Response to questions posed by the Food and Drug Administration (FDA): *Cyclospora cayetanensis* in Produce

National Advisory Committee on Microbiological Criteria for Foods[1]

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[1] Participating agencies include the U. S. Department of Agriculture, Food Safety and Inspection Service; U.S. Department of Health and Human Services, Food and Drug Administration, and Centers for Disease Control and Prevention; U.S. Department of Commerce, National Marine Fisheries Service; and U.S. Department of Defense, Veterinary Service Activity. Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture and other participating agencies.

Executive Summary of Findings

Cyclospora cayetanensis (C. cayetanensis) is a coccidian protozoan parasite, belonging to the phylum Apicomplexa, order Eucoccidiorida, family Eimeriidae, described between 1993 to 1994 as a newly identified human gastrointestinal pathogen. Within the genus *Cyclospora*, only *C. cayetanensis* is known to infect humans, however, recent advances in genomics separated *C. cayetanensis* into 3 species, with two new species that are also parasitic to humans (*Cyclospora ashfordi* sp. *nov*. and *Cyclospora henanensis* sp. *nov*.) recently proposed.The parasite produces oocysts that are resistant to harsh environmental conditions and to many chemical treatments commonly used to reduce the presence of bacterial pathogens in the specialty crop production environment and in agricultural inputs (e.g., agricultural water). *C. cayetanensis* is the etiologic agent of cyclosporiasis, its host range is limited to humans. Detected in association with human illness in many parts of the world, *C. cayetanensis* previously was considered to be a pathogen acquired during childhood in developing nations. In the United States, cyclosporiasis was previously associated with international travel or consumption of contaminated imported foods. In recent years, the U.S. has seen an increase in cases and positive samples associated with produce, both as raw agricultural commodities and fresh-cut produce, grown in the U.S. However, laborers with the history of recent travel to countries where *C. cayetanensis* is endemic have not been ruled out as the sources of the pathogen in these outbreaks. Since 2016, the number of cyclosporiasis cases has increased approximately 3-fold, often linked to the consumption of leafy herbs and ready-to-eat salads. Fecal contamination from symptomatic or asymptomatic carriers is, ultimately, the only known source of *C. cayetanensis*. A hypothesis that *C. cayetanensis* has become endemic in the production regions of the U.S. remains to be robustly supported, therefore in the meanwhile, farm workers with a history of recent travel to areas where the parasite is common are the likeliest source of the pathogen. *C. cayetanensis* likely spreads via the fecal-environment-oral route when sanitation controls break down. Efforts have been made to develop molecular detection methods for *C. cayetanensis* in both food vehicles and environmental water. However, due to the high degree of synteny between *C. cayetanensis* and its close relatives that are not pathogenic in humans, results of some environmental surveys that relied solely on the PCR-based detection of ribosomal RNA genes have been called into question. There remain significant knowledge and data gaps that hamper the implementation of effective measures to prevent the contamination of produce with the oocysts

of this parasite. Awareness of the factors that can contribute to *C. cayetanensis* contamination of domestically grown and imported produce is key to developing an effective prevention and management strategy.

RECOMMENDATIONS:

1. To facilitate future research (e.g., validation of surrogates, studies on environmental persistence and attachment) and identification and validation of control strategies, the Committee urges development of a practical method to propagate *C. cayetanensis* oocysts under laboratory settings.

2. Because of the limited availability of *C. cayetanensis* oocysts, research with surrogates – and specifically with the close relative *Eimeria* – can be informative for identifying control strategies and learning about persistence in the production environment.

3. Method development for the detection of *C. cayetanensis* in food and environmental samples should include the evaluation of multiple genetic targets representing different regions of the genome. Modifications to current molecular methods for the detection of *C. cayetanensis* should be thoroughly validated for impacts on specificity before using modified methods on food or environmental samples. Conversely, detection methods should be designed to be robust, reproducible and tolerant of minor modifications in the methodologies (e.g., brand of equipment or reagents, minor deviations in PCR conditions, etc.) without sacrificing specificity.

4. Given the likeliest source of the parasite in the food production environment (workers with a history of recent travel to areas where infections with *C. cayetanensis* are common), preventative measures should center around clear sanitation guidelines, ensuring on-site capacity for implementing sanitation protocols (i.e., readily accessible hand washing stations with soap, etc.) and periodic training of the employees.

Charge from FDA to NACMCF

Background

Cyclospora spp. are protozoan parasites in the phylum Apicomplexan that can parasitize different species of mammals with remarkable host-specificity. *Cyclospora* has a complex life cycle and can only multiply within the infected hosts. Among the *Cyclospora* species, only *Cyclospora cayetanensis* is known to infect humans; all other species are associated with infections in other animals. This parasite is characterized by environmentally-hardy oocysts that are shed in stool by the infected persons. These oocysts are shed unsporulated and are not infectious. Once released into the environment, unsporulated oocysts require approximately 7 to 14 days under certain environmental conditions to sporulate and become infectious. The

oocysts are thought to be transferred to the surface of foods through environmental routes (e.g., through human fecal pollution carried by agricultural water) subsequently infect the host after produce is consumed. Once consumed, the sporulated oocysts replicate in the human gastrointestinal tract and continue the infection cycle as unsporulated oocysts are shed in stool. The cycle continues as human fecal pollution again contaminates the environment. A limitation to widespread *Cyclospora cayetanensis* research is the inability to directly culture or propagate the organism. Researchers rely solely on acquired oocysts to conduct research. Some work has been done to use surrogate organisms to mimic the life cycle of *Cyclospora cayetanensis*, however with limited positive results.

A positive *C. cayetanensis* finding is indicative of the presence of human fecal contamination, as humans are the only known reservoir. Cyclosporiasis is characterized by symptoms such as explosive diarrhea, vomiting, fatigue, and weight loss. *C. cayetanensis* has become a major public health and food safety concern during the last few years. Outbreaks of cyclosporiasis affect thousands of individuals in the U.S. annually, with a steady increase in reported cases over recent years. In 2020, CDC reported 1,241 laboratory-confirmed cases of cyclosporiasis in people who had no history of international travel. In 2019 and 2018, there were 2,408 and 2,299 cases reported each year, respectively. Comparatively, between 2000–2017, the total number of cases reported for cyclosporiasis in the US was 1,730. Additionally, cyclosporiasis typically results in symptomatic illness in the general population regardless of age in the US, whereas in endemic areas, young children and immunocompromised individuals are most at risk for severe illness. Outbreaks of cyclosporiasis generally occur during the warmer months of May – September for the northern hemisphere, and November – March for the southern hemisphere. Historically, these outbreaks have been linked to ingestion of contaminated berries, fresh cilantro, basil and, more recently, ready-to-eat bagged salads.

Several efforts have been implemented to develop molecular detection methods for *C. cayetanensis* in both food vehicles and environmental water. These methods have been used to assist epidemiological investigations and surveys to estimate the prevalence of *C. cayetanensis* in commodities and growing regions. Despite these scientific efforts, there are still several significant knowledge and data gaps that hamper the implementation of effective measures to prevent the contamination of produce with the oocysts of this parasite.

Charge Questions:

FDA is seeking information on the factors that can contribute to *C. cayetanensis* contamination of domestically grown and imported produce, and recommendations for developing an effective prevention and management strategy.

1. What is known about the prevalence, incidence, and burden of disease of cyclosporiasis in the U.S. and internationally?
    a) Are there specific segments of the U.S. population that may be at higher risk for infection? What is the geographic distribution of cases in the U.S.?

b) What is the diversity of *Cyclospora cayetanensis* genotypes in the US and internationally?
c) What factors (e.g., food safety practices, location of the farms) may contribute to contamination with *Cyclospora cayetanensis*?
d) Are certain factors (e.g., type of food, seasonality, where the food is produced, degree of hand contact during growing and harvesting) more significant than others?

2. How does the seasonality, incidence and prevalence of cyclosporiasis compare throughout the United States and internationally and what factors may contribute?
   a) Extrinsic factors that may influence sporulation and survival (e.g., extrinsic factors influencing sporulation and survival);
   b) Environmental factors influencing movement (e.g., rainfall);
   c) Other?

3. What sampling data exists for *Cyclospora cayetanensis* in food products and environmental samples, domestically and internationally?
   a) What trends have been observed?
   b) What methods of detection were used?

4. What types of foods have been attributed to outbreaks of cyclosporiasis domestically and internationally and what (if any) contributing factors, sources or routes of contamination that have been identified?

5. Is monitoring for *Cyclospora cayetanensis* by testing food products, agricultural environment and agricultural inputs being applied as a management strategy currently (e.g., by industry, government)?
   a) Are there best practices for monitoring for the presence of *Cyclospora cayetanensis* in agricultural production (including matrices [e.g., water, product], frequency, timing of sample collection (pre- vs. post-harvest), and sample numbers)?
   b) Has monitoring led to development and implementation of effective preventive measures? If so, how effective have they been?

6. What are available approaches for characterizing the relatedness of different strains of *Cyclospora cayetanensis* (e.g., subtyping)?

7. What are currently available test methods (and comparative sensitivity/specificity) for detecting and/or isolating *Cyclospora cayetanensis* in different matrices (e.g., food, water, environmental samples)? What type of validation has the method(s) undergone? What are the matrices for which the methods have been validated?

8. What information exists on assessing viability of oocysts?

9. What preventive measures exist for the control of *Cyclospora cayetanensis* (e.g., using filtration)?

a) How effective have they been?
b) What are the impediments to development of effective preventive measures for *Cyclospora cayetanensis* and how can they be overcome?

10. What is known about *Cyclospora cayetanensis* persistence/survival in food, such as produce, and the environment (e.g., soil, water, food contact surfaces)?

11. What is known about transfer and attachment of *Cyclospora cayetanensis* from environmental samples (water and soil) to produce?

12. What other coccidian parasites could serve as a surrogate research model for *Cyclospora cayetanensis* behavior (e.g., for evaluation of control measures)?

13. Are there indicator organisms that can be used to determine the likely presence or absence of *Cyclospora cayetanensis* in various matrices?

14. What is known about the role of vectors (such as non-human organisms), if any, in the transmission of *Cyclospora cayetanensis*?
15. What role do farm workers play in the transfer of *Cyclospora cayetanensis* contamination during pre-harvest, harvest and post-harvest handling? Are there particular approaches that would result in selective identification of the serotypes of public health concern?
    a) How might farm workers serve as both sources and routes of contamination (such as through contamination of agricultural water, or transfer of contaminated soil to food contact surfaces or produce)?
    b) What are strategies that have been utilized to mitigate the contamination from farm workers? Have efforts to mitigate contamination from farm workers been successful?

16. Are there practices for the maintenance and conveyance of wastewater, septage or human waste that may increase the incidence of *Cyclospora cayetanensis* contamination? Are there practices that may be useful in the management of waste to reduce the potential for contamination by *Cyclospora cayetanensis* (e.g., third-party toilet service or municipal wastewater treatment)?
    a) Which wastewater, septage, and human waste treatments in the U.S. are effective against *Cyclospora cayetanensis*? Which treatments may not be effective against *Cyclospora cayetanensis*?
    b) Does municipal water treatment adequately reduce, control or eliminate *Cyclospora cayetanensis*?
    c) Can effective municipal water treatments systems be scaled to treat agricultural water used in produce production?
    d) How do practices compare for domestic growers versus international growers who export to the U.S.?
17. What elements or points in the parasite's life cycle are potential targets of strategies to disrupt its progression, eliminate or destroy oocysts, stop dissemination into the environment, and prevent food contamination?

a) What are control measures that should be evaluated for effectiveness against *Cyclospora cayetanensis*? Including control measures that can be applied to the environment and/or foods that may be consumed in the raw form.
b) What is a recommended protocol for evaluating the effectiveness of control measures against *Cyclospora cayetanensis*?

18. What are the relevant factors, available data, and data gaps needed to develop an informative quantitative risk assessment model for *Cyclospora cayetanensis* contamination and risk of illness?

COMMITTEE RESPONSES

Approach by the committee:

A number of comprehensive reviews of peer-reviewed literature on *Cyclospora* have been published recently and consulted by this committee (e.g., REFs). However, in this rapidly evolving field, a reliance on only peer-reviewed publications was deemed limiting by this Committee. Therefore, in addition to the peer-reviewed studies accessible via PubMed, the committee consulted scientific reports (such as those found in the databases of completed or ongoing research projects found in the USDA CRIS database and in the database maintained by the Center for Produce Safety), the Committee accessed documents released by federal agencies into the public domain and heard semi-structured testimonies from academic, federal and industry researchers working on *C. cayetanensis* and other parasites. Results of these findings are presented in this report.

The committee notes an on-going conversation about nomenclature of *Cyclospora* and a proposal to separate *C. cayetanensis* into 3 species (with the addition of *Cyclospora ashfordi* sp. *nov*. and *Cyclospora henanensis* sp. *nov*.). All three of these species are parasitic to humans (Barratt et al. 2023 same as: https://pubmed.ncbi.nlm.nih.gov/36560856/). However, because all prior research functionally defined *C. cayetanensis* as the only member of the genus responsible for the human cyclosporiasis, and all prior publications referred to this parasite as *"Cyclospora cayetanensis"* or "*C. cayetanensis*" the rest of this report will continue to refer to these organisms as *"Cyclospora cayetanensis"* or "*C. cayetanensis*".

Finally, the committee notes recent studies from academic and federal laboratories that demonstrated striking limitations of the detection of *C. cayetanensis* relying solely on the PCR primers designed to amplify 18S regions of the organisms rRNA genes. When environmental isolates amplified with these primers were sequenced, the majority of them (>90%) revealed amplification of closely related *Eimeria* spp. parasitic in various animals, but not humans. Therefore, throughout this report, when discussing environmental and food samples (and unless a secondary positive identification step was performed), this report discusses the detection of amplicons in a PCR reaction, not the presence of *C. cayetanensis* nor a presumptive presence of the parasite, regardless of the conclusions drawn by the authors of the original publications at the time of the original publication.

The committee organized the charge questions into 5 groups: (1) Sources and Routes (Q4, Q11, Q14 and Q15); (2) Prevalence/Persistence and indicators (Q1, Q2, Q10 and Q13); (3) Analytical Methods (Q3, Q6, Q7 and Q8); (4) Control Strategies and surrogates (Q5, Q9, Q12, Q15b, Q16 and Q17); and (5) Relevant Factors & Data Gaps (Q18).

Sources and Routes

Q4: Foods associated with outbreaks

What types of foods have been attributed to outbreaks of cyclosporiasis domestically and internationally and what (if any) contributing factors, sources or routes of contamination that have been identified?

Cyclosporiasis outbreaks have been linked to fresh fruits and vegetables, and foods containing them. From a total of 70 outbreaks reported globally, 55 were caused by fresh produce. The fruits and vegetables most frequently related to *Cyclospora* infections were: raspberries (34%), basil (31%), cilantro (10%) and salad mixes (10%). Sugar snap peas, lettuce, blueberries, blackberries, carrots, mangos, mint, scallions, mixed vegetable trays, and fruit salads were also associated with cyclosporiasis outbreaks (in some investigations, a single food vehicle was not identified.) During the summer months cyclosporiasis increases in both endemic and non-endemic regions. Research is needed to identify factors that influence the transmission of the parasite to fresh fruits and vegetables.

According to estimates from the CDC from 2011, the number of infections transmitted via food contaminated with *C. cayetanensis* per year is >11,000, which result in ~11 hospitalizations each year (Scallan et al. 2011). The incidence determined by FoodNet has increased markedly in the last five years with as many as 1.51 cases per 100,000 in 2019 (CDC, 2023 Pathogen Surveillance, FoodNet). In contrast, from 2006 to 2016 this incidence ranged from 0.03 to 0.09. In the US, the first cyclosporiasis outbreak happened in hospital workers in Illinois in 1990, which the authors attributed to the tap water in a storage tank that may have experienced a pump failure (Huang et al. 1995). While this report is often cited as the first case of domestically acquired cyclosporiasis, it is important to note that the diagnosis was based solely on a microscopic observation of spherical bodies 8-11 um in diameter, and neither the methodology nor key epidemiological data linking the outbreak to the water tank were reported. Further, in light of the currently prevailing hypothesis of the route of transmission, questions about the eventual source of the parasite in the tank of presumably chlorinated city tap water also remain unanswered.

Most studies indicate that the fecal--oral route via transmission through contaminated water and/or food is most likely for *C. cayetanensis* (1). The direct fecal-oral transmission is less likely given the observation that fecally shed oocysts (which are themselves not known to be infectious) need to sporulate into infectious spores in response to a yet unknown environmental or chemical cue. Therefore, the route of transmission is more accurately described as “fecal-environment-oral”. In the absence of known vertebrate or invertebrate vector (see discussion on vectors below), the only reasonable routes of transfer involve fecally-contaminated

agricultural water or fecally contaminated deposits on or in direct vicinity of the harvested product.

Multiple studies have been conducted by FDA, academic researchers and the industry to determine presence of *C. cayetanensis* in various water sources; these studies included samples from irrigation water, contaminated crop protectant sprays, or contaminated wash waters (Almeria et al. 2019). Almeria and coworkers summarized 20 different studies from several countries in which amplicons resulting from a PCR reaction using primers to detect *C. cayetanensis* or presumptive *Cyclospora* in water were reported. Thirteen of those studies used microscopy and seven reported a PCR method for detection of *C. cayetanensis*. However, even when DNA amplifiable with the *C. cayetanensis* ribosomal RNA PCR primers was detected in the irrigation canals, no conclusive evidence linking presumptive positives with an on-going outbreak was established. Finally and importantly, it should be noted that the primers developed to amplify fragments of *C. cayetanensis* 18S rDNA and often used in environmental surveys have a very high degree of cross-reactivity with orthologous genes from closely related *Eimeria* spp. that are not pathogenic to humans, which resulted in the vast majority (>90%) of PCR-positive environmental samples having been confirmed as belonging to various Eimeria spp. by sequencing.

Most recorded *Cyclospora* foodborne outbreaks have been linked to the consumption of fresh fruits and vegetables (Almeria, Cinar and Dubey 2019, Hadjilouka and Tsaltas 2020). The first documented case of transmission via a food product was reported in 1995 when raspberries imported from Guatemala were linked to 45 cases in the U.S. (Herwaldt 2000). It was not determined if the contamination came from direct human contact (e.g., worker hands), animals, or indirect human contact through contaminated water from poorly constructed or maintained wells, or from run off during the rainy season. Insecticides or fungicides mixed with contaminated water were also suspected. However, during the testing period, no positive results for *C. cayetanensis* were obtained from any of the environmental samples (Herwaldt and Ackers 1997). Prior to the first raspberries outbreak, water had been the only known vehicle for transmission of the parasite and to this date no food category other than fresh or fresh-cut produce has been associated with this parasite (Almeria 2019, Almeria et al. 2019).

From 1990 to 2021, more than 55 outbreaks have been reported in the U.S. (Almeria et al. 2019). In the last decade, five outbreaks were caused by fresh produce imported from Mexico, including three events due to contaminated cilantro (CDC 2018c; CDC 2020; CDC 2021). More than 70 outbreaks in different parts of the world have been reported since 1989, and from those, 55 have been suspected or confirmed to be linked to fresh produce (Almeria et al. 2019) and Table 1). From those outbreaks in which the vehicle was identified between 1995 to 2019, basil consumption was reported in 34% of outbreaks and raspberries were the vehicle in 31% of events (Hadjilouka and Tsaltas 2020). Cilantro was the third individual fresh produce commodity most frequently associated with cyclosporiasis outbreaks, and different salad mixes caused eight outbreaks (Almeria et al. 2019a) (Almeria 2019, Almeria et al. 2019). Other fruits and vegetables reported to be linked to *C. cayetanensis* transmission include snow snap peas, blackberries, blueberries, salad mixes, fruit mixes, scallions, carrots, and mangos.

Table 1. Most common food attribution of recorded *C. cayetanensis* outbreaks.

Food vehicle	No. of outbreaks (confirmed and suspected)	Years	Countries where the cases were reported	Size of outbreaks (No. cases)
Raspberries	12	1995, 1996, 1997, 1998, 2001, 2009	Canada, USA, Spain,	13 -1,465
Basil	11	1997, 1999, 2001, 2004, 2005, 2006, 2007, 2010, 2018, 2019	Canada, USA,	28 - 582
Salad mixes (including vegetable trays and coleslaw)	8	1997, 2000, 2001, 2013, 2016, 2018, 2020, 2021	Canada, Germany, Mexico, USA,	25 - 711
Cilantro	6	2003, 2004, 2013, 2014, 2015, 2018	Canada, USA	8 - 546
Berry/fruit mixes	6	1997, 1998, 1999, 2009, 2019	Canada, USA	8 – 104
Lettuce	2	2010, 2014,	Australia, USA,	227, 266
Snap peas	2	2008, 2009	Sweden, USA	4, 18

(Almeria et al. 2019a; CDC, 2020; CDC, 2021)

The first year when *Cyclospora* infections were suspected to have a domestic origin in the U.S. was in 1997, when 185 cases were reported after attending an event and consumed contaminated basil (CDC 1997). In 2001, 17 cases of cyclosporiasis were reported in British Columbia, Canada. The investigation found that 11 of 12 (92%) cases had consumed Thai basil, which had been imported from the U.S. (Ortega and Sanchez 2010). In 2017,from more than 1,060 cases of laboratory-confirmed cyclosporiasis (CDC 2017), 597 of those patients reported no international travel. In 2018, several outbreaks were recorded (CDC 2018a)(CDC 2018b) including an event associated with pre-packaged mixed vegetable (broccoli, cauliflower, carrots, and dill dip) trays. While the specific vehicle of transmission was not identified, these produce items appeared to have been grown domestically. The second major outbreak in 2018 with domestically produced vegetables involved 511 laboratory-confirmed cases in 15 states, caused by romaine lettuce and carrot salads served at a fast-food chain and produced by a fresh cut processor company (CDC 2018b).

In addition to those two outbreaks, there were clusters of cases linked to cilantro and basil reportedly grown in the U.S. In 2019, from 2,409 cyclosporiasis cases distributed among multiple restaurant and event clusters, only 10% of the patients were linked to consumption of fresh basil imported from Mexico (CDC 2019). In 2020, another multi-state outbreak that

involved 701 cases was caused by salad mixes containing iceberg lettuce, carrots, and red cabbage, distributed by the same fresh produce company from 2018 (CDC 2020). In 2021, 1,020 confirmed cases were reported with no history of international travel, including two outbreaks of 40 and 130 illnesses, respectively, in which the patients reported consuming different leafy greens (CDC 2021).

Pasteurized foods or foods thoroughly heated before consumption have not been associated with cyclosporiasis in the U.S. From the 154 outbreaks listed in the National Outbreak Reporting System (NORS) from 1971 to 2021, none of them lists food that was subjected to processing other than cutting and bagging. (CDC, 2023). Shellfish have been proposed to concentrate oocysts from contaminated waters. Controlled laboratory studies with fresh-water clams (*Corbicula fluminea*) showed that 48 to 100% of the clams retained *Cyclospora* oocysts for up to 13 days (Graczyk et al. 1998). In surveys of natural exposure of invertebrates to *C. cayetanensis,* filter feeder shellfish such as mussels and clams were found to be positive for oocysts of *C. cayetanensis*)(Aksoy et al. 2014, Ghozzi et al. 2017). Although, the review authors concluded that shellfish were unlikely to be significant to the epidemiology of cyclosporiasis because the mollusks did not travel large distances, it was noted that sampling shellfish for *C. cayetanensis* oocytes may be more efficient than sampling large volumes of water (Totton et al. 2021).

Factors, sources, or routes of contamination

For this report, we distinguish associations with environmental conditions between countries/regions where *C. cayetanesis* is endemic (and transmission is via fecal-environmental-oral route) versus those where cases of cyclosporiasis are linked to exotic introductions (via travel, or interactions with imported product).

Seasonality has been identified as one of the factors affecting the incidence of *Cyclospora* infections in the areas where *C. cayetanensis* is endemic (Li et al. 2020). In most countries, especially in the Northern hemisphere, during the summer months the cases increase markedly, but other climate factors such as rainfall seem to differ in some regions of the world (Almeria et al. 2019). The seasonality of traditionally non-endemic countries, such as the U.S., has resembled seasonal patterns of endemic countries from which produce is exported or those of popular travel destination, such as Mexico. Increased incidence between May to September has continued in the U.S. in the last four years in domestically acquired outbreak cases (CDC 2018a, CDC 2021). This coincidence in the seasonality of the presumptive domestically acquired cyclosporiasis cases is curious, but it is unclear whether it coincides or correlates with increased summer travel, migration of seasonal labor force, import of certain commodities to supplement domestic production during “shoulder seasons” or some other factor not yet accounted for. Intriguingly, a study by Barratt et al. (2022) suggests that distinct genotypes (or species) of *Cyclospora* are responsible for partially overlapping seasonally occurring outbreaks of cyclosporiasis.

In countries where cyclosporiasis is endemic, consumption of contaminated water has been consistently identified as the most important risk factor for infections (Almeria et al. 2019a).

Studies in Venezuela and Nepal have also reported a relationship between exposure to soil contaminated with human feces, exposure to livestock, and consumption of fruits and vegetables (Bhandari et al. 2015, Chacín-Bonilla 2008). Bhandari et al. (2015) is the only report that found a group of patients in which the OR was significant for exposure to livestock. This observation seemingly contradicts the prevailing hypothesis of the host range for *C. cayetanensis* and may mask an underlying livestock management practice where exposure to *C. cayetanensis* is likely. In the U.S., the majority of cases used to be linked to ingestion of imported fresh produce or to international travel, but in recent years the proportion of cases that do not have an identified connection with international origin is increasing (CDC 2018a, CDC 2019, CDC 2021).

Since 2018, as the implicated crops have been predominantly grown in the U.S., leafy greens have emerged as one of the most common vehicles, as compared to earlier years when imported produce was more frequently associated with outbreaks (CDC 2018C). Despite the periodic seasonal, occurrence of outbreaks every year, the means by which fresh produce becomes contaminated have not been elucidated. The possibility that infected field laborers were the source of food contamination has not been ruled out, nor was the parasite isolated from the crop production environment or irrigation water on farms supplying the produce implicated in outbreaks. In one of the few cases of traceback investigation on implicated farms, the FDA detected signals using primers designed for the amplification of the *C. cayetanesis* 18S ribosomal rRNA genes in a sample from a water management canal that may have supplied irrigation water to one of the farms in Florida (FDA 2020). The PCR method used at that time was an FDA-validated method, but the investigators were not able to genetically match the amplicons from the environmental isolations with clinical cases. For advancing knowledge of sources and routes of contamination, it is essential that fully validated detection protocols are applied in future prevalence and incidence studies.

Q11: Transfer and attachment

What is known about transfer and attachment of *C. cayetanensis* from environmental samples (water and soil) to produce?

A literature survey was completed on the detection, epidemiology and control of *C. cayetanensis* on produce, water and soil. The review indicated that out of 38 studies, 13 were conducted on produce, 24 were conducted on water and only one study was conducted on a soil sample. (TOTTON; O'CONNOR; NAGANATHAN; MARTINEZ *et al.*, 2021). The CDC has conducted multiple epidemiology studies during or after an outbreak period and have yet to conclusively determine if transfer is primarily from direct contact of contaminated surfaces or worker's hands or indirect contact from food contact surfaces or water sources such as irrigation water, protective sprays or wash water. The lack of conclusive results about source transfer represents a major knowledge gap and more studies are needed to better understand whether/how *C. cayetanensis* oocysts are transferred from water and/or soil to produce. The attachment of *C. cayetanensis* to plant surfaces is not fully understood but it may be enhanced by the physical structure of the plants and surface adhesive structures produced by the parasite(Tefera et al. 2018). The physical attachment of better studied parasites to non-host

surfaces may offer models that shed light on attachment and transfer of *Cyclospora* to/from plants.

Cyclospora oocysts are considered comparatively more "sticky" than *Cryptosporidium* oocysts, due to specific adhesins (proteins present on the surface of bacteria or fungi that help attach to biotic or abiotic surfaces). (Tefera et al. 2018). *Cyclospora*, *Toxoplasma*, *Eimeria* and other parasites in the Apicomplexa phylum use adhesins to promote recognition, attachment, and invasion of the host cells. The parasite could produce modified versions of the naturally occurring surface glycans in plants to increase affinity and specificity. It is suggested that susceptibility is partially determined by the surface molecules on the parasite and host that act in a concerted receptor-ligand manner (Boulanger MJ et al, 2010).
In Chandra, et al 2014, oocysts were dislodged with water from basil leaves, but were more efficiently recovered using acidified water or surfactant. This result may suggest that some parasite surface structures were involved in the covalent or physical attachment to plant surfaces. Given that little is known about *Cyclospora* attachment, a potential opportunity is to conduct a comparative analysis with *Eimeria* and what is known about attachment of *Eimeria* to animal cells. *Eimeria* attachment mechanism has been extensively studied, but attachment to animal cells almost certainly involves different mechanisms than attaching to plant cells (Fuller and McDougald, 2002). This is an opportunity for further research to determine whether this is an example of a mechanism used to attach to both plant and animal hosts. However, given the currently limited availability of *C. cayetanesis* of oocysts, these studies may need to be de-prioritized.

Q14: The role of vectors
What is known about the role of vectors (such as non-human organisms), if any, in the transmission of *C. cayetanensis*?
C. cayetanensis is known to infect only humans, and humans are the only known naturally occurring host for *C. cayetanensis*. However, the involvement of animals could not be discounted in the epidemiology of cyclosporiasis associated with fresh produce. Exposure to domestic animals/livestock has been implicated as a risk factor for cyclosporiasis. Living closely with birds, guinea pigs, rabbits (Bern et al. 2002), poultry (el-Karamany, Zaher and el-Bahnasawy 2005), and cattle (Bhandari et al. 2015) were found to be a possible hygienic factor associated in elevated incidence of cyclosporiasis. From the set-up of these correlative studies, it is unclear whether it is interactions with the livestock per se that increased the risk, or whether there were hygiene practices masked by the structure of the observations.

When *C. cayetanensis* was first recognized as an infective agent in human outbreaks, surveys were conducted in an attempt to determine whether there was a zoonotic source of the parasite. Garcia-Lopez et al, (García-López, Rodríguez-Tovar and Medina-De la Garza 1996) found what was assumed to be *C. cayetanensis* oocysts in fecal samples pooled from 600 4–6 week old chickens and a second pooled fecal sample of 50 6-8 week old chickens. The identification was based on oocyst morphology, positive acid-fast staining, positive autofluorescence under UV light and sporulation after 10 days of incubation. The authors hypothesized that it could have been a related organism, and – in retrospect – this was the likeliest conclusion, with the

researchers having almost certainly had observed a closely related *Eimeria* spp., a common poultry parasite.

Yai et al,(Yai et al. 1997) reported on two cases of dogs with unexplained diarrhea that yielded characteristic *Cyclospora* oocysts using light microscopy. The authors suggested that this contact with dogs may be important in human cyclosporiasis. Zhao et al, 2021 provided a literature review of animal surveys for *Cyclospora*-like organisms in a variety of animals including dogs, birds, cattle, insects, poultry, non-human-primates, rodents, sheep/goats, and shellfish. A variety of methods were used in these studies to identify *Cyclospora*-like organisms ranging from light microscopy with staining to distinct types of PCR. Although *Cyclospora*-like oocysts were observed microscopically or samples were positive using PCR in these studies, infection of any animal by *C. cayetanensis* was not confirmed.

A wide range of primates, reptiles, rodents and insects may serve as hosts to 19 different species of *Cyclospora* (Onstad et al. 2019, Giangaspero and Gasser 2019). Incidents have been reported of *Cyclospora* found outside of the primary host organism such as in shellfish and non-host primates in the wild and captivity, although infections of the non-host organisms were not demonstrated (Graczyk, Ortega and Conn 1998, Li et al. 2015, Marangi et al. 2015, Chu et al. 2004). Eberhard et al, identified three different *Cyclospora* species from oocytes in baboon and monkey stool samples. However, sporulated oocytes could not be identified due to the preservation process (Eberhard et al. 1999). Marangi and colleagues used primers targeted to a 116 bp region within *Cyclospora*'s ITS-2 gene. Infection of these animals by *C. cayetanensis* was not confirmed by biopsy of the small intestine or it was not performed in any of these studies (Totton et al. 2021). Several experimental studies attempted to infect other animal species with *C. cayetanensis*. The results of those experiments suggested that after 4-6 weeks of infection, there were no signs of infection indicating that any of the animals tested were susceptible to infection with *C. cayetanensis* (Eberhard et al. 2000). In reviewing surveys of natural exposure of vertebrates to *C. cayetanensis*, no publications were found that examined fish, reptiles or amphibians' exposure to *C. cayetanensis* oocysts. (Totton et al. 2021)
The hypothesis that *C. cayetanensis* is transmitted by coprophagous animals (as paratenic or transient hosts) was tested using a soil nematode model. Huamanchay et al. (Huamanchay et al. 2004) reported that while a soil nematode *Caenorhabditis elegans* was able to ingest *Cryptosporidium parvum* oocysts, oocysts of *C. cayetanensis* were not ingested by the nematode. The authors hypothesized that the observed difference was due to the much larger size of the *Cyclospora* oocysts. Despite this outcome, the authors noted that there was possibility that other nematodes may be able to ingest *C. cayetanensis* oocysts and that the role of other free-living nematodes in the mechanical transport of *C. cayetanensis* oocysts from the soil to fresh product needs to be investigated. The fact that coprophagous animals present in crop production environment (dogs, coyotes and some birds) are also a host to their own host-adapted close relatives of *C. cayetanensis* complicates interpretation of the surveys given difficulties in interpreting PCR and microscopy data without a confirmatory sequencing step.

Totton et al, (Totton et al. 2021) reviewed the role of animal vectors in the epidemiology of cyclosporiasis. In the review of natural or experimental studies of infection of animals by *C.*

cayetanensis, the authors included only studies that were specific to *C. cayetanensis* and used PCR in non-laboratory studies to identify DNA consistent with *C. cayetanensis*. They used this method of selecting studies to be included in the review because a variety of *Cyclospora* species infect animals and identification by microscopy is not sufficient to accurately identify *C. cayetanensis*. The authors also recommended that future studies use PCR coupled with DNA sequencing to confirm *C. cayetanensis* because PCR primers may cross-react with other protozoa leading to misidentification. Solarczyk (2021) also reviewed the zoonotic implications of *Cyclospora* and recommended using morphometric analysis along with sporulation analysis as a primary method in zoonotic surveys. These reports clearly stress the importance of primer design and specificity for *C*. *cayetanensis* to minimize false positives. Since insects such as houseflies are attracted to human feces, insects could be an area for future research (Totton et al. 2021).

Q15: The role of farm workers

What role do farm workers play in the transfer of *C. cayetanensis* contamination during pre-harvest, harvest and post-harvest handling? Are there particular approaches that would result in selective identification of the serotypes of public health concern?

a) **How might farm workers serve as both sources and routes of contamination (such as through contamination of agricultural water, or transfer of contaminated soil to food contact surfaces or produce)?**
b) **What are strategies that have been utilized to mitigate the contamination from farm workers? Have efforts to mitigate contamination from farm workers been successful?**

Hygienic practices of farm workers are a key focus area for prevention of the transmission of cyclosporiasis. Farm workers may be temporary seasonal workers hired for weeding, irrigation, harvesting and packing of fresh produce items in many agricultural regions of the United States. These farm workers may have been asymptomatic during the harvest period. Therefore, it is critical that farmworkers are well trained in appropriate hygienic practices, that necessary equipment is available including well-managed toilet facilities, gloves and aprons, and that there is an awareness of nearby sources of potential human fecal contamination into farm water sources.

Because *C. cayetanensis* is a host-limited parasite, human fecal contamination is the only ultimate source of oocysts in the production and processing environment. In the areas where *C. cayetanensis* is endemic, oocysts are likely common in agricultural water. In most crop production environments of the United States, feces of laborers who are symptomatic or asymptomatic carriers of *C. cayetanensis* are likely sources of the oocysts in the production or processing environment.

In 2012-2015 between April 1 and August 31, the CDC and State Health Departments identified multiple outbreaks traced to cilantro harvested from farms in Puebla, Mexico. Since none of the outbreaks were confined to a single farm, pack date, ship date and/or lot code, the FDA concluded that the contamination was from a larger source (Abanyie et al. 2015). Suggested sources of the parasite included fecal contamination of growing areas, irrigation of fields with

water contaminated with sewage, cleaning, or cooling produce with contaminated water, poor hygienic practices of workers that harvest and process the produce, and lack of adequate cleaning and sanitizing of equipment that encounters the product. Inspections of 11 farms and pack houses found human feces and toilet paper in the growing fields and around facilities; inadequately maintained and supplied toilet and hand washing facilities (no soap, no toilet paper, no running water, no paper towels) or a complete lack of toilet and hand washing facilities; food-contact surfaces (such as plastic crates used to transport cilantro or tables where cilantro was cut and bundled) were visibly dirty and not washed; and water used for purposes such as washing cilantro was vulnerable to contamination from sewage/septic systems (Graczyk et al. 1998). In these cases, the transfer was either from direct contact with human feces in the field, on supplies, workers hands and/or contaminated wash water. An increase in the chance for cross-contamination over a larger volume was observed after the cilantro was cut or chopped (Abanyie et al, 2015) (8).

Fresh produce growers, harvesters, processors, and shippers need to be aware of potential mechanisms for fresh produce to be contaminated with *C. cayetanensis* and the best practices to manage the potential risk. Farm workers can be carriers of *Cyclospora* and may or may not be symptomatic and aware of their illness, although conducting surveys of laborers in the United States will require satisfactorily addressing ethical and legal concerns. Food safety programs at growing operations that are intended for *Cyclospora* should include training for workers handling fresh produce on general hygiene, “sick worker” policies, personal protective equipment (gloves, boots, aprons, etc.) as well as management of sanitary facilities (permanent or temporary), assessment of agricultural water for potential human waste contamination, and appropriate handling of tools and equipment. There are numerous resources available to fresh produce operations and the respective supply chain that provide training materials, best practices, and assessment tools for mitigation of food safety risks associated with *Cyclospora* (REFs).

Cyclospora oocysts shed in the feces of an infected person require maturation (sporulation) outside of the host (in the environment) to become infective. Once contaminated feces are in the production environment, they can contaminate water and soil which could serve as potential routes of contamination. Fresh produce growers, harvesters, processors, and handlers must be aware that human waste can enter water systems, especially open water sources, overhead or furrow irrigation, ditches in which water can accumulate, and sewage system infiltration. Other potential sources of human waste contamination include recreational vehicles and portable toilets near a growing field (REFs). It remains to be determined how effective against *C. cayetanensis* are chemicals typically used in portable toilets, or chlorine (or other sanitizers) used to treat agricultural water. Therefore, it’s critical that a growing operation complete an assessment of surrounding land uses, the management of nearby permanent or portable toilets as well as other possible points of contamination from human feces, such as boots or clothing to build a comprehensive prevention plan.

Prevalence/Persistence and Indicators

Q1: Prevalence, incidence, and burden

What is known about the prevalence, incidence, and burden of disease of cyclosporiasis in the U.S. and internationally?

a) **Are there specific segments of the U.S. population that may be at higher risk for infection? What is the geographic distribution of cases in the U.S.?**
b) **What is the diversity of *C. cayetanensis* genotypes in the US and internationally?**
c) **What factors (e.g., food safety practices, location of the farms) may contribute to contamination with *C. cayetanensis*?**
d) **Are certain factors (e.g., type of food, seasonality, where the food is produced, degree of hand contact during growing and harvesting) more significant than others?**

The response below highlights the distribution of *C. cayetanensis* infections and illness outbreaks both in the U.S. and internationally. Many cases of cyclosporiasis illness in the US are associated with people who have traveled to other countries. Other domestic illnesses have not been associated with specific geographical areas in the US. There is evidence that young or immunocompromised people are more susceptible to infection than the general population in the U.S. The question (1b) of diversity of *C. cayetanensis* genotypes is discussed in the Question 6 response. The factors (e.g., food safety practices, location of the farms) that may contribute to contamination with *C. cayetanensis* (Question 1c) are discussed with the responses to Questions 4, 14 and 15. Additional question responses discuss factors that can prevent contamination. Factors that may be more significant for increasing the incidence of cyclosporiasis or detection of *Cyclospora* (Question 1d) are discussed in the Question 2 response and elsewhere in this report.

Distribution of *C. cayetanensis* infections and illness outbreaks internationally. At least 54 countries have documented *C. cayetanensis* infections and 13 of them have recorded cyclosporiasis outbreaks. Although *Cyclospora* appears to have a worldwide distribution, detailed epidemiological information on this pathogen group is still scarce for most countries around the world. Most of the information concerning the epidemiology of *Cyclospora* is from travelers and inhabitants of areas where this protozoon is endemic, such as Haiti, Guatemala, Peru, and Nepal. Ortega and Sanchez (2010) summarized data and information from 198 publications in a review article. They summarized that the clinical presentation is different in areas of endemicity, where asymptomatic infections are more frequent with younger children reporting more severe clinical symptoms, and infections to be milder and severity of disease to be milder as children got older. For example, the prevalence of *Cyclospora* in children in Peru with ages from 1 to 2.5 years was 18%, whereas the prevalence was 6% in children with ages from 1 month to 1.5 years. The authors hypothesize that the difference in prevalence rates for *Cyclospora* in these studies reflect the age at which children were exposed to the parasite, most likely from foods (Ortega et al. 1993). The prevalence of *Cyclospora* in Nepalese children aged 6 to 60 months who also had diarrhea was 5%, while only 2% of asymptomatic children had cyclosporiasis (Hoge et al. 1995).

Giangaspero and Gasser (2019) provided an assessment of the prevalence of *C. cayetanensis* infection in humans determined using coprological or molecular tests. They report higher prevalence rates of cyclosporiasis in endemic countries with 5.6 % in China, 9.2% in Nepal, 17.4% in Turkey and up to 22% in India. Similarly, prevalence rates of 7.9% in Haiti, 10.8% in Brazil, 24.2% in Venezuela and up to 41.6% in Peru were reported for Latin America. Among African countries, prevalence rates of 10% in Egypt and 7.2% in South Africa were reported. Lower prevalence rates were reported in non-endemic countries, from 1.9% in Canada to 0.1% in the Czech Republic, to 2.6% in Germany although there was a much higher prevalence rate of 27.5% in Italy in 2015. In areas where *Cyclospora* is not endemic, infections are symptomatic, with some reports of severe clinical manifestations (REFs).

C. cayetanensis infections are commonly reported in endemic areas with low-socioeconomic levels, although large outbreaks have also been documented in developed countries. Among susceptible populations, the highest prevalence has been documented in immunocompetent individuals with diarrhea (Li et al. 2020). The disease is self-limiting in most immunocompetent patients, but it may present as a severe, protracted, or chronic diarrhea in some cases, and the parasite may colonize extra-intestinal organs in immunocompromised patients (Mansfield and Gajadhar, 2004). Authors also report a very low incidence rate of *Cyclospora* in malnourished children and people with HIV/AIDS which seem to contradict the findings of other published reports. (Pratdesaba et al. 2001). Ramezanzadeh et al. (2022) concluded that the prevalence of *C. cayetanensis* infections among people living with HIV and/or AIDS is higher, and this sub-population is more prone to gastrointestinal disease and diarrhea due to infection.

Epidemiological studies conducted in Guatemala at three raspberry farms, two of which were involved in the 1996 cyclosporiasis outbreak in the U.S., showed that children were five times more likely to show cyclosporiasis than adults, and AIDS patients reported higher rates of infection. Infections were more common in the warmer months, coinciding with the spring raspberry harvest. The overall prevalence of *Cyclospora* was 2.3% with higher detection between May and August, with the highest indigence rate of 6.7% in June. High levels of fecal contamination were noted in the rivers from May to July with estimates of 15,000 or more oocysts per 10 liters (Bern et al. 1999). Curiously, Pratdesaba et al. (2001) reported no cases of *Cyclospora* in fecal samples of raspberry farm workers in Guatemala in a one-year study.

Distribution of *C. cayetanensis* infections and illness outbreaks in the U.S. Hall et al. (2012) summarized data regarding laboratory-confirmed cases of *Cyclospora* infection in the U.S. reported during 1997-2009 via the Foodborne Diseases Active Surveillance Network (FoodNet), which gradually expanded to include 10 sites (Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, and selected counties in California, Colorado, and New York) that represent approximately 15% of the US population (Hall et al. 2012). A total of 370 cases were reported during this period, with 70.3% (260) of cases from residents of Connecticut (134 [36.2%]) and Georgia (126 [34.1%]), which accounted for 29.0% of the total FoodNet population under surveillance.

About a third of the 1,110- laboratory confirmed sporadic cases of cyclosporiasis in the U.S., recorded by the CDC from 1997 to 2008, were linked to persons with a known history of international travel who might have become infected while traveling outside the continental United States. The majority (278 [69.8%]) of onset or diagnosis dates for domestically acquired cases occurred during April-August (Hall et al. 2011). The authors report that some of these cases were outbreak-associated but were not linked to other cases, in part because of a lack of molecular tools. Overall, the case-patients' median age was 44 years (range: 3 months-96 years); 50.5% were female, 47.2% were male, and the sex was unknown for 2.3%. Gender does not have a significant effect on the *Cyclospora* infection rate in different geographical areas. In endemic areas where water and food sanitation are poor or non-existent, cyclosporiasis seems to be particularly affecting children (Bern, 2002).

A total of 372 case-patients (33.5%) had a documented history of international travel during the two-week period before symptom onset or diagnosis, 398 (35.9%) reported no international travel, and 340 (30.6%) had an unknown travel history. Among the 398 case-patients classified as having domestically acquired cases, 124 persons (31.2%) lived in Florida, and 64 persons (16.1%) lived either in NYC (49 persons) or elsewhere in New York state (15 persons). The extent to which the geographic concentration reflects higher rates of testing, more sensitive testing methods, or higher exposure/infection rates is unknown. Of note, cyclosporiasis is a reportable disease in 43 states, the District of Columbia and New York City (CDC, 2022). Casillas et al. (2019) reported that five of the ten outbreaks of cyclosporiasis investigated during this period were linked to foods of domestic and international origin. They indicate that many of the sporadic domestically acquired cases might have been associated with identified or unidentified outbreaks, and the potential associations were not detected with the available epidemiological information .

The five-year surveillance data from the U.S for the period 2011- 2015 shows seasonal increases in reported cases of cyclosporiasis during spring and summer months. Barratt et al. (2022) suggest that distinct genotypes (or species) of *Cyclospora* may be responsible for the outbreaks occurring earlier and later in the summer, this report is discussed in more detail below.

Q2: Seasonality, incidence, and prevalence

How does the seasonality, incidence and prevalence of cyclosporiasis compare throughout the United States and internationally and what factors may contribute?

a) **Extrinsic factors that may influence sporulation and survival (e.g., extrinsic factors influencing sporulation and survival);**
b) **Environmental factors influencing movement (e.g., rainfall);**
c) **Other factors?**

Cyclosporiasis exhibits a seasonal pattern globally. In the U.S., the peak season occurs from May to August. The seasonality of infections varies geographically, and infections can be more prevalent in dry seasons or in rainy seasons. Detection frequencies of *Cyclospora* oocysts throughout the year can vary and may not correlate to patterns of seasonal infections. The

factors contributing to the seasonality of cyclosporiasis are not fully known, and the variations across regions cannot be attributed to a single common factor, although recent evidence suggests that distinct genotypes (or species) of *Cyclospora* may be responsible for the outbreaks occurring in different seasons (Barratt et al., 2023 REF). The sporulation and survival of *Cyclospora cayetanensis* can be influenced by various external factors. While the application of some cold and hot temperatures affects sporulation and survival, exposure to some commonly used pesticides and antimicrobial chemicals has been shown to have a limited effect.

Environmental factors influencing seasonality of incidence or prevalence of cyclosporiasis. *C. cayetanensis* infection is remarkably seasonal worldwide (REF). This seasonality varies by region, most likely due to human activities, environmental contamination, and the optimal sporulation conditions in each area. The reasons for the apparent absence of symptomatic human infection for prolonged periods, where the parasite is present in the environment, and which biological conditions are needed for the survival of the parasites during these prolonged periods is unknown. Factors such as rainfall, temperature, humidity, and perhaps photoperiod could affect the seasonality, which clearly cannot be related to rainfall alone, as there is a marked seasonal variation in very dry environments (REF). The incidence of *C. cayetanensis* infection increases in warm periods of maximal rainfall in countries such as Guatemala, Honduras, Mexico, Jordan, Nepal or China (REF). These conditions contribute to the contamination of water supplies with *Cyclospora* oocysts (REFs). However, infection is more prevalent in the absence of rain, during the drier and hotter months of the year in Peru and Turkey. In Haiti, infections occur during the driest and coolest times of the year, or during the cooler wet season in Indonesia. In India, clinical cases were more frequent in the summer before the rainfall period (REF). Therefore, it is difficult to explain a common factor for the differences observed in seasonality.

In a study conducted in Colombia by Frickmann et. al., (2021), fewer individuals (2/16, 12.5%) reported gastrointestinal symptoms in the rainy season compared to the dry season (6/15, 40%) despite higher parasite loads in the rainy season. A considerable prevalence of *C. cayetanensis* in Colombian indigenous people persists in the dry season. Low proportions of gastrointestinal symptoms along with higher parasite loads make colonization likely rather than infection, and the data between environmental detection of the parasite and clinical presentation in the population remains unexplained.

Cyclosporiasis cases are reported throughout the year in the U.S., but there is an increase in domestically acquired cases from May to August. During non-outbreak periods between 1992 and 1995, the rate of *Cyclospora* endemic infection in the general population of North America and the United Kingdom was less than 0.5% (REF). However, there were variations in the prevalence of infection across different regions within the U.S. Between 1997 and 2009, out of a total of 370 laboratory-confirmed cases of *Cyclospora* infection reported through the Foodborne Diseases Active Surveillance Network, the majority (70.3%) were concentrated in Georgia and Connecticut. In the period from 2004 to 2009, 37.8% (70/185) of the cases were classified as domestically acquired. It is important to note that while cyclosporiasis is not considered endemic in the U.S., there is a possibility of localized areas with

low-level endemicity (REF). Additional research into domestic prevalence, environmental contamination, and endemicity could be considered.

The factors contributing to the seasonality of cyclosporiasis are not fully known, and the variations across regions cannot be attributed to a single common factor. There is still uncertainty regarding the absence of symptomatic human infection during certain periods when the parasite is present in the environment and the specific biological conditions required for parasite survival during these periods. In non-endemic industrialized nations, individual cases and outbreaks are primarily linked to international travel and the consumption of contaminated imported produce from endemic regions. *Cyclospora* oocysts have been detected in produce outside the typical seasonality of cyclosporiasis cases in the US, indicating the potential year-round presence of oocysts in certain produce. However, the detection of oocysts does not necessarily imply their sporulation or infectivity, nor does it guarantee illness if consumed.

Barratt et al (2023) suggested that, at least in part, the seasonality of domestic outbreaks can be explained by the distinct genotypes (or species) of *Cyclospora,* with the Lineage A being responsible for the outbreaks appearing earlier in the season and peaking around June, and Lineage B most prevalent in outbreaks later in the season peaking around July. While this is an intriguing hypothesis, it is important to note that each of these "domestic" lineages included at least one isolate common to areas of Mexico and/or Central America where the majority of the US seasonal labor force originates. These genomic data should be analyzed in the systems context which includes seasonal crop production patterns.

Extrinsic factors that may influence sporulation and survival of oocysts. *Cyclospora* oocysts are formed in enterocytes, excreted unsporulated in feces, and require sporulation to become infective to a host. Transmission usually occurs through the ingestion of oocysts found in fecally-contaminated water or produce. Direct person-to-person transmission is unlikely as the excreted oocysts are not infectious, requiring sporulation to take place outside the host before becoming infective. The median incubation period is approximately one week, during which the organism invades the enterocytes of the small intestine (REF). It is worth noting that oocysts from some patients with severe diarrhea may not undergo sporulation (REF).

The sporulation and survival of *Cyclospora cayetanensis* can be influenced by various external factors. For example, under laboratory conditions at temperatures of 22°C and 30°C, sporulation of *Cyclospora* oocysts stored in deionized water or potassium dichromate typically takes place within 7–14 days outside the host. However, exposure of oocysts to temperatures of 37°C for 4 days or 50°C for 1 hour has been observed to induce sporulation. Conversely, storage at 4°C or 37°C for 14 days delays sporulation, with only 12% of human- and baboon-derived *Cyclospora* spp. sporulating under such conditions. Interestingly, oocysts that were stored at 4°C for one to two months sporulated when subsequently stored for six to seven days at 30°C (REF).

The effects of temperature, including freezing and heating conditions, on the sporulation of *C. cayetanensis* were investigated in dairy products and basil. Sporulation was observed in

these matrices at 23°C, but extreme temperatures led to the inactivation of oocysts. No sporulation occurred at temperatures of -70°C, 70°C, and 100°C for both water and basil samples. Similarly, dairy products did not exhibit sporulation when cooked at 70°C, frozen at -70°C for 1 hour, or exposed to -15°C for 24 hours. Basil kept at -20°C for two days and water stored for four days also did not support oocyst sporulation. Additionally, the use of recommended concentration levels of pesticides, including fungicides and insecticides, or combinations of these products did not affect the sporulation of *C. cayetanensis*. Due to the limited understanding of the mechanisms triggering sporulation in *Cyclospora cayetanensis* oocysts and the factors influencing their survival, it would be prudent to investigate factors that affect sporulation and survival in similar parasites. Particularly, the examination of surrogate organisms in future challenge studies could provide valuable insights into the study of *Cyclospora cayetanensis* (REF).

Q10: Persistence/survival in food and the environment

What is known about *Cyclospora cayetanensis* persistence/survival in food, such as produce, and the environment (e.g., soil, water, food contact surfaces)?

C. cayetanensis oocysts have been detected in several types of water including chlorinated and unchlorinated drinking water, food/agricultural process water, wastewater, recreational waters, and well water. Furthermore, this organism has been detected in areas where soil can contact human feces and in areas where there is a lack of personal hygiene. Further research is needed to elucidate survival times and sporulation rates in water, soil, and food or agriculture process environments.

Water and soil contaminated with fecal matter may act as a vehicle of transmission for *C. cayetanensis* infection. In endemic areas, drinking water has been determined as a risk factor for cyclosporiasis. *C. cayetanensis* oocysts have been detected in several types of water—including chlorinated water, and wastewater in endemic areas and in non-endemic areas—which suggests the potential spread of the parasite via drinking and recreational water (Almeria et al. 2019) (Rabold et al. 1994) (Kwakye-Nuako et al. 2007). Oocysts can pass through physical barriers and are not affected by chlorine and other water disinfectants (Mansfield and Gajadhar, 2004). Studies conducted in Guatemala concluded that significant risk factors for cyclosporiasis, among children <2 years of age, were drinking untreated water and soil contact. These studies also found that among 182 people in the cohort, four farm workers had asymptomatic cyclosporiasis (Bern et al. 1999).

Exposure to recreational water contaminated with *C. cayetanensis* oocysts may also be a source of infection (Bilung et al. 2017). Nine percent of water samples (20 out of 233) collected along a river in Spain over a one-year period tested positive for *Cyclospora* spp. with 17/20 positive in a qPCR with primers amplifying 116-bp fragments in the internal transcribed spacer 2 (ITS-2) gene (Lalonde and Gajadhar, 2008) (Galvan et al. 2012). Nine of 48 samples of influent and effluent water from wastewater treatment plants in Arizona showed the presence of *C. cayetanensis*. The authors reported that they did not determine the efficacy of the removal of *Cyclospora* in the treatment process (Kitajima et al. 2014). These studies show that fecally contaminated water could be a potential source of *Cyclospor*a contamination.

Soil is a potential and possibly important mode of transmission and source of infection for *C. cayetanensis*. Some studies have included contact with contaminated soil as a risk factor for *C. cayetanensis* infections, in both developing and developed countries (Mansfield and Gajadhar, 2004). In Venezuela, for example, most cases of *C. cayetanensis* were clustered in the areas of extreme poverty where living in a hut, not having a toilet, and having contact with soil contaminated with human feces were strongly associated with infection. *C. cayetanensis* was more prevalent where agricultural work and lack of hand washing were present. In Italy, soil was found to be positive for oocysts (11.8% positive samples, 6/51) (Giangaspero et al. 2015a). Higher rates of infection have been noted in additional areas where risk factors such as deficient sanitary facilities, poor personal hygiene, and soil contaminated with human feces were present.

Q13: Indicator organisms

Are there indicator organisms that can be used to determine the likely presence or absence of *C. cayetanensis* in various matrices?

An indicator organism is a microorganism or group of microorganisms that may indicate a possible presence of a pathogen of concern, that are typically present in much lower numbers than indicators or that conditions under which an indicator increases in numbers may favor pathogen growth (Busta, et al., 2003). Indicator organisms for parasites such as *C. cayetanensis* are difficult to identify. Since *C. cayetanensis* can only originate from human feces, an indicator of human fecal pollution is likely to provide a practical solution. The committee acknowledges the multitude of studies on advantages and also limitations of indicators of human fecal contamination, as well as at least a dozen existing and at least that many proposed indicators of human fecal contamination.

A study by Mattioli et al. attempted to correlate the presence of a fecal indicator bacteria, *Escherichia coli,* and human-specific fecal molecular markers, Bacteroides HF183 and crAssphage with the presence of *C. cayetanensis* in the crop production environment (REF). However, while this study detected the presence of some of these markers of human fecal pollution*, C. cayetanensis* was not detected in any of the samples. This outcome should not be considered discouraging as indicators often overestimate the potential for the presence of fecally-shed human pathogens. Given that essentially nothing is known about persistence of *C. cayetanesis* in the environment and given that oocysts of the parasites are currently in a limited supply to conduct correlational or comparative studies with well-characterized indicator organisms, it is unclear how productive efforts to identify a “perfect indicator” for the presence of *C. cayetanensis* would be. Collectively, these results indicate that future efforts should continue to programs on risk-based management, not on efforts to manage hazards, whether potential or perceived as potential.

Analytical Methods

Isolation, Concentration, Detection and Confirmation

As discussed throughout this report, *C. cayetanensis* is parasite with a host range that is limited to humans, while many other animals host very closely related organisms that are nonpathogenic in humans. This, therefore, highlights the primary challenge with the isolation, concentration and detection of *C. cayetanensis*: any *Cyclospora* isolated from a human fecal sample is almost certainly *C. cayetanensis* (because humans act as "biological concentrators" of the parasite), however, environmental isolates of *Cyclospora* could have originated from a nearly infinite number of potential hosts of non-human parasites. Because a *Cyclospora* from a human sample is almost certainly *C. cayetanensis*, a fairly generic target (such as 18S ribosomal RNA gene) for the typing at the genus level is practically sufficient (to distinguish from other eukaryotic or procaryotic causes of gastrointestinal symptoms). However, using 18S ribosomal RNA genes as targets for environmental samples has led many researchers to erroneous conclusions about prevalence of *C. cayetanensis* in environmental samples collected in regions where the parasite is not endemic. These limitations were highlighted by recent studies of Mattioli (REF), and a retrospective re-analysis of samples previously thought to be *C. cayetanensis* by Ortega (REF). A nearly 90% false-positive rate for PCR-based assays using a common method targeting 18S ribosomal RNA genes highlights the need for a more robust and reproducible method for the detection of the parasite to the species level in environmental samples.

In the absence of a robust, specific and reproducible single-step method for the detection of *C. cayetanensis*, there remains the need for confirmatory molecular methods of the PCR-positive samples from implicated foods and potential contamination sources. A genotyping system for *Cyclospora* based on eight genetic markers has been applied to human clinical samples (Almeria et al. 2019). This approach, although helps to discriminate between clinical cases, still requires development for food sampling and improved cluster detection, and does not alleviate the concern that only a genus-level detection is practically sufficient for clinical samples, while at least species-level (and ideally strain-level) detection is required for environmental samples.

Whole-genome sequencing is impractical for routine molecular surveillance of *C. cayetanensis* outbreaks because of the inability to culture the organism (which makes it difficult to obtain sufficient DNA mass from samples) and due to its large genome (44 megabases). To address these issues, researchers have focused on the development of new methods based on potential genomically-derived markers for strain-level identification (Nascimento et al. 2020, Gopinath et al. 2018). One approach has been to apply bioinformatic analyses to public mitochondrial genome assemblies to create a reference genome which can then be used in the application of subtyping *C. cayetanensis* strains during foodborne outbreak investigations (Nascimento et al. 2020). In addition, it is worth exploring other options for the detection and differentiation of C. cayetanensis such as infrared-functionalized microbalance sensor (Santin and Tetard, CPS REF).

Q3: Sampling data

What sampling data exists for *Cyclospora cayetanensis* in food products and environmental samples, domestically and internationally?

a) **What trends have been observed?**

b) What methods of detection were used?

Summary of Question 3 Response

Currently, there are no international standards for testing for *C. cayetanensis* in the environment and food products. Since the FDA BAM Chapter 19c detection method was validated, most studies have used either this method or a modified methodology, however, this method targets 18S ribosomal RNA genes, and the limitations of this approach have been discussed in this report. However, the prevailing consensus is that more methods need to be developed that are able to isolate the small numbers of oocysts from environmental samples, in addition to the various food matrices. Even when *C. cayetanensis* is detected in environmental samples, additional confirmatory testing is required due to the significantly high number of false positives from cross- reactions with related parasites that are not pathogenic in humans.

Q3a). What trends have been observed?

As discussed earlier in this report, data collected from regions and countries where *C. cayetanensis* is endemic should not be co-interpreted with the data from the regions where the parasite has not established endemically. Seasonal trends and epidemiological trends for the areas where *C. cayetanensis* is endemic have been discussed elsewhere in this report. Epidemiological trends in the US have been discussed in response to other questions.

Prior to the reports of Mattioli (REF) and Ortega (REF), detection of 18S ribosomal RNA gene amplicons in environmental samples has been interpreted to indicate the presence of *C. cayetanensis* in an environmental (primarily, water) sample. Given an alarming (~90%) false positive rates of a common PCR-based method for the detection reliant on primers for 18S ribosomal RNA genes, discussing trends based on the results of studies in which a definitive confirmatory step (such as amplicon sequencing) was not performed is premature.

Q3b). What methods of detection were used?

Currently, there are no recognized International Organization for Standardization methods for detecting *C. cayetanensis* in foods and the environment. Most studies have been conducted using BAM 19b, BAM 19c, or a modification. (Lalonde, L. et al 2022). Current 18S-based methodologies do not appear to be sufficiently sensitive to distinguish *C. cayetanensis* from *Eimeria or Isospora* species. (Mattioli et al. 2022)

The Center for Produce Safety (CPS) sponsored a study, from January 2020 through April 2022, to examine the sources and prevalence of *C. cayetanensis* in irrigation water, harvested produce (using spent packinghouse water in dump tanks as a proxy for the produce), and municipal wastewater in the Southeastern Coastal Plains region in Georgia. (Mattioli 2022).

At the start of the project in 2020, the researchers collected samples from the surface-fed holding ponds once a month during the fallow and growing periods and twice a month during harvesting. In 2021, the sampling frequency was increased to twice a month during the fallow and growing seasons. The researchers collected weekly samples from the spent packinghouse water in the dump tanks, the spent water being used as a proxy for harvested produce. The samples were either filtered onsite or, if the turbidity was too high, the samples were sent to the

CDC. Municipal wastewater sludge samples were taken from the thickener sludge and from the return activated sludge from the aeration basin, Dead-end Ultrafiltration was used to concentrate holding pond water samples and continuous flow centrifugation was used to concentrate dump water from the packinghouses. Sludge and portable toilet samples were concentrated via centrifugation. All samples underwent DNA extraction followed by quantitative PCR (qPCR). BAM Chapter 19C defines a positive as any sample that has at least one of the three qPCR replicates below a C_q of 40. The researchers deviated from the cutoff C_q value in BAM 19C. Instead, they used a $Cq_{of} \leq 37$, to reduce the number of false positives. This should have increased (not decreased) the sensitivity of the method by ^3. Samples with at least one replicate with a $C_q \leq 37$ were submitted to the CDC Parasitic Branch. This was useful to eliminate false-positive results. Of the 217 samples from eight surface-fed holding ponds, 18S rRNA amplicons were detected in 59 (27%). 18S rRNA amplicons were detected in only one of 46 (2%) dump tank water samples. No 18S rRNA amplicons were detected in the 37 samples from the on-farm portable toilets. Of the total of 76 sludge samples, 18S rRNA amplicons were detected in nine (20%) sludge from the thickener and nine (30%) return activated sludge. However, of the samples submitted for amplification, only one sample matched *C. cayetanensis* haplotypes from clinical specimens, which indicated low level community shedding. Despite positive 18S rRNA amplicon detections, their sequencing failed to confirm amplicons as those belonging to *C. cayetanensis*. Furthermore, positive qPCR detection in irrigation pond samples was not associated with human fecal contamination (Mattioli et al. 2022), consistent with the fact that the detection of amplicons resulted from cross-reactivity with non-human isolates of *Cyclospora* relatives.

Giangaspero et al conducted the first comprehensive molecular survey, over a two-year period from 2012 to 2014, looking for *C. cayetanensis* in southern Italy. They examined water (treated water from the municipal treatment plants, drinking water, and well water used for irrigation), eight types of vegetables and fruits (cucumber, lettuce, fennel, celery, tomato, melon, endive, and chicory), farm soil from the bases of the selected vegetables and fruits, and human fecal samples, that had been submitted to the main area hospital. The water samples were filtered through a 1 µm yarn-wound cartridge filter which was backflushed three times then concentrated using centrifugation. Likewise, soil and produce samples were placed in suspension, centrifuged and filtered through double gauze and the filtrate again centrifuged. All sedimented pellets were subjected to Percoll-sucrose flotation and DNA extraction. The samples were tested using qPCR-coupled single strand conformation polymorphism (SSCP) analysis and DNA sequencing. Giangaspero et al detected *Cyclospora* DNA in 21.3% of treated water samples and 6.2% of well water samples but did not detect *Cyclospora* DNA in drinking water samples. Detection rates in soil and produce samples were 11.8% and 12.2% respectively. (Giangaspero et al. 2015c). The survey did not use controls, therefore, as seen with other studies, it is difficult to determine if the positive samples cross-reactions and, therefore, false positives.

Table 2. CPS Report Results, Mattioli et al (2022)

Sample source	n	18S rRNA detected	% Detection	Confirmed C. cayetanensis
Irrigation ponds	217	59	27	0
Packinghouse dump tanks	46	1	2	0
Municipal wastewater sludge (RAS/REC)	46/30	9/9	20%/30 %	7
Portable toilets	37	0	0	0

Table 3. Southern Italy Survey Results, Giangaspero et al (2015)

Sample source	n	% Detected *Cyclospora* DNA
Well water used for irrigation	16	6.2
Treated water from municipal treatment plants	94	21.3
Drinking water	3	0
Farm soil surrounding produce	51	11.8
Produce (vegetable and fruit crops)	49	12.2
Human feces (submitted to main hospital)	40	27.5

Q6: Approaches for characterizing

What are available approaches for characterizing the relatedness of different strains of *C. cayetanensis* (e.g., subtyping)?

Clearly understanding relatedness of strains and species of *C. cayetanensis* has at least two practical implications. First, there is a need to define genomic targets for the specific detection and differentiation of strains of *Cyclospora* capable of causing human illness, as currently available tools based on the amplification of 18S rRNA genes fail to do so reproducibly and robustly. Second, the question of endemicity of *C. cayetanensis* in the U.S. remains open (with environmental sampling data being called into question and the uncertainty with the interpretation of sewage data as the main argument for endemicity). Robust and conclusive molecular evidence will be required to address it. For example, does clustering of *C. cayetanensis* from domestic outbreaks with seemingly random isolates from a number of countries where the pathogen is endemic and most agriculture labor force originate argue for the "exotic introduction" hypothesis for the origin of the parasite in each outbreak? If *C. cayetanensis* has established endemically in some areas of the U.S., how soon should we

expect genome-level separation of the “US isolates”, given that the parasite has a diploid genome and undergoes sexual reproduction? The presence of distinct regional clusters is supported by current studies (Barratt REF), however, it remains to be elucidated whether it took years or decades for these regionally-distinct genotypes to evolve. Given that *C. cayetanensis* can only reproduce inside the human host and given a relatively low prevalence of human cyclosporiasis in the U.S., the temporal scale of evolution of the geographically distinct strains may be longer than what is expected for areas where the pathogen is endemic and cycles rapidly through human populations.

Recently, whole genome assemblies, complete mitochondrial and apicoplast genomes of *C. cayetanensis* have become available (Cinar et al. 2016). Cluster analysis of specific *C. cayetanensis* apicoplast genomes revealed tight grouping of *C. cayetanensis* with *Eimeria* and *Toxoplasma*, separated from distant species such as *Plasmodium* and *Babesia*. Single nucleotide polymorphisms (SNPs) and identified DNA sequence repeats may be useful as genetic markers for identification and differentiation of *C. cayetanensis* isolates found and could facilitate outbreak investigations (Cinar et al. 2016) The mitochondrial genome and apicoplast genomes of *C. cayetanensis* have a high similarity to *Eimeria* spp., which has clearly complicated PCR-based detection of the parasite in environmental samples. The chromosome genome of *C. cayetanensis* has important differences that help to differentiate this organism from other apicomplexans. Human *C. cayetanensis* isolates from around the world have noticeable geographic clusters. *C. cayetanensis* genotyping methods, using targeted amplicon sequencing, are useful for epidemiological trace-back investigations (Cinar et al. 2020). Molecular typing of *C. cayetanensis* in produce and clinical samples can distinguish between case clusters and may be helpful for cyclosporiasis outbreak investigations. (Zhang, et al. 2021).

To supplement the epidemiological data with genetic information, (Yanta et al. 2022) genotyped isolates from stool samples in 169 Canadian cyclosporiasis cases which occurred between 2010 to 2021. An eight-marker targeted amplicon deep (TADS) scheme specific to *C. cayetanensis* as previously described by the U.S. Centers for Disease Control and Prevention (CDC) was used. Their study focused on evaluating the genotyping performance and genetic clustering of the Canadian *C. cayetanensis* isolates and reports that genotype information was successfully collected with at least part of one of the markers in the TADS assay for 97.9% of specimens, and 81.1% of cyclosporiasis cases met the minimum requirements to genetically cluster into 20 groups. The authors conclude that examining cyclosporiasis cases genetically will be a valuable tool for supplementing epidemiological outbreak investigations and further research is required to expand the number of discriminatory markers to improve genetic clustering. From March 2018 to October 2020, a total of 3459 *C. cayetanensis* genotypes were sequenced from fecal specimens collected from patients who received a diagnosis of cyclosporiasis in the U.S. or Canada, and from 4 specimens collected before 2018 ((Nascimento et al. 2020); (Barratt et al. 2021), (Barratt et al. 2022)).

Barratt et al., (2023) reported genotyping thousands of US isolates and 1 from China (strain CHN_HEN01) and revealed two lineages. Their retrospective examination of epidemiologic data revealed associations between lineage and the geographical distribution of U.S. infections plus

strong temporal associations. With the multiple lines of evidence for speciation within human infecting *Cyclospora*, the authors provide an updated taxonomic description of *C. cayetanensis* and describe two novel species as etiological agents of human cyclosporiasis: *Cyclospora ashfordi* sp. nov. and *Cyclospora henanensis* sp. nov. (Apicomplexa: Eimeriidae). The Barratt et al. (2023) study may be the first study suggesting the existence of two "US species/genotypes" of *Cyclospora*, a potential evidence that the parasite is becoming endemic in the United States. However, a critical examination of the conclusions raises the following questions: (1) Lack of panmixia between the two "US genotypes" cannot be interpreted using the Hardy-Weinberg principle: because *C. cayetanensis* undergoes sexual and asexual reproduction only within a human host for Hardy-Weinberg principle to apply random and multiple co-infections with multiple strains must occur. While this is possible in the regions where the parasite is endemic, it is not the case in the United State. (2) Existence of regional genotypes in the Midwest and New York (areas that experience prolonged freezing temperatures) would be a deviation from what is assumed to be known about the climatic zones where *C. cayetanensis* thrives. (3) The prevalence of *C. cayetanensis* Lineage A in Georgia (at the levels observed in New England) does not fit with the observation that Lineage B is prevalent in neighboring states. Finally, it should be noted that each of the "US lineages" included isolates most closely related to those isolated from Mexico and Guatemala. The same clustering of the US isolates with those from Mexico and Guatemala (where a significant number of the non-permanent US ag laborers originate) was reported by Leonard et al (2023). In contrast, in both studies (Barratt 2023 and Leonard 2023), isolates from Asian neighboring countries (Nepal, China and Indonesia) cluster tightly and separately. It is unknown whether the researchers would have reached the same conclusion if more genomes from clinical samples in Mexico and Central America were included into the study. These early studies are interesting, however, it is premature to use them as genetic evidence for the endemicity of *Cyclospora cayetanensis* in the United States. A clear understanding of the length of time required for speciation (or evolution of dominant genotypes) of *Cyclospora* will be required to interpret these studies.

With the recent advances in sequencing technologies such as next generation sequencing (NGS) and availability of efficient genome assembly programs, whole genome assemblies, complete mitochondrial and apicoplast genomes of *C. cayetanensis* have become available (Cinar et al. 2016). Whole-genome sequence data from *C. cayetanensis* protozoa enabled the development of a multilocus sequence typing (MLST) tool for characterizing isolates in outbreak investigations. The high resolution of the typing tool and the apparent presence of geographic clusters might facilitate the identification of outbreaks and infection sources. (Guo et al. 2016). One method based on MLST has been recently developed by CDC researchers. This method relies on the amplification of 8 genetic markers followed by deep sequencing and bioinformatic analysis of the data. This method has been used to characterize haplotypes of *C. cayetanensis* for molecular epidemiology purposes (Nascimento et al. 2020, Barratt et al. 2021, Barratt et al. 2022). This method has been implemented at FDA to be used on characterization of *C. cayetanensis* haplotypes of DNA extracted from produce and water that is found to be positive for the presence of *C. cayetanensis*. In 2021 this MLST approach was applied to environmental samples collected from a canal in FL. Characterization of *C.*

cayetanensis haplotypes using this approach was possible in 6 of the 8 samples analyzed (FDA/CDC report from August 13th, 2021- unpublished data). This was a follow up of the work done as part of investigation to identify the root cause of the 2020 bagged salad outbreak. In 2022 FDA will begin to apply the CDC genotyping method, with modifications, to *C. cayetanensis* collected from produce and water samples, which will enable the linkage of human illness to suspect food items19.

Q7: Current available test methods for detecting and/or isolating
What are currently available test methods (and comparative sensitivity/specificity) for detecting and/or isolating *C. cayetanensis* in different matrices (e.g., food, water, environmental samples)? What type of validation has the method(s) undergone? What are the matrices for which the methods have been validated?

Detection methods

C. cayetanensis is an unculturable parasite, therefore all analytical methods used for the detection and characterization of *C. cayetanensis* in different types of samples rely on microscopy techniques, detection of the parasite's DNA (e.g., PCR methods), and/or DNA sequencing analysis of suitable genetic markers.

In clinical settings, *Cyclospora* infection is diagnosed by examining stool specimens using various microscopy techniques and/or by PCR assays designed to detect the parasite in stool (CDC 2023). Symptomatic patients are known to at times shed low numbers of *Cyclospora* oocysts, therefore sample preparation techniques to concentrate the oocysts, such as the formalin-ethyl acetate sedimentation technique, are typically used to increase the chances of detection (CDC 2023). Smears of the resulting sediment can be stained and examined microscopically using modified acid-fast or modified ("hot") safranin techniques, although *Cyclospora* oocysts may not uniformly stain and appear either stained or unstained in microscopic fields when using the modified acid-fast technique. Alternatively, an ultraviolet (UV) fluorescence microscope (set at 330-365 nm or 450-490 nm) can be used to view *Cyclospora* oocysts since they autofluorescence (CDC 2023). Although these microscopy techniques are effective for clinical diagnosis, they cannot distinguish oocysts of *C. cayetanensis* from morphologically identical oocysts of other *Cyclospora* species which may be present in food, environmental, or other zoonotic samples (Eberhard, Pieniazek and Arrowood 1997, Eberhard et al. 1999).

C. cayetanensis oocysts are expected to be present in exceedingly low numbers in food and environmental samples, when present at all. Unlike clinical samples, food and environmental samples are expected to contain significant background populations of other parasites, including non-pathogenic species of *Cyclospora* and closely genetically related Apicomplexan parasites, such as species of the genus *Eimeria*. Due to the limitations of microscopy techniques to detect *C. cayetanensis* oocysts in food and environmental samples, molecular methods, such as PCR, represent the most feasible approach for detection in these matrices (Lalonde and Gajadhar 2008, Murphy et al. 2017, Durigan, Murphy and da Silva 2020, Kahler et al. 2021, Barlaam et al.

2021, Lalonde, Oakley and Fries 2022). Sample preparation techniques, such as flocculation, floatation, filtration, and centrifugation, have been used alone or in combination to concentrate oocysts and improve the chances of detecting *C. cayetanensis* in food and environmental samples. Challenges to developing molecular detection methods for *C. cayetanensis* in food and environmental samples include matrix complexity (including the potential presence of PCR inhibitors), the inability to culture the organism *in vitro*, and the lack of genomic sequences for *C. cayetanensis* and other closely related organisms (Balan et al. 2023). Currently, FDA has validated two methods for the detection of *C. cayetanensis*, one that is specific for detection in fresh produce and another that is specific for detection in agricultural water. These methods employ various sample preparation techniques followed by a quantitative real-time PCR (qPCR) targeting the 18S rRNA gene with a species-specific probe and an internal amplification control.

The current FDA method for the detection of *C. cayetanensis* oocysts on fresh produce (Chapter 19b of the FDA Bacteriological Analytical Manual (BAM)) (FDA 2023a) was validated in a collaborative study to detect as few as five oocysts inoculated onto 25 g samples of cilantro or 50 g samples of raspberries (Murphy et al. 2017). This method uses a procedure to recover inoculated oocysts from produce previously demonstrated to significantly improve the recovery of *C. cayetanensis* oocysts from basil and lettuce and a commercial DNA extraction kit (Shields, Lee and Murphy 2012, Murphy et al. 2017). The collaborative study included a comparison of the nested PCR from FDA's previous method with the qPCR in the current method. Although the nested PCR detected *C. cayetanensis* at the 5-oocyst inoculation level in a few more inoculated samples than the qPCR method, analysis of uninoculated samples using the nested PCR resulted in a false-positive rate of 2.6% for cilantro samples and 5.0% for raspberry samples, whereas there were no false-positives observed for the qPCR (Murphy et al. 2017). The performance of the current FDA method for the detection of *C. cayetanensis* oocysts on fresh produce has since been verified for other produce matrices, such as shredded carrots, basil, parsley, cilantro, blackberries, strawberries, blueberries, shredded cabbage, romaine lettuce, spring mix, coleslaw, and green onions (Almeria et al. 2018, Lalonde et al. 2022).

The current FDA method for the detection of *C. cayetanensis* oocysts in agricultural water (Chapter 19c of the FDA BAM) (FDA 2023a) was validated in a multi-laboratory study to detect as few as six oocysts in ten liters of irrigation water (Murphy et al. 2017, Durigan et al. 2020). The previous FDA BAM method (Chapter 19a) was found to be ineffective at handling agricultural waters with high turbidity during a *C. cayetanensis* outbreak in 2013 (Durigan et al. 2019, FDA 2023a). The current method uses hollow fibers in a dead-end ultrafiltration (DEUF) configuration to recover inoculated oocysts from large volumes of agricultural water. A DNA purification step was added after DNA extraction to overcome PCR inhibitors commonly found in agricultural waters and optimize performance (Durigan et al. 2020). In addition, the qPCR was evaluated using a panel of DNA samples from selected foodborne bacterial and parasitic pathogens: *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Eimeria acervulina*, *Eimeria tenella*, *Eimeria maxima*, *Entamoeba histolytica*, *Giardia duodenalis*, *Blastocystis hominis*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Salmonella* spp., *Escherichia coli*, and *Trypanosoma cruzi* (Durigan et al. 2020). The purification method was used on 6 samples from open water sources in Maryland, and PCR-based detection targeting 18S rDNA using a modified FDA's BAM, chapter 19B (REF) method. In 3/6 samples, amplicons were detected (Ct

values ranging between 33 and 36, vs Ct values from 27 to 31 for stool samples from patients that were used as positive controls). However, amplicons resulting from environmental samples were not sequenced, with questions about specificity of detection (in light of studies of Mattioli and Ortega (REFs)) remaining unresolved.

Alternative to the BAM qPCRs, a nested PCR assay targeting the 18S rRNA gene has recently been described for the detection of *C. cayetanensis* oocysts in fresh berries and soil from berry farms in Mexico (Resendiz-Nava et al. 2020). The primer sets used for the nested PCR reaction were the same as used in the BAM qPCR method for fresh produce. Sensitivity of the nested PCR (established only with blueberries) was as few as 50 oocysts per 50 g berry sample, and Sanger sequencing and phylogenetic analysis was used on the amplicon from the second PCR to confirm the presence of *C. cayetanensis*. To further promote sensitivity, bovine serum albumin was added to the PCR reactions to offset potential inhibitory substances commonly present in environmental samples (Resendiz-Nava et al. 2020). When evaluated by the nested PCR assay, 16.6% (1/6), 36.4% (4/11) and 20.0% (1/5) of blueberry, blackberry, and farm soil samples, respectively, tested positive for *C. cayetanensis* and Sanger sequencing of the nested amplicons matched database sequences of *C. cayetanensis* (Resendiz-Nava et al. 2020). Although nested PCRs may allow for sensitive detection, the transfer of amplicon from the first PCR reaction to the second PCR introduces additional opportunity for cross contamination within the laboratory.

Targeting the 18S rRNA gene for qPCR detection of *C. cayetanensis* has several advantages and challenges. The 18S rRNA gene is conserved among the coccidian group and *C. cayetanensis* has been previously estimated to contain 18 copies per genome (Murphy et al. 2018). Considering that a sporulated *C. cayetanensis* oocyst in the environment would contain four genomes, targeting the numerous copies of the 18S rRNA gene allows more sensitive detection than targeting a single-copy gene. However, given the conserved nature of the 18S rRNA gene among coccidia/Apicomplexa, even slight modifications to the qPCR described in the FDA BAM methods for fresh produce and agricultural waters may negatively affect the assay's specificity. For example, one study demonstrated that some modifications to the qPCR master mix from that described in the FDA BAM method for the detection of *C. cayetanensis* on fresh produce resulted in cross-reactivity with several *Eimeria* spp. and *Isospora suis*, whereas strict adherence to the method verified specificity on various types of produce (Lalonde et al. 2022). This highlights the importance of conducting robust validation of any modifications to the FDA BAM methods for the detection of *C. cayetanensis* before attempting to use the modified method for evaluating food or environmental samples.

PCR methods targeting the mitochondrial genome

Recently described methods for detecting *C. cayetanensis* in fresh produce and agricultural water have included PCR assays targeting the *C. cayetanensis* oxidase gene (*Cox3*; a multi-copy gene) located within the mitochondrial genome. Two such methods are available on the FDA's website as "Other Analytical Methods of Interest to the Foods Program" (FDA 2023b). These two methods are virtually identical to the FDA BAM methods for the detection of *C.*

cayetanensis in fresh produce and agricultural water except the qPCR assay targeting the 18S rRNA gene in the BAM methods has been replaced with the Mit1C qPCR assay (which also includes an IAC). As such, the Mit1C qPCR is positioned as a stand-alone detection assay to be used as an alternative to the current BAM qPCR. The target for the Mit1C assay (a 205 bp region) was identified *in silico* using BLAST searches against available sequences of *C. cayetanensis* and other genera/species (e.g., *Eimeria* spp. and *Isospora* spp.) in the Apicomplexa phylum (Shipley, Arida and Almeria 2022). The Mit1C qPCR assay was validated in a single-laboratory study to detect as few as 5 *C. cayetanensis* oocysts in 25 g samples of cilantro or romaine lettuce, and 50 g samples of raspberries (Balan et al. 2023). In the study, the Mit1C qPCR demonstrated specific amplification when used to evaluate cilantro and romaine lettuce samples spiked with oocysts of two *Eimeria* spp. (*E. acervulina* and *E. tenella*) alone or at a 2:1 ratio with *C. cayetanensis* oocysts (Balan et al. 2023). However, the genetic diversity of food and environmental samples extends beyond the mitochondrial genome sequence data currently available and the scope of this study, therefore further evaluation of Mit1C qPCR specificity is needed.

A flotation concentration method using saturated sucrose solution followed by the FDA BAM qPCR assay (targeting the 18S rRNA gene) was recently investigated for isolation and detection of silt loam soil samples inoculated with *C. cayetanensis* oocysts (Shipley et al. 2022). Additionally, this study compared the flotation method to three commercial DNA isolation kits and compared detection using the Mit1C qPCR with the BAM qPCR. The flotation method resulted in greater sensitivity of detection than the three commercial DNA isolation kits, and the method was reported capable of detecting 10 oocysts in 10 g of soil (Shipley et al. 2022). The Mit1C qPCR was only evaluated at the 100 oocysts per 10 g soil inoculation level, however when compared to the BAM qPCR the Mit1C qPCR achieved detection at lower Ct values indicating better detection (Shipley et al. 2022). In the study, all inoculated samples evaluated by the Mit1C assay tested positive and all negative control (uninoculated) samples tested negative.

Detecting parasites from various types of soil samples has historically been difficult, therefore further studies using the flotation concentration method followed by either the BAM qPCR or the Mit1C qPCR for the detection of *C. cayetanensis* oocysts in samples of silt loam soil, sandy clay loam soil, and a commercial potting mix were performed (Arida, Shipley and Almeria 2023). Similar to the previous study, both qPCRs provided specific detection of as few as 10 oocysts per 10 g sample (both silt loam and sandy clay loam soils) with the Mit1C qPCR achieving detection in a higher (but not statistically significant) number of samples but at a significantly lower Ct value (reported as Cq) (Arida et al. 2023). Modification to the flotation method was required to optimize recovery and detection of *C. cayetanensis* oocysts from the potting mix with both the BAM qPCR and Mit1C qPCR detecting as few as 20 oocysts in 5 g samples. It should be noted that the unseeded (negative control) sample of potting mix returned an “undetermined” result by the Mit1C qPCR assay. Amplicons from the Mit1C qPCR on soil samples inoculated with 100 oocysts were successfully sequenced to confirm alignment with *C. cayetanensis*, however such attempts at sequencing amplicons from the samples inoculated at 20 and 10 oocysts were unsuccessful (Arida et al. 2023).

Additionally, a conventional PCR (Mit3PCR) has also been recently described for the detection of *C. cayetanensis* in food and water samples that targets a 182-bp fragment of the *Cox3* gene (the same mitochondrial target as the Mit1C qPCR assay) (Durigan et al. 2022). Mit3PCR was proposed to be used complementary to the FDA BAM methods to confirm BAM qPCR-positive samples, with any amplicon bands generated by the Mit3PCR subsequently analyzed by DNA sequencing (Durigan et al. 2022). The sensitivity of the mit3PCR method was confirmed to be equivalent to that of both FDA BAM qPCR methods (unpublished data from a single laboratory validation performed by FDA in 2018). The specificity of mit3PCR was evaluated using a panel of DNA samples from selected foodborne bacterial and parasitic pathogens: *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cyclospora papionis*, *Eimeria acervulina*, *Eimeria tenella*, *Eimeria maxima*, *Entamoeba histolytica*, *Giardia duodenalis*, *Blastocystis hominis*, *Plasmodium falciparum*, *Neospora caninum*, *Toxoplasma gondii*, *Salmonella* spp., *Escherichia coli*, and *Trypanosoma cruzi*. No cross reactivity with this DNA panel was observed. In addition, the specificity of the 182-bp region amplified by the mit3PCR was also confirmed by sequence comparison with other Eimeriidae species. Therefore, the sequences of any amplicons generated due to cross-reaction with background taxa in mixed samples could be used to resolve the specificity by comparison with a database of mitochondria genomes (Durigan et al. 2022). However as noted previously, the genetic diversity of environmental samples extends beyond this limited DNA panel as well as the mitochondrial genome sequence data currently available.

Future method development and evaluation for the detection of C. cayetanensis *in food and environmental samples*

The close genetic relatedness of *C. cayetanensis* with other coccidia/Apicomplexa, such as other *Cyclospora* spp. and *Eimeria* spp., and the limited genomic sequences of coccidia currently available have clearly complicated the development of DNA/RNA-based detection methods with the desired degree of specificity and robustness for widespread laboratory use. For these reasons, the specificity of genetic targets for detecting *C. cayetanensis* that are identified in silico should be evaluated using a robust exclusivity panel of DNA from closely related coccidia as well as a wide range of food and environmental samples. As discussed in sections above, the current BAM qPCR methods require strict adherence to the defined PCR conditions/procedures for specific detection of *C. cayetanensis*. Any modifications to PCR conditions/procedures in established official methods should be thoroughly evaluated to determine if the modification(s) negatively affected specificity before the method is further developed by the addition of steps to confirm and/or genetically characterize PCR-positive samples. Continued research to identify additional genetic targets for specific detection of *C. cayetanensis* in food and environmental samples should be continued as well as the development of detection methodology that is more tolerant to minor modifications without sacrificing specificity.

Future method development for the detection of *C. cayetanensis* in food and environmental samples should include the evaluation of multiple genetic targets representing different regions of the genome. However, secondary genetic targets to confirm initial PCR-positive samples

should also be specific for *C. cayetanensis* to avoid amplification bias when testing complex samples expected to contain a diverse population of coccidia/apicomplexan protozoa, such as food and environmental samples. DNA sequencing analysis (e.g., NGS or Sanger sequencing) of one or more amplicons should be considered for further confirmation of the presence of *C. cayetanensis* and/or characterization at the species or haplotype/genotype levels (Durigan et al. 2020, Kahler et al. 2021, Lalonde et al. 2022). However, amplicon sequencing is unlikely to be a useful method when a required management decision needs to be done quickly (i.e., whether or not to harvest a field within the next 3-5 days).

At the time of this Committee report, the International Standards Organization (ISO) has approved a project proposal within TC34/SC9 to develop an international standard for detection of *C. cayetanensis* in fresh leafy green vegetables and berry fruits, with possible application to other fresh produce (ISO 2023). Although the approved ISO proposal does not include the determination of *C. cayetanensis* to the genotype or haplotype level, the development of this international standard should consider the recommendations of this report for future method development, specifically the inclusion of multiple genetic targets representing different regions of the genome and the use of DNA sequencing of secondary target amplicons to further confirm the presence of *C. cayetanensis*.

Q8: Viability of oocysts

What information exists on assessing viability of oocysts?

The viability of recovered oocysts is needed to assess the public health risks of foodborne transmission. Currently, the viability of Cyclospora oocysts can only be assessed by analysis of the sporulation rates of the oocysts (Almeria et al. 2019). The sporulation viability of oocysts refers to the ability of the oocysts to undergo sporulation, which is the process of forming sporozoites, the infective stage of the parasite. Sporulation was often used as an indicator of viability of C. *cayetanensis* oocysts. Assessing the sporulation viability provides information on the ability of the oocysts to develop into the infectious form.

Compared with the development of detection techniques for Cyclospora, the assessment of viability of Cyclospora oocysts was much slower. One of the barriers for the assessment of viability of oocyst was that the sporulation of Cyclospora takes much longer than other protozoan parasites, with an incubation of 7 to 12 days at 25 to 30°C.

Microscopy-based technique was used to assess the sporulation viability of oocysts of Cyclospora (reference). Some other methods, like vital dye assays (e.g., DAPI) (Almeria et al. 2019) and Electrorotation method (Dalton et al. 2001), which was either not commercially available or still at the research stage (Almeria et al. 2019).

Some molecular methods such as PCR (polymerase chain reaction) or qPCR (quantitative PCR) were useful to detect oocysts and to investigate the source of contamination. In the past, those methods cannot be used to verify viability of oocysts. A recent study by Tucker and colleagues (Tucker et al. 2021) reported that genes differentially expressed in *E. acervulina* during sporulation, in mature and immature oocysts were identified and their homologs were

detected in *C. cayetanensis.* It is reasonable to hypothesize that these could be useful targets for mRNA-based assays for viability of the propagules.

Another promising development of assessment of viability of Cyclospora oocysts was to use artificial intelligence and machine learning to speed detection of *Cyclospora cayetanensis*' infectious life stage. Researchers are using a library of images of viable and nonviable oocysts to "teach" a machine to make the differentiation by robotic microscopy and image analysis (reference).

Control Strategies and Surrogates

Q5: Current monitoring and management strategies
Is monitoring for *Cyclospora cayetanensis* by testing food products, agricultural environment and agricultural inputs being applied as a management strategy currently (e.g., by industry, government)?

a) **Are there best practices for monitoring the presence of *Cyclospora cayetanensis* in agricultural production (including matrices [e.g., water, product], frequency, timing of sample collection (pre- vs. post-harvest), and sample numbers)?**
b) **Has monitoring led to development and implementation of effective preventive measures? If so, how effective have they been?**

Summary response. Currently, widespread environmental monitoring of agricultural environments and agricultural inputs, even in endemic areas, is not routinely conducted. The challenge, with environmental monitoring, lies in the low prevalence of in the environment and low recovery rate for oocysts and unreliable detection of *C. cayetanensis* DNA with current testing methodologies. The limiting factor for environmental monitoring is the lack of commercially available rapid test kits, that are low cost and can detect very low oocysts concentrations. In the interim, emphasis should be on, and enforcing, improved worker hygiene and sanitary practices, to include toileting habits, and routine testing of irrigation water supplies for human fecal contamination at the farm and packing facilities.

Q5 a) In regions where *C. cayetanensis* is endemic, a more comprehensive set of best practices could be put in place to monitor for *C. cayetanensis* in the production environment (CODEX ref). We recognize that even in the areas where *C. cayetanensis* is endemic, in the small number of studies that have been done so far, amplicons resulting from PCR reactions aimed at detecting 18S rDNA from *C. cayetanensis* were detected in only 1-4% of locally grown produce (Barlaam et al., 2021, Caradonna et al., 2017, Giangaspero et al., 2015, Ortega et al., 1997, Sim et al., 2017). 18s rDNA amplicons were detected by Resendiz-Nava et al in 20% of soil samples from Mexican farms; and by Giangaspero in 12% soil samples and 6 to 21% of well and municipal treated water. (Resendiz-Nava et al 2020, Giangaspero et al 2015). Resendiz-Nava et al confirmed the presence of *C. cayetanenis* using Sanger sequencing and phylogenic analysis, comparing the amplicons with 18S rRNA genes from GenBank archived *C. cayetanensis*

genome sequences. Giangaspero et al used single-strand conformation polymorphism and the BLAST tool to compare amplicons against known reference sequences.

Given the low level of prevalence in food samples even in the areas where *C. cayetanensis* is endemic, risk-based sampling would be advisable. The Code of Hygienic Practice for Fresh Fruits and Vegetables, Codex Alimentarius CXC 53-2003, provides guidance in accordance with Good Agricultural Practices and Good Hygienic Practices to control hazards, beginning with primary production at the farm. *C. cayetanensis* is listed among the microbiological pathogens of concern. Risk-based and risk-appropriate testing of agricultural water may be advisable, however, this Committee is not convinced that testing specifically for *C. cayetanensis* using existing methodologies and abundance of closely related organisms that are not pathogenic to humans is more practical than the risk-based tests for the presence of human fecal pollution. Growers should consider testing irrigation water for microbial and chemical contamination for identified risks, at a frequency determined by water source, with the consideration risk of environmental contamination, such as flooding, the type of irrigation or application method, and the use of manure, biosolids, and natural fertilizers.

Growing operations should consider evaluating hazards posed by the agricultural workers who may be symptomatic or asymptomatic carriers and consider medical examinations as appropriate. Agricultural workers should be encouraged to report diarrheal diseases and incentivized to seek treatment. Growing operations should emphasize and reinforce, training in health, hygiene, and sanitation. Adequate number of functional sanitary toileting facilities and handwashing stations close to work areas in the growing fields and packinghouses should be available. (Codex Alimentarius, CXC 53-2003) Codex Alimentarius Guidelines CAC/GL 88-2016 provides guidelines to control food-borne parasites, although *C. cayetanensis* is not specifically mentioned by name. (Reference is Guidelines on the Application of General Principles of Food Hygiene to the Control of Foodborne Parasites, CAC/GL 88-2016, Codex Alimentarius International Food Standards, Food and Agricultural Organization of the United Nations and World Health Organization, Adopted 2016).

However, in the areas where *C. cayetanensis* is not endemic (such as production areas in the continental U.S.), proposed sampling and preventative measures must recognize extremely low detection rates in environmental and food samples. Therefore, environmental testing programs must take into consideration that humans are the only documented vector for *C. cayetanensis*, and contamination with human waste or sewage is the likeliest source of the parasite in the production or processing environment. If testing of the final product is considered, a testing method needs to be developed to address the following criteria:

- Reliable and cost-effective detection of *C. cayetanensis* in samples in the presence of closely related organisms that are not pathogenic to humans
- Quick laboratory turn-around time to recognize the fact that commodities previously linked to the outbreaks of illness are highly perishable, and final product testing is typically done on the already harvested commodities
- Ideally, tests should be sufficiently sensitive to allow for a single-step detection, with only an occasional need for a second step validation of rare positives.

- Given the low prevalence of *C. cayetanensis* in domestic environmental samples and the final product, a method for detecting small numbers of oocysts from large volumes of wash water, for example, needs to be developed.
- When sampling and testing are done for root cause analysis, an appropriate number of samples from a diversity of sites, such as water source, irrigation water, farm soil, and field portable toilet facilities should be considered.
- Given the low prevalence of *C. cayetanensis* in areas where it is not endemic, a negative result from a routine test for *C. cayetanensis* does not conclusively establish absence as the number of oocysts may be below current detection levels. Therefore, testing should include indicators of human fecal pollution.

b. Has monitoring led to development and implementation of effective preventive measures? If so, how effective have they been?

There is little published information regarding monitoring programs. In 2017, the Canadian Food Inspection Agency implemented a national routine surveillance for *C. cayetanensis*, using the BAM Chapter 19b method, in imported and domestic fresh leafy greens, herbs, and berries (Chacin-Bonilla and Santin 2023). The Canadian Centre for Foodborne and Animal Parasitology detected *C. cayetanensis* in 0.28% of the survey samples. Chacin-Bonilla and Santin, reiterated the recommendations in Codex Alimentarius CXC 53-2003, where the focus should be on agricultural worker hygiene and sanitary practices. Resendiz-Nava et al (2020) recommend monitoring at the farm and packing facilities. The issue with monitoring at the farm and packing facilities remains the low prevalence of *C. cayetanensis* in the environment and low recovery rate for oocysts and detection of *C. cayetanensis* DNA with current testing methodologies.

Q9: Preventive measures

What preventive measures exist for the control of *Cyclospora cayetanensis* (e.g., using filtration)?

a. How effective have they been?

b. What are the impediments to development of effective preventive measures for *C. cayetanensis* and how can they be overcome?

Measures to control or eliminate *C. cayetanensis* in food products have generally not been identified (Erickson and Ortega 2006, Kniel et al. 2007, Erickson and Ortega 2006, Ortega and Sanchez 2010). The resilient nature of the oocyst bilayer cell wall structure and its larger size (7.5-10µm) could make filtration a practical and promising approach to eliminating the risk from an environment assuming that filtration rates and useful life of filters meet the throughput requirements. To date, no filtration systems have been constructed to eliminate *C. cayetanensis* while also providing the speed and filtering capacity needed to eliminate the oocyst in high-volume and high-speed production systems (Erickson and Ortega 2006, Kniel 2020). Given the challenges of filtration, other treatments for food systems, water, and irrigation systems have been studied (Erickson and Ortega 2006, Kniel et al. 2007, Erickson and Ortega 2006). The oocysts' sporulation ability has been evaluated by treating them with chemicals common in food processing such as chlorine, peracetic acid, and chlorine dioxide; the resilient oocyst cell walls have proven mostly resistant to such treatments (Ortega et al. 2008, Ortega

and Sanchez 2010, Ortega et al. 2008, Malka and Park 2021, Praeger, Herppich and Hassenberg 2018). Post-harvest treatments and processing conditions of temperature (high/low), UV, ozone, high-pressure processing (HPP) have all been evaluated to eliminate the risk for consumers; most treatments have been unsuccessful, impractical, or only evaluated on surrogate organisms with unknown understanding of how *C. cayetanensis* oocysts may react (Kniel et al. 2007,Ortega and Sanchez 2010, Guo, Huang and Chen 2019, Kniel et al. 2007, Erickson and Ortega 2006, Kniel et al. 2007). Extreme temperatures (60°C for 1 h or 70°C for 15 min, 100°C or -70°C temperature treatment) were found to be successful to inhibit sporulation; however, these temperatures are not practical for the foods often associated with risk; fresh produce, berries, herbs, etc. (Erickson and Ortega 2006, Kniel et al. 2007, Almeria et al. 2019, Ortega and Sanchez 2010). On-going research on *C. cayetanensis* oocysts with more novel treatment options is needed to identify effective and functional treatment options for the food industry (Kniel 2020, Erickson and Ortega 2006, Malka and Park 2022).

Preventative Measures. Potential means to prevent future illness from *C. cayetanensis* are to physically remove oocysts that may be found in food, water, and agricultural production environments, and/or to render *Cyclospora* oocysts that may be present in a food or production environment non-infectious (Erickson and Ortega 2006). The spherical Cyclospora oocyst bilayer cell wall serves as a strong protective barrier minimizing susceptibility to challenging environmental conditions and common antimicrobial treatments (Kniel et al. 2007). The physical structure of the Cyclospora oocyst is highly resistant to degradation in the environment from heat, sunlight, cold, and other environmental pressures (Erickson and Ortega 2006). The characteristics that allow for environmental survival also render them challenging to eliminate with mitigations in the food industry (Kniel et al. 2007, Erickson and Ortega 2006). Previous research has relied upon using oocyst sporulation as an indication of whether oocysts remain infectious post treatment or surrogate experiments with related parasites since no effective in vitro or in vivo methods have been discovered to be able to test infectivity directly (Ortega and Sanchez 2010). Below are brief summaries of preventative measures and treatments that have been explored for efficacy against consumer exposure from *C. cayetanensis* within the food system.

Fresh produce represents a high percentage of foods associated with past *Cyclospora* outbreaks; numerous events attributed to processed salads, berries and herbs (Temesgen et al. 2021). Products consumed fresh represent a challenge for food safety due to the limited number of approaches available to control microbial risk while maintaining the attributes demanded by the consumer (e.g., freshness, texture, color) (Kniel et al. 2007). In addition to the lack of many mitigation methods for fresh produce, managing parasite risk is further complicated in that many foodborne parasites such as *Giardia*, *Cryptosporidium* and *Cyclospora* oocysts have been observed to harbor physical structures that facilitate adherence to surfaces; consequently, physical removal from food surfaces is difficult (Temesgen et al. 2021).

Filtration. Filtration relies upon the physical removal of oocysts from a sample or environment as opposed to rending the oocyst noninfectious; it is an approach reliant on elimination either from water wash systems that may be in a production environment, or from water distribution and irrigation systems used for crop production. *Cyclospora* oocysts' size (7.5-10μm) make

them a more favorable candidate for physical separation methods than bacteria and viruses which are smaller and subsequently more difficult to physically remove from an environment or process (Erickson and Ortega 2006). A 2020 study using *Cryptosporidium* parvum (4.5µm oocysts) and *Eimeria tenella* (19-22µm) as surrogates for *C. cayetanensis* found success in removing oocysts from contaminated pond water using sand and also studied zero-valent iron (ZVI) filtration for combined physical removal and oocyst inactivation (Kniel 2020). Sand filtration physically captures oocysts, while using ZVI in combination with sand filtration is intended to impact oocyst viability. Cryptosporidium parvum in this study represented a parasite closer in size to *C. cayetanensis* (7.5-10µm), observed a 4.3 log reduction using a ZVI sand column versus sand only (Kniel 2020). *Eimeria tenella*, a much larger oocyst, obtained a 6-log reduction for the combined ZVI sand column, and only a 2.3 log reduction by sand alone (Kniel 2020, CPS Ref). Filtration remains a promising approach to control *Cyclospora cayetanensis* within water systems; however more research is needed to identify systems to effectively remove oocysts while also accommodating the volume of water needed in industrial wash and irrigation networks.

Washing. While industry and produce consumers have long relied on washing to clean produce items prior to consumption, these treatments have historically been utilized to remove soil, insects, and other substances prior to consumption, but were not designed for the removal of microbial risks. Research on produce wash systems has shown that microorganism removal efficacy can vary immensely based on matrix, target organism(s), and whether a combination of washing (physical application) with other treatments such as chlorination, chemical treatment, ozone, etc. are applied) (Temesgen et al. 2021). In 2021, researchers studied the potential of three consumer wash systems (running water, 1.75% acetic acid solution, washing followed by a salad spinner) applied to fresh blueberries and raspberries inoculated with *C. parvum, C. cayetanensis, G. duodenalis* oocysts to better understand if washing just prior to consumption would be sufficient to remove foodborne parasite risk (Temesgen et al. 2021). Results indicated that 80% of the *C. parvum* and *G. duodenalis* oocysts were removed by each of the washing methods on either berry matrix; raspberries being more difficult to remove oocysts than blueberries. *C. cayetanensis* across all treatments was found to remain on the berries to a much greater percent than the other parasites studied (Temesgen et al. 2021). Cyclospora's greater adherence led the researchers to hypothesize that the adherence may be related to the specific adhesions found within this organism's oocyst cell structure that are unique compared to the other parasites studied (Temesgen et al. 2021). Industrial wash systems have not yet been studied in respect to *C. cayetanensis* and is an area requiring further research.

Chemical Sanitizers (chlorine/peracetic). Fresh produce industry relies on chemical sanitizers to prevent cross-contamination of the product during post-harvest processing. Chlorine is one of the most common and universally effective sanitizers used in the food and agriculture industries (Erickson and Ortega 2006, Malka and Park 2022), Suslow, Trevor. 1997). Unlike bacteria and viruses which are susceptible to destruction following chlorine treatment, Cyclospora oocysts have not been shown to be susceptible to common chlorine treatments used for disinfection and sanitization within the food industry (Erickson and Ortega 2006, Malka and Park 2022).

Chlorine Dioxide. Chlorine dioxide (ClO2) in gaseous and aqueous forms has been found to be a useful tool in the food and fresh produce industry due to its bactericidal effects on bacterial foodborne pathogens, its efficacy over a wider range of pH values (pH 3-8) than other disinfectant systems, and unlike other chlorine-based sanitizers it does not create dangerous halogenated byproducts ((Ortega et al. 2008, Malka and Park 2021, Praeger, Herppich and Hassenberg 2018), FDA. 2008). However, oocysts of *C. cayetanensis* artificially inoculated onto basil and lettuce were able to withstand the exposure to gaseous chlorine dioxide at 4.1 mg/liter for 20 min without losing the ability to sporulate (Ortega et al. 2008, Ortega and Sanchez 2010) .

Temperature. Cyclospora oocysts treated in the laboratory with high temperatures, 60°C for 1 h or 70°C for 15 min, 100°C were found to no longer be capable of sporulation (Erickson and Ortega 2006, Almeria et al. 2019). In one study, researchers observed that a slight reduction in temperature to 50°C for 1 hour-maintained *C. cayetanensis* sporulation abilities (Erickson and Ortega 2006). Application of high temperature could be a means of inactivation for oocysts; however, such treatments would not be practical for fresh produce treatments as item quality would degrade and no longer meet market expectations (Kniel et al. 2007).

Past cyclosporiasis outbreaks have been associated with consumption of foods that have been stored refrigerated and frozen, providing an indication that freezing and cold temperatures alone may not be sufficient to inactivate *Cyclospora* oocysts (Ortega and Sanchez 2010). Experimentally, *Cyclospora* oocysts remain capable of sporulation following -15°C treatment for 24 hours in dairy matrices, and -20°C for 48 hours on basil and for 4 days in -20°C water samples (Ortega and Sanchez 2010, Almeria et al. 2019). In one study, -70°C temperature treatment of basil and water samples inoculated with Cyclospora oocysts was also found to be successful in prohibiting oocyst sporulation (Ortega and Sanchez, 2010). These extreme temperatures would not be practical with current industry practices, nor product expectations for consumers. While no studies were found that specifically researched refrigeration temperatures consistent with cold-chain and consumer storage, one reference noted that Cyclospora oocysts stored at 4°C for one or two months were capable of sporulating following a 30°C exposure for 6-7 days (Almeria et al. 2019).

UV. Ultraviolet (UV) light consists of short wavelengths of light (250-270nm) that has been used for its antimicrobial properties on bacteria, viruses, yeasts, molds and parasites in a variety of food, water and produce matrices ((Guo, Huang and Chen 2019, Kniel et al. 2007). While direct exploration of *Cyclospora* has not been completed, UV treatment of a closely related bird parasite Eimeria acervulina yielded variable outcomes that were dependent on the UV exposure and the inoculum level of oocysts (Kniel et al. 2007).

Ozone. No published research to date has been completed to *C. cayetanensis* response following ozone exposure and further research is needed to determine if ozone would be effective on preventing sporulation, however in prior laboratory studies ozone treatments were

effective against *Giardia lamblia* and *Cryptosporidium parvum* (Erickson and Ortega 2006). (Include Khalifa REF from Q17)

High pressure processing (HPP). HPP treatment with 550 MPa at 40°C for 2 minutes was found effective to inactivate oocysts on fresh basil and raspberries inoculated with *Eimeria acervulina*, a potential Cyclospora surrogate (Kniel et al. 2007). Findings on surrogates are suggestive that HPP may be effective at rendering *Cyclospora* oocysts nonviable on food matrices where HPP is a potential application.

Future needs. As evident from the studies and treatments referenced in the preceding sections, direct research for *C. cayetanensis* is limited, and many conclusions and hypotheses on efficacy of treatments for *Cyclospora* have been drawn from studies completed on related parasites and surrogates. Major impediments for identifying or developing preventative measures against *Cyclospora* include the limited availability of *Cyclospora* oocysts for researchers, the inability to culture oocysts in the laboratory, and lack of consensus on the most appropriate surrogates for this organism. When considering the collection of studies and approaches that have been completed regarding preventative measures against *Cyclospora*, few studies have yet to identify commercially viable measures against this parasite for the food and produce industry. Of the studies that identified promising measures (e.g., 70-100°C, HPP, filtration) most would not be practical within the produce industry's supply chain. Given the percentage of cyclosporiasis outbreaks associated with fresh produce items, the lack of identified treatments amenable for fresh produce is problematic and warrants further exploration. Future research is needed to better understand the organism and ultimately help identify measures to reduce consumer risk from this organism. For this research to be successful, solutions must first be found regarding *C. cayetanensis* oocysts availability and/or identifying the most appropriate surrogates for this organism.

Q12: Possible surrogates
What other coccidian parasites could serve as a surrogate research model for *Cyclospora cayetanensis* behavior (e.g., for evaluation of control measures)?
Three other Apicomplexan parasites have been proposed as surrogates for *C. cayetanensis*: *Eimeria*, *Toxoplasma* and *Cryptosporidium*. *Eimeria* is probably the best surrogate because of its taxonomic closeness, the existence of an animal model and the extensive molecular tools available. *T. gondii* has also been widely characterized, but safety issues for lab workers, the public concern for use of cats for research, taxonomically farther and different life cycles limit their equivalence. *C. parvum* has less similarities to be considered a representative surrogate of *C. cayetanensis*.

Several publications have provided a set of criteria to guide researchers in the selection of surrogate organisms. Busta et al (2003) distinguished between indicator and surrogate organisms, defining the latter as a unique tool that is specifically utilized to evaluate the effects and responses of a target organism to selected processing treatments. A list of twelve ideal traits for potential surrogate microorganisms was outlined in the same publication. Those included:

Nonpathogenic Inactivation characteristics and kinetics useful to predict those of the target organism. Similar responses to pH, temperature and oxygen as the to the target microorganisms when exposed to raw fruit and vegetable. Growth characteristics that are consistent and stable Cultivated easily to obtain relatively high-count populations Inoculum population changes very little from preparation to utilization. Easily quantified using rapid, sensitive, inexpensive detection systems Easily differentiated from other microorganisms. Similar attachment characteristics to those of the target microorganisms. Genetically stable so results can be reproducible, by multiple laboratories. Does not have persistence characteristics to become a spoilage organism in the environment or products where it is applied. Susceptibility to injury similar to that of target pathogen.

Harris et al. (Harris et al. 2012) and Harris et al. (Harris et al. 2013) provided similar recommendations to the use of surrogates for agricultural water and un-treated soil amendments of animal origin to be used in fresh produce fields. These included: “(i) similar characteristics to those of the pathogen of concern such as growth, inactivation kinetics, attachment capacity, susceptibility to sublethal stress injury, and resuscitation; (ii) inducible stress tolerance resistance traits (pH, heat, desiccation, osmotic pressure, etc.); (iii) ease of detection; and (iv) differential or unique phenotypic and/or genotypic traits from background isolates” (Harris et al. 2012). The latter reference also included a compilation of surrogate microorganisms reported in the literature, but none of these included parasite surrogates. It should be noted that among bacteria several surrogate strains may meet most of them, for parasite surrogates it is challenging to identify organisms according to those desirable traits.

The concept of surrogate microorganism was also defined by Sinclair et al in a more general scope as: “an organism, particle, or substance used to study the fate of a pathogen in a specific environment” (Sinclair et al. 2012). This paper outlined a detailed conceptual decision framework for selecting a surrogate and listed four possible types of surrogate benchmarking and validation experiments. Based on this set of proposed experiments, validation of surrogates can only occur if both the potential surrogate and the target microorganisms can be compared under the same experimental conditions. The other benchmarking options described depend on whether the target organism can be reliable grown and detected and if the surrogate organism is known to have the greatest resistance of its category.

Cyclospora cayetanensis is a coccidian protozoa classified under the family Eimeriidae (Ortega 2019). *Eimeria* species are the coccidian parasites more closely related to Cyclospora (Dubey, Khan and Rosenthal 2022) and both genera have a fecal-oral cycle and they infect predominantly one host. Eimeria species are economically relevant parasites because they infect poultry, cattle and other livestock causing significant losses to agriculture (Thompson and Rosenthal 2020). Eimeria species have been extensively characterized and there have been as many as 1,800 species identified that infect multiple vertebrate species (Burrell et al. 2020). Interestingly, not a single Eimeria species has been reported to infect humans, but because *C. cayetanensis* is so genetically similar, it was considered as the “human Eimeria.” *E. tenella* and *E. acervuline* are the two most common poultry coccidia that their life cycle, pathogenesis, and

invasion mechanism have been extensively described (Venkatas and Adeleke 2019). Because of those similarities and the lack of limited laboratory tools to study Cyclospora, Eimeria is considered the best surrogate for *C. cayetanensis*.

In the most recent review paper that addressed the potential of *Eimeria* as a *Cyclospora* surrogate, Tucker and coworkers conducted a systematic assessment that supported its utilization (Tucker et al. 2022). That analysis recognized that the two major limitations to make significant research progress to address the public health threat that *C. cayetanensis* represents are the scarcity of oocysts and the lack of a viable animal model. According to the same study, *Eimeria acervulina* and other poultry parasites meet several of the desirable criteria for a surrogate organism described above. Another recently published study, the genomic and genetic closeness of *E. acervulina* with *C. cayetanensis*, was also confirmed using gene expression in maturing oocysts (Tucker et al. 2021). Because of the evidence presented these two papers, there should be little doubt that *E. acervulina* is the most viable surrogate.
In addition to *Eimeria* species, *Toxoplasma gondii*, another coccidian parasite in the family Sarcocystidae has also been investigated as *Cyclospora* surrogate (Lee and Lee 2001, Dubey et al. 1998). Lee and Lee reported that a gamma irradiation dose greater than 1 kGy was necessary for the complete inactivation of 650 *E. acervulina* oocysts inoculated on fresh raspberries (Lee and Lee 2001). Those results were similar to previous studies conducted with *T. gondii* and *E. tenella* (Dubey et al. 1998, Gilbert et al. 1998).

Toxoplasma gondii was used as a surrogate for *C. cayetanensis* on raspberries (Assurian et al. 2020). *T. gondii* as a *Cyclospora* surrogate poses several advantages that include its extensive characterization, well defined models of gene annotation, availability of in vitro and animal study models and a large network of scientist (Anderson, B., personal communication). An additional positive aspect for using this parasite as surrogate is the high resistance of its oocysts to inactivation, which can serve as a safety factor in developing interventions against *Cyclospora*. In contrast, adoption of *T. gondii* as surrogate, presents serious challenges. Public concern about the use of cats for research has led to a reduced availability of oocysts for research. Because it is a human parasite, its utilization in research may be a risk for laboratory workers. The lack of evolutionary relatedness and different life cycles are probably the strongest argument against the use of *T. gondii* as *Cyclospora* surrogate.

The availability of a well-tested animal model for Eimeria presents one of the factors that favors adoption of this parasite as *Cyclospora* surrogate (Tucker et al. 2022). In 1995, a detailed protocol to grow and recover *Eimeria* oocysts from less than 5-day-old baby chicks was published (Shirley, 1995). In addition to requiring the use of very young chicks, this method is based on the use of coccidia-free animals. Because the different *Eimeria* species impact different sites in the chicken GI tract, the recovery of merozoites was well described. This method continues to be used to this date by researchers developing in-vitro cell culture methods (Marugan-Hernandez et al. 2020). Chicken epithelial and kidney cell lines have been tested with promising results and in the near future, a chicken-free method for harvesting oocytes may be available for researchers (Bussière et al. 2018).

Tefera (2021) suggested using *C. parvum* as a surrogate for *C. cayetanensis*. Infections by *C. cayetanensis* can also lead to coinfections of opportunistic pathogens (OP) such as *Cryptosporidium*, therefore, if suspected, screening for *C. cayetanensis* when there is presence of OP should be considered. *Cryptosporidium* presents some favorable traits to be considered a surrogate such as extensive characterization, availability of oocysts, developed tools and similar tolerance levels. However, its life cycle differences, large taxonomic separation, and multiple hosts limit the extrapolation of *C. parvum* to C. *cayetanensis*.
Given current limited availability of *C. cayetanensis* oocysts, validation of surrogates remains challenging.

Table 4. Comparison of *Eimeria*, *Toxoplasma gondii* and *Cryptosporidium parvum* as possible surrogates for *Cyclospora cayetanensis*.

Trait	Eimeria	*Toxoplasma gondii*	*Cryptosporidium parvum*
Taxonomic family	Emeriidae	Sarcocystidae	Cryptosporidiidae
Existing animal model	Baby chicks	Cats, controversial	Multiple
Life cycle	Very similar	Different	Different
Lab worker risk	Small	Significant	Moderate
Oocyst tolerance	Similar	More tolerant	More tolerant
Number of hosts	Single	Multiple	Multiple

Q16: Maintenance and conveyance of wastewater
Are there practices for the maintenance and conveyance of wastewater, septage or human waste that may increase the incidence of *C. cayetanensis* contamination? Are there practices that may be useful in the management of waste to reduce the potential for contamination by *C. cayetanensis* (e.g., third-party toilet service or municipal wastewater treatment)?

a) **Which wastewater, septage, and human waste treatments in the U.S. are effective against C. cayetanensis? Which treatments may not be effective against C. cayetanensis?**
b) **Does municipal water treatment adequately reduce, control or eliminate C. cayetanensis?**
c) **Can effective municipal water treatments systems be scaled to treat agricultural water used in produce production?**
d) **How do practices compare for domestic growers versus international growers who export to the U.S.?**

The low prevalence of Cyclospora in U.S. wastewater impedes our understanding of the effectiveness of current wastewater treatments against these protozoa. Available information suggests that physical removal methods, such as sand-filtration, as well as exposure to sunlight (UV) lead to measurable reductions of parasite load. Improvements in detection methods as well as studies with surrogate protozoa may assist our understanding in the future. Although

agricultural water sources may serve as a potential contamination route, there is little information on what practices a grower can deploy to reduce risk beyond physical removal.

a) Which wastewater, septage, and human waste treatments in the U.S are effective against *Cyclospora cayetanensis*?

Although parasites, including *C. cayetanensis*, are considered wastewater-associated pathogens, there is minimal evidence that current wastewater treatment practices are sufficiently effective to address the potential health risk. Parasite occurrence and concentration, including *C. cayetanensis*, in wastewater can vary greatly and is highly dependent on many factors, including source, season, human population demographics and population prevalence rates. Generally, in the U.S., the prevalence and concentration of *C. cayetanensis* is expected to be low (Zarlenga and Trout 2004). This low prevalence makes it difficult to measure the effectiveness of the many and varied wastewater, septage and human waste treatment processes in use across the US. Limited data are available specific to *C. cayetanensis* reduction related to chemical, biological and physical wastewater treatments. Chemicals commonly used in water treatment, such as chlorine or chlorine dioxide gas, have limited effect on C. cayetanensis.(Ortega et al. 2008)

Research on physical methods, such as sand filtration, support effectiveness in reducing *Cyclospora* spp. oocysts. For example, a study in rural areas of Nepal measured the impact of diarrheal disease following introduction of sand-filtered drinking water. With respect to *C. cayetanensis*, the researchers noted 88.2% removal rate of oocytes (confirmed by microscopy) in water samples and a reduction of 4.9% in diarrheal disease within the community. 10
While the research on inactivation of *C. cayetanensis* in wastewater, septage and human wastewater could be important, it will be hampered by the lack of available oocysts. The use of various surrogates is more practical, but it is important to recognize that no surrogate will respond to treatment exactly as the target organism. On-going studies indicate that *Eimeria* spp. exhibit reasonable sensitivity to some common treatments (REF).

b) Does municipal water treatment adequately reduce, control or eliminate C. cayetanensis?

Naganathan, et. al (2022) 16 completed a comprehensive review and meta-analysis of *C. cayetanensis* in water. Their search identified 33 articles which met the researchers' criteria for inclusion in their analysis of the prevalence of *Cyclospora cayetanensis* in different types of water. In brief, the authors, demonstrated that when all analyses are combined (92 prevalence estimates from 33 studies), *Cyclospora cayetanensis* prevalence was estimated to be 6.9% in global water samples. The authors noted constraints on their analyses including a bias toward datasets from endemic areas, and the use of data from studies in which only a single step detection was used (which likely to have overestimated the numbers of *C. cayetanensis* in samples). However, despite constraints they estimated that household or drinking water prevalence was 5.12%, and water used for irrigation had the highest prevalence at 17.1%.
A study of two wastewater treatment plants in Arizona found *C. cayetanensis* in both the influent and effluent. (Kitajima et al. 2014) Nine of the 48 water samples collected from 2011-2012 were

positive for *C. cayetanensis* using a novel qPCR technique; however, the authors did not determine the removal efficiency of the wastewater treatment plants. The authors concluded that existing regulations for water treatment are insufficient to protect the public from *C. cayetanensis* because there are no regulations for managing *C. cayetanensis* in drinking water or wastewater. Further work is needed to understand the specific wastewater treatment practices that would demonstrate sufficient effectiveness to benefit public health in the context of the contributing population.

c) Can effective municipal water treatment systems be scaled to treat agricultural water used in produce production?

Water scarcity is a major public health problem which impacts billions of people around the globe and is an issue that is only becoming worse due to climate change. Parasite removal can vary considerably in wastewater treatment plants (WTP) for reasons previously discussed. A five-year study of the efficacy of pathogen removal for a California water reuse facility supported a very low prevalence of Cryptosporidium *spp.* oocysts, Giardia s*pp.* cysts, and Cyclospora *spp.* oocysts in the effluent, 2.3, 0.3 and 0.034 cysts/L, respectively. Importantly, these concentrations were not considered a health risk. 17 Water reuse considerations are critical to the evaluation of the effectiveness of municipal water treatment systems and the downstream applications of reclaimed water, including agricultural applications. In the aforementioned study, the expectation was that the water intended for agriculture meet California Title 22 standards consistently. Assuming that this standard is sufficient to manage the potential health risk, then it provides a model for other municipal systems to consider.

Finally, our understanding of the effectiveness of municipal water treatment systems and their applicability to treat agricultural water is limited by the methodology applied to data gathering. There remain important considerations for the specificity and sensitivity of the detection methods and until resolved there will be limitations to scientific approaches to evaluations of not only the risk presented by agricultural water for specific crops and corresponding irrigation systems but also effective risk reduction measures.

Q17: Prevention in food

What elements or points in the parasite's life cycle are potential targets of strategies to disrupt its progression, eliminate or destroy oocysts, stop dissemination into the environment, and prevent food contamination?

a) What are control measures that should be evaluated for effectiveness against *Cyclospora cayetanensis*? Including control measures that can be applied to the environment and/or foods that may be consumed in the raw form.

b) What is a recommended protocol for evaluating the effectiveness of control measures against *Cyclospora cayetanensis*?

Control measures for *C. cayetanensis* should target both the sporulated and unsporulated forms. Control strategies should start with measures at the farm and food production levels with the provision of proper handwashing and toileting facilities for workers. The detection methods used will need to also be evaluated in the presence of *Cyclospora* spp. that are not human

pathogens to ensure that any positive samples represent contamination specifically by C*. cayetanensis*.

Because the sporulated oocyst is the infectious form of the parasite, reduction or prevention of oocyst sporulation may be a way to control *C. cayetanensis*. However, because the unsporulated oocyst can become sporulated, the best control strategy would target both stages of the parasite lifecycle to reduce or prevent dissemination of *C. cayetanensis*. Furthermore, there is currently no standardized way to distinguish between the unsporulated and sporulated *C. cayetanensis* oocyst except microscopy. However, a molecular technique that relies on detection of differentially expressed genes between mature and immature oocysts in model organism *E. acervulina* was recently published (Tucker et al. 2021). That same study reported that *C. cayetanensis* has genes similar to differentially expressed genes identified in *E. acervulina* during sporulation (Tucker et al. 2021). Nonetheless, the identified genes from the Tucker et al. study would need to be validated for the capacity to discriminate between unsporulated and sporulated *C. cayetanensis* before they could be used.

Because no non-human reservoir for *C. cayetanensis* has been identified, the most appropriate point at which reduction of parasite contamination is likely to succeed is in the environment around produce production. Environmental controls that can be implemented include inspecting delivery vehicles and packaging materials for cleanliness, inspecting produce for damage and filth, removing foreign matter, and maintaining records that allow traceback (Guidance for industry, 2008). Proper toileting facilities and hand washing procedures for field workers and food handlers should also decrease the contamination of the environment and food with *C. cayetanensis*. Field workers that are sick should be able to stay home until they have recovered. Produce that is to be cut should be washed to reduce microbial contamination from the surface onto cut surfaces (Guide for industry, 2008). Because water washes were shown to dislodge oocytes from market produce, it is reasonable to hypothesize that washes can reduce the load on the product [i](Duedu et al., 2014; Ortega et al. 1997). Water used for cleaning produce must comply with all Federal, State, and local requirements. In addition, if water is reused, the cleanest water should be used in the final wash step (Guide, 2008). The use of temperature to prevent oocyst sporulation may require too extreme of temperatures to be practical (Sathyanarayanan and Ortega 2006). Therefore, a strategy to reduce, destroy or eliminate oocysts prior to produce reaching the consumer is important.

a) **What are control measures that should be evaluated for effectiveness against *C. cayetanensis*? Including control measures that can be applied to the environment and/or foods that may be consumed in the raw form.**

Control measures that have been shown to be effective against other parasites could be evaluated for *C. cayetanensis*, however we note earlier studies with chlorine, ClO_2, or UV had limited success (Gaafar, 2007; Ortega et al., 2008). In contrast, treatment with magnesium oxide (MgO)-coated particles reduced sporulation of unsporulated oocysts, and viability of sporulated oocysts by 50% when used at 10 mg/ml for 24 hours (Hussein et al., 2018). Pesticides such as captan, benomyl, zineb, malathion, and diazinon did not reduce *C. cayetanensis* sporulation when used as directed (Sathyanarayanan and Ortega 2004).

Although, microwave-based heating of *C. cayetanensis* suspended in water resulted in about a 1/4 to 1/3 reduction in sporulation when temperature of the water reached approximately 50°C after 10 seconds of heating, but temperatures of 95°C reached after 30 seconds of heating did not eliminate sporulation (Ortega and Liao 2006).

Various wash solutions have been tested to determine which would allow recovery from basil artificially inoculated with *C. cayetanensis* to allow detection by laboratory –based methods (Chandra, Torres and Ortega 2014). The solution that was the best at recovering the parasite was a 1% HCl-pepsin solution, better even than Alconox detergent. It is unclear from the publication what fold-reduction such a treatment would allow since the data were reported as the number of samples positive for *C. cayetanensis* after the wash. However, such solutions could be tested specifically as control measures in the future. Some studies on washing methods have already been conducted. For example, running water removed roughly 40% of the *C. cayetanensis* load from raspberries whereas washing inside a salad spinner or using a vinegar wash removed more than 80% of the parasite (Temesgen et al. 2021). However, for blueberries all three wash methods were greater than 95% effective (Temesgen et al. 2021). The parasite was detected by RT-PCR with the internal transcribed spacer 1 (ITS-1) region as the target (Temesgen et al. 2021, Temesgen, Tysnes and Robertson 2019). These studies suggest that washing by the food preparer should result in reduced parasite load.

The lack of *in vivo* or *in vitro* methods to test *C. cayetanensis* viability has prompted researchers to use surrogate parasites, such as *Eimeria* or *Toxoplasma* species to evaluate other treatments. For example, *Toxoplasma* oocysts irradiated with ≥0.4 kGy sporulated, excysted, and infected cells but were not infectious in mice (Dubey et al. 1998). It was recommended, therefore, that 0.5 kGy be used to kill coccidian oocysts on fruits and vegetables (Dubey et al., 1998). However, inactivation of *Eimeria acervulina* oocysts required 1 kGy (Lee and Lee, 2001). The use of high-pressure processing (HPP) demonstrated some effectiveness against the surrogate *E. acervulina* and Toxoplasma (Kniel et al, 2007, Lindsay et al., 2008), but has not been tested against *C. cayetanensis* to our knowledge. Additionally, the practicality of using HPP for berries is doubtful.

b) What is a recommended protocol for evaluating the effectiveness of control measures against *C. cayetanensis*?

Given the low levels of *C. cayetanensis* in the final product, establishing reasonable targets for reduction are challenging. A further complication is the lack of information on the infectious dose (though suspected to be low). However, it is important that a set of reasonable preventative or control measures be put in place to minimize or mitigate the risks of this pathogen in commodities that have been associated with outbreaks of human illness. Validation of control measures is complicated by the lack of *C. cayetanensis* oocysts readily available for research. Currently, oocysts are taken from clinical fecal samples. However, more robust studies will require consistent access to oocysts. The use of *C. cayetanensis* oocysts for experiments requires the development of approaches for generating oocysts under laboratory conditions. Once there is a reliable source of *C. cayetanensis*, methods for control and detection can be tested. For detection of the organism, it would be ideal to ascertain not only

absence/presence but also whether the organism is viable. To test detection methods, food products are spiked with a known quantity of *C. cayetanensis,* and the RT_PCR methods already described are likely adequate in the absence of native background. However, when the research moves to food products with unknown levels of *C. cayetanensis*, it will be important to distinguish *C. cayetanensis* oocysts from any possible Cyclospora contamination of other species. Such studies could initially be done with food products spiked with both *C. cayetanensis* and *Cyclospora* from other animals such as chickens or dogs.

Q15b: Strategies use to mitigate the contamination from farm workers
What are strategies that have been utilized to mitigate the contamination from farm workers? Have efforts to mitigate contamination from farm workers been successful? What environmental indicators may be helpful in verification of mitigation practices?
Currently, mitigation for *C. cayetanensis* includes increased hygiene and protective gear for farm workers. It is not clear if those efforts have been successful, as testing is not routinely done. Testing for reduction in fecal contamination indicators would be the most practical method to verify mitigation practices.

At present, the primary strategy to mitigate contamination of fresh produce by *Cyclospora cayetanensis* has been to focus on prevention via farm worker training including the topics of personal hygiene, clean clothing and other protective gear, such as gloves and boots, equipment management and appropriate sanitary maintenance of toilet facilities.
Routine water testing for fecal coliforms and/or other markers of human fecal contamination can also be used as an indicator of potential risk regarding the presence of other bacterial, viral or parasitic pathogens. Some operations may also use routine health evaluations and clinical testing for Cyclospora as a mitigation strategy for the worker populations, in growing regions outside of the United States. In a recent paper, L. Chacin-Bonilla and M. Santin (Chacin-Bonilla and Santin 2023) proposed that in developed countries, there is a likelihood that endemic population foci of *Cyclospora* infections may exist, most likely in socially and economically disadvantaged communities, such as rural farm-worker communities, thus, raising concerns regarding transmission issues. The authors believe there would be benefit in exploring the potential for endemic foci to better define the sources of infection, routes of spreading and potentially environmental contamination including produce fields, water sources and animals.

Relevant Factors and Data Gaps – What we know and what we don't know.

Q18: Relevant factors, available data, and data gaps for quantitative risk assessment
What are the relevant factors, available data, and data gaps needed to develop an informative quantitative risk assessment model for *C. cayetanensis* contamination and risk of illness?

In developing a framework for controlling this parasite (and other foodborne pathogens), it is important to consistently rely on risk-based and not on hazard-based approaches. Risk-based and risk-appropriate measures have been the hallmark of the US regulatory process and management approaches. A hazard-based approach to regulation should be avoided.

In assessing the risk of cyclosporiasis and establishing an actionable risk assessment framework, there are significant data gaps pertaining to sources of *C. cayetanensis* in the crop production environment and its routes of transmission, persistence in the crop production environment (especially in the areas where it is not endemic), the utility of indicators, accuracy of analytical methods, control strategies and applicability of surrogates to develop control measures.

Fresh produce represents a high percentage of foods associated with past *Cyclospora* outbreaks; numerous events attributed to processed salads, berries and herbs (Temesgen et al. 2021). Products consumed fresh represent a challenge for food safety due to the limited number of approaches available to control microbial risk while maintaining the attributes demanded by the consumer (e.g., freshness, texture, color) (Kniel et al. 2007). In addition to the lack of many mitigation methods for fresh produce, managing parasite risk is further complicated since the oocysts of many foodborne parasites, such as *Giardia*, *Cryptosporidium* and *Cyclospora,* have been observed to harbor physical structures that facilitate adherence to surfaces; consequently, physical removal from food surfaces is even more difficult (Temesgen et al. 2021). Further evaluation of the risks to public health for cyclosporiasis illnesses and the detection and control of *Cyclospora cayetanensis* in food, water and the environment can be enhanced by addressing many of the data or research gaps listed below.

Sources and routes of contamination.

- *C. cayetanensis* is a host-limited parasite, and human fecal contamination is the main (if not only) source of the oocysts. However, it is not known and critically needed to be understood for how long oocysts remain viable and infectious under the diversity of conditions associated with the fresh produce value chain.
- Measures of endemicity: A defined criteria and measures are needed to standardize designations of endemicity and non-endemicity transmission. This may also aid in the identification of pockets of endemicity and assess trends.

Prevalence and persistence of *C. cayetanensis*.

- There appears to be a seasonal pattern in outbreaks where *C. cayetanensis* is endemic, but no specific climate-linked condition has been identified. Even though non-endemic in the US, global trade results in products and ingredients imported throughout the year. Therefore, year-round vigilance is important, even though no outbreaks in winter have been reported in the US.
- Approaches for the mitigation of the risk of transmission of *C. cayetanensis* will differ in the areas where it is endemic vs non-endemic. In areas where *C. cayetanensis* is not endemic (such as many of the production areas in the continental US), it will be important to focus mitigation efforts on the likeliest sources of the *C. cayetanensis* oocysts (i.e., human vectors). We note the importance of global trade, and the fact that even domestically acquired infections may be ultimately linked to products or ingredients originating from areas where it is endemic.
- The persistence and prevalence of *C. cayetanensis* oocysts in the post-harvest environment is an especially notable data gap.

Indicators for *C. cayetanensis*.

- As discussed earlier, we acknowledge that no perfect biological or chemical indicator exists for *C. cayetanensis*. However, given the low prevalence of *C. cayetanensis*, even in the areas where it is endemic, there is a need to establish a reasonably reliable indicator. Meanwhile, validated indicators of human fecal pollution can serve as convenient indicator.

Analytical methods.

- The low prevalence of *C. cayetanensis* in environmental samples (including finished product) represents a statistical challenge. Therefore, a method to concentrate oocysts from large volumes of water is needed.
- A lack of availability of oocysts to serve as a positive control can hinder laboratory detection method development.
- Given the diversity of *Cyclospora*-like organisms that are not known to be pathogenic to humans but share significant homology with target DNA sequences used for the pathogen detection, there is a need to develop a tool for *C. cayetanensis* detection using a simple, reproducible, and robust method. Given that the infectious dose of *C. cayetanensis* is not known, a qualitative detection method for non-clinical samples may be sufficient.
- Methods to determine infectivity or viability of oocysts are lacking, but improved quantitative or qualitative methods for detection of oocysts may have a greater public health impact.

Control strategies and mitigation.

- *C. cayetanensis* appears to be resistant to common chemical interventions widely used in the fresh produce industry. Therefore, additional antimicrobial processes or chemicals should be evaluated.
- In the absence of sufficient information on the sources of the pathogen and its routes of transmission in the areas where it is not endemic, additional efforts to develop and implement GAPs aimed at specific/likely routes of transfer should be considered (i.e., Produce can be contaminated due to little or no washing, contamination by food handlers, crop irrigation with untreated water, and contaminated soil.)
- In the absence of validated control strategies, a focus on preventative approaches is warranted. Post-harvest processing to potentially control *C. cayetanensis* and others.
- Further work is needed to understand the specific wastewater treatment practices that would demonstrate sufficient effectiveness to benefit public health in the context of the contributing population.
- *Eimeria* spp. (another parasitic protozoa) are the most appropriate surrogate organisms known at the time of this report's writing. These and other organisms should be further studied for use as a surrogate for *Cyclospora* or *C. cayetanensis*. A lack of reliable access to *C. cayetanensis* oocysts hampers further efforts, and a method for culturing oocysts in the laboratory will significantly advance efforts to control it.

Risk assessment framework. A risk characterization will need to integrate elements of (1) hazard identification, 2) exposure assessment, and 3) hazard characterization into an estimation of the adverse effects likely to occur in a given population, including attendant uncertainties. An

infectious dose of *C. cayetanensis* is not known, and this may be difficult to determine. Excreted organisms are not infectious and require maturation for 7 to 14 days in the environment. Furthermore, the impact on infectivity is unknown for both the “age” of oocysts and the food/water matrix source of contamination. Immunocompromised individuals are at a greater risk of infection or illness, and there appears to be immunity in people who have had *C. cayetanensis* as children resulting in asymptomatic infections.

C. cayetanensis has been detected in chlorinated water, wastewater, irrigation water, and produce processing wash water. Foodborne illness outbreaks globally have been linked to the consumption of fresh fruits and vegetables. Collaboration should be encouraged between food and agricultural industries, academia, states, and local and foreign partners to promote research and share data to better understand the prevalence of *C. cayetanensis* in agricultural water and soil.

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Appendix A: Glossary

Term	Definition
18S	A component of the eukaryotic ribosomal ribonucleic acid (RNA). Detection of the 18S rRNA is used as an indication of the presence of a species. The sequence of the 18S rRNA gene is used to determine relatedness among organisms.
Apicomplexa	A group that derives its name from the apical complex, a collection of anterior structures that allow the parasite to invade host cells and establish themselves therein.
Apicoplast	The apicoplast is a secondary plastid organelle unique to most species within the phylum Apicomplexa that is essential for survival.
apicoplast genome	Apicoplasts contain their own DNA (35kb circular DNA) that shares sequence similarities with plastids (organelles found in the cells of photosynthetic organisms like algae and plants).
Cyclospora cayetanensis	A single-celled parasite that is human-specific and transmitted through food or water contaminated with human feces. The causative agent of cyclosporiasis.
cyclosporiasis	An intestinal disease caused by *Cyclospora cayetanensis* characterized by watery diarrhea. Diarrhea may be persistent in some individuals.
coliform	Intestinal bacteria that are indicators of fecal contamination
Eimeria	A genus of parasites that includes some species that cause coccidiosis (diarrhea) in animals.
endemic	A geographical location in which an organism is present consistently.
HPP	high-pressure processing or high-hydrostatic-pressure processing
indicator	An organism(s) whose presence is used to suggest the presence of a pathogen.
In silico	Detected by computer search rather than by experimental procedure in the laboratory
mitochondrial genome	The mitochondria (organelle that provides energy to the cell) contains its own DNA that is separate from the DNA held in the nucleus.
MLST	Multilocus sequence typing: a technique in which the DNA sequences of parts of several genes are used to divide organisms into different groups.

prevalence	The fraction or percent of the samples positive for the assessed parameter.
qPCR	Quantitative PCR
reference genome	A complete assembly of the DNA sequence from a representative organism. The sequence is available in a digital database for comparison with newly derived sequence data.
risk-based sampling	A method that prioritizes sampling of products considered as having a greater likelihood of being positive. This is as opposed to random sampling.
root cause analysis	A process used to find the cause of a problem so that solutions may be identified.
sporadic case	An illness not associated with an outbreak.
surrogate	An organism used to estimate the activity of a pathogen.

Appendix B: Additional Tables

Table 1

Method	Advantages	Disadvantages
PCR	High sensitivity compared to culture and staining (Liu et al. 2019) Ability to test for anti-microbial resistance (Liu et al. 2019) Quickly performed in 3-7 hours (Giangaspero et al. 2015b)	Potentially lower specificity compared to culture and staining (Liu et al. 2019) Need for a narrow list of causative agents to use specific primers (Liu et al. 2019) Supply costs, machinery fees, training expenses (Lalonde et al. 2022)

	Increased ability to detect fewer common organisms such as viruses (Kahler et al. 2021) Shown to be more cost-effective with selective use than culture and staining (Giangaspero et al. 2015b)	Becomes less cost-effective when performed with a multi-organism PCR approach (Craighead et al. 2021)
Flow Cytometry	Can handle large quantities of specimens (Quintero-Betancourt et al. 2002) Automated(Quintero-Betancourt et al. 2002) Relative sensitive (Duhain et al. 2012)	Very slow (Quintero-Betancourt et al. 2002) Often not necessary, since there are other alternatives(Quintero-Betancourt et al. 2002) Nucleic acid dyes might not be as reliable as infectivity studies in predicting the inactivation of oocysts following treatment (Duhain et al. 2012)
Microscopy: was it successfully used to diff viable/infectious from non-infectious?	Relatively simple technique (Masangkay F. R., 2019) Possible to count the number of parasites (Masangkay F. R., 2019) More useful than rapid diagnostic tests (Sathyanarayanan L. & Ortega Y., 2007)	Requires a level of skill (Masangkay F. R., 2019) They often lead to false-positive or false-negative results (Sathyanarayanan L. & Ortega Y., 2007)

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	PCR	Flow Cytometer	Microscopy
Pre-treatment	Fresh products were purchased a maximum of 24 hours prior to use, those fresh products were sampled and weight in bags with a microperforated filter. The bags before sealing need to dry at room temperature for about 3 to 4 hours and are finally stored overnight at 4 °C prior to processing. Water was stored at -80 °C, then the contaminated water was filtered using a cheesecloth and centrifuged at 2125 x g for 30 minutes, all the water that was in the top was discarded. All the bottom particles were mixed using a pipette.	Fresh products are washed with ethanol, the inoculum was placed on the surface of the green peppers' pieces, those pieces were placed inside sterile tubes, to be dried at 4 °C for 1 hour each tube before treatment. Water, there is no filter to pretreat water, just the use of a centrifuge	Fresh products were washed for approximately 6 to 7 minutes, and each vegetative sample was eluted y vigorous agitation followed by sonication for 30 minutes. The supernatant was discarded, and the pellets were washed by centrifugation. Samples of water was collected using a sterile polyethylene cup attached. The collected water samples were placed inside an ice chest. To be transported processing within 24 hours.
Quantities	Three studies were conducted involving different ranges in analysis. The first one did not have confirmation of the result, the second involved different studies in which PCR had the	We could not find ranges or numbers of analysis	There were several studies in which it was determined that the range and number of analyses was made by

	highest result. Finally, the third study had similar results to the second one		comparison between two or more variables
Sensitivity	High sensibility including for fresh and frozen fruits	Can detect cells between 1 and 15 microns in diameter, although it is possible to detect particles outside of this range (0.2 -150 microns) using specialized systems (Rowley, 2010)	Low sensitivity in a range of 40% and 50% (Omoruyi, Nwodo, Udem, & Okonkwo, 2014)

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