gene copies of Cyclospora /liter	Air temp (°C)	Water temp (°C)	Relative humidity (%)	pH	Turbidity (NTU)
Canal								
arithmetic	78.37*[a]	1373.31*[a]	29.16[a]	25.58[a]	22.17[a]	30.31[a]	8.49[a]	9.31[a]
geometric	13.27*[a]	850.42*[a]	108.03[a]					
% positive	99.21% (126/127)	100% (127/127)	12.60% (15/119)					
Effluent								
arithmetic	99.06[b]	186.97[b]	1319.43[b]	22.20[b]	26.89[b]	37.21[b]	7.54[b]	12.26[a]
geometric	2.46[b]	8.95[b]	563.12[b]					
% positive	40.91% (36/88)	60.23% (53/88)	36.58% (30/82)					
Influent								
arithmetic	2390.84[c]	2390.83[c]	366076.27[c]	22.53[b]	27.05[b]	37.20[b]	8.29[ab]	190.41[b]
geometric	2202.88[c]	2203.14[c]	152215.18[c]					
% positive	98.98% (82/83)	100% (83/83)	55.42% (46/83)					

* indicates a significant difference in comparing Arizona (Region 1) and Texas/New Mexico (Region 2) in water type, *P*-value < 0.05.
[abc] different letters indicate that within Region 1 there are statistically significant differences between each microbial and physiochemical parameter in all three sample types, with a *P*-value < 0.05. If the letters are the same there is no statistical difference.

Table 2. Texas/New Mexico overall findings in Canal, Effluent, and Influent samples

	E. coli **(MPN/100 ml)**	**Total Coliforms (MPN/100 ml)**	**Gene copies of Cyclospora /liter**	**Air temp (°C)**	**Water temp (°C)**	**Relative humidity (%)**	**pH**	**Turbidity (NTU)**
Canal								
arithmetic	133.69*[a]	2420.00*[a]	113.23[a]	N/A	27.68[a]	N/A	8.52[a]	305.07[a]
geometric	81.55*[a]	2420.00*[a]	1445.19[a]					
% positive	100% (57/57)	100% (57/57)	5.26% (3/62)					
Effluent								
arithmetic	79.65[b]	299.65[b]	2271.10[b]	N/A	28.09[a]	N/A	7.38[b]	7.35[b]
geometric	2.62[b]	23.40[b]	1366.74[b]					
% positive	30.77% (12/39)	79.49 (31/39)	25.64% (10/39)					
Influent								
arithmetic	2420.00[c]	2420.00[c]	155412.81[c]	N/A	26.77[a]	N/A	7.34[b]	162.08[c]
geometric	2420.00[c]	2420.00[c]	141842.43[c]					
% positive	100% (39/39)	100% (39/39)	46.15% (18/39)					

* indicates a significant difference in comparing Arizona (Region 1) and Texas/New Mexico (Region 2) in water type, *P*-value < 0.05.
[abc] different letters indicate that within Region 2 there are statistically significant differences between each microbial and physiochemical parameter in all three sample types, with a *P*-value < 0.05. If the letters are the same there is no statistical difference.