CYCLOSPORA TECHNICAL BULLETIN

Prepared for United Fresh, sponsored by Shenandoah Growers, Inc.

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OVERVIEW

Cyclospora is an emerging pathogen of concern for the fresh produce industry, first reported in the mid-1990s, but now linked with one or more outbreak in 17 of the last 20 years. Food borne illness outbreaks attributed to this pathogen have been linked to numerous imported fresh items including raspberries, basil, cilantro, scallions, and bagged salad mix and seem to consistently peak between May and July, however cases have been recorded in 8 different months. Given this pathogen's apparent consistent emergence, produce industry actors, the CDC and FDA all seek to improve their understanding of risk factors, contamination pathways, and mitigation opportunities.

KEY POINTS

- *Cyclospora* is a parasite, not a bacterium
- Antimicrobials that are effective against bacteria (e.g., chlorine) are not effective against *Cyclospora*
- *Cyclospora cayetanensis* is the species that causes human illness
    - Illness onset occurs several days (sometimes >10) after consumption, and typically presents as diarrhea that can last several weeks
    - Hospitalization is not generally required
    - Unlike outbreaks caused by other foodborne pathogens, cyclosporiasis cases cannot be genetically linked to each other, food vehicles or their sources due to the lack of real-time, validated molecular typing tools
        - Outbreak identification relies on epidemiological data (what people reported eating), not molecular similarity
- Since the organism cannot be grown in a laboratory, it is difficult for researchers to acquire enough parasite to study it
- Since it only grows inside the human host, clinical samples (feces) contain high levels of the pathogen
    - Clinical methods have only recently begun to screen for cyclosporiasis, demonstrating that there is a high burden of illness
- If contaminated, food and water samples seem to have very low levels and the organism will not grow in food or water, necessitating the evaluation and concentration of large sample volumes
    - Testing methods are primarily restricted to research purposes
        - Methodology continues to evolve, including the validation of different food and environmental matrices, and the inclusion of appropriate positive and negative controls
    - Commercial testing methods are very limited and expensive, relative to other pathogen tests
        - Only one US lab currently has ISO 17025 accreditation for *Cyclospora* testing

TECHNICAL REVIEW AND DISCUSSION

Capacity, Ongoing Projects, Remaining Questions

There are 3 published methods summarized in the FDA's Bacteriological Analytical Manual (BAM)[1] for detection of the primary species of *Cyclospora* that poses human pathogenic risk, *Cyclospora cayetanensis.* Published in 2004, 2017, and 2020 and referred to as 19a, 19b, and 19c these published methods constitute the FDA's preferred laboratory procedures for *C. cayetanensis* detection. 19a, or 'Detection of *Cyclospora* and *Cryptosporidium* from Fresh Produce: Isolation and Identification by Polymerase Chain Reaction (PCR) and Microscopic analysis' is the oldest method and is functionally obsolete at this time due to two issues[2]. First, some of the reagents required became commercially unavailable in 2012 (a big problem for outbreaks in 2013-2016). Second, it relies on a time consuming and laborious nested, PCR amplification method that directs successive identification of 6 primer sequences to differentiate between *C. cayetanensis* and other closely related species that share some of the segments including other non-pathogenic *Cyclospora* species and *Eimeria spp.* This makes it prone to false positives. While the PCR portion of the method is supplemented by microscopic analysis, other *Cyclospora* species are morphologically identical, which leaves this method inappropriate for monitoring or detection of *C. cayetanensis* in either water or on fresh produce.

For these reasons, FDA researchers developed method 19b, 'Molecular Detection of *C. cayetanensis* in Fresh Produce Using Real-Time PCR' in July 2016[3]. This method uses a much more accurate real-time PCR method that can detect lower concentrations more typical of contamination levels on fresh produce (as opposed to waterborne *C. cayetanensis*). Other advantages of 19b over 19a include increased sensitivity, specificity, and throughput, and decreased analytical time. Washing, extraction, and analysis can all be completed within a single day, or over the course of a 2-day period. This method has undergone multi-laboratory validation on fresh basil[4] and parsley[5] (2017), bagged pre-cut romaine[6] (2018), blackberries[7] (2020), and processed vegetables (shredded carrots[8], 2017; and cabbage[9], 2020). This new revised method also includes use of a new detergent that better recovers parasitic protozoa from food. Like 19a, it includes critical genetic sequencing necessary to definitively identify *C. cayetanensis* to species but does NOT require the amplification steps used in 19a that made 19a more vulnerable to false positives. Method 19b does not include verification via microscopy.

Although method 19b is an improvement over 19a, there are still challenges that limit its routine commercial use. Compared to 19a, 19b has a shorter analytical timeframe (also due to the elimination of the amplification step) and use of a commercial DNA extraction kit less prone to user error and false positives than the filter method

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[11] *FDA. 2021. Bacteriological Analytical Manual (BAM). Available from: https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam*

[2] *FDA. 2004. 19a. Available from: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19a-detection-cyclospora-and-cryptosporidium-fresh-produce-isolation-and-identification*

[3] *FDA. 2017a. 19b. Available from: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19b-molecular-detection-cyclospora-cayetanensis-fresh-produce-using-real-time-pcr*

[4] FDA. 2017b. Basil Matrix Extension Study Results. *https://www.fda.gov/media/107201/download*

[5] *FDA. 2017c. Parsley Matrix Extension Study Results. https://www.fda.gov/media/107206/download*

[6] *FDA. 2018. Bagged pre-cut romaine lettuce salad: Matrix Extension Study Results. https://www.fda.gov/media/132498/download*

[7] *FDA. 2020a. Blackberry Matrix Extension Study Results. https://www.fda.gov/media/144684/download*

[8] *FDA. 2017d. Carrot Matrix Extension Study Results. https://www.fda.gov/media/107213/download*

[9] *FDA. 2020b. Bagged Shredded Green Cabbage Matrix Extension Study Results. https://www.fda.gov/media/144685/download*

employed in 19a. The laboratory facility requirements for 19b are relatively simple: bench space for produce washing, hood for DNA extraction, centrifuge, and an Applied Biosystems™ 7500 Real-time PCR. Extracted samples need to be stored at 4 °C and analyzed within 2 days, or they can be frozen at -20 to -80 °C and stored to be analyzed by real-time PCR later. The temperature sensitivity and time requirements make extraction in one lab and shipment to another lab for analysis challenging/somewhat impractical. A key limitation to commercial / industry accessibility of this method is the availability of oocysts to serve as a positive control. As we gain a greater molecular understanding of the various species of *Cyclospora* and related parasites, it is possible that the method will continue to be updated.

Development of 19b addresses the need for detection of foodborne *C. cayetanensis*, however in outbreak situations there is often a need or desire to determine the route of contamination, which motivated development of 19c[10], a method for detection in water. While the relative likelihood of direct human contamination relative to waterborne contamination routes is still an area of active research and debate, there is interest in determining whether contamination via irrigation water or wash water is the root cause of some outbreaks. Testing agricultural water samples for *C. cayetanensis* poses numerous challenges related to the small sediment and debris frequently present in irrigation water, particularly from surface ponds that may pose a greater contamination risk. This turbidity and sediment rendered the filtration method in 19a useless in a 2013 outbreak investigation, motivating the search for and development of 19c, which uses dead-end ultrafiltration (DEUF) to isolate and identify *C. cayetanensis* from agricultural water. This method, which employs hollow fiber filters less prone to clogging and mobile turbidity testing is appropriate for testing agricultural water and detecting concentrations of *C. cayetanensis* down to 6 oocysts per 10 liters of agricultural water. Filtered samples can also be stored at 4°C for up to 1 week, enabling refrigerated shipment of samples taken in the field to relatively distant laboratory facility for further processing and analysis with real-time PCR. Both single lab and multi-lab verification of 19c has been completed, though publication of the method to the BAM just occurred in July 2020, so there is need for additional commercialization of this new method.

Although these two new methods developed and published in the last 5 years represent significant and valuable advances for detecting and investigating both foodborne and waterborne *Cyclospora*, there are still numerous remaining questions, challenges, and work needed to better understand and manage this pathogen. Almeria et al. (2019)[11] performed an exhaustive review of *C. cayetanensis* epidemiology and research and highlight numerous remaining questions and needs. Notably, they highlight the need for more information on, “infective dose, when sporulation takes place, persistence in the environment, the role of water and soil on transmission and/or about the existence of potential reservoirs.” One major barrier to advances in these areas is the lack of animal models or in vitro culture systems specific to *C. cayetanensis*. Previous research that attempted to infect non-human species with *C. cayetanensis* has not been effective, suggesting that the pathogen is specific to humans. However, research underpinning this interpretation is from 1995-2008, prior to development of more accurate, species-specific PCR-based methods. Therefore, there are remaining questions around inferred host specificity and whether other species could play a role in the spread of *C. cayetanensis*.

Molecular research and a soil-based method could be valuable to understand whether non-human vertebrates or even free-living soil-borne organisms could be playing a role in spreading this pathogen (e.g., Ortega and Sanchez

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[10] *FDA. 2020c. BAM 19c: Dead-end Ultrafiltration for the Detection of Cyclospora cayetanensis from Agricultural Water. https://www.fda.gov/media/140309/download*

[11] *Almeria, Cinar, Dubey. 2019. Cyclospora cayetanensis and Cyclosporiasis: An Update. Microorganisms https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6780905/*

2010[12]). Beyond understanding reservoirs and spread in the environment through research employing more accurate molecular methods that could definitively identify *Cyclospora* to species, there is need for additional method development that could support not just species-specific identification but genotyping to support outbreak investigation source identification, as well as further multi-laboratory validation of 19b on other types of produce. Produce growers need greater commercial access to recently developed methods, particularly in non-US growing locations, as well as new research to enable more responsive management to this pathogen.

KEY INDUSTRY CONTROLS

Because the chemical antimicrobials typically used in the produce industry are not effective against *Cyclospora*, produce growers and handlers should focus on prevention of contamination, with an eye toward the management of human waste. This includes attention to handwashing, sanitation of restrooms and portable toilets, avoiding use of sewage sludge, managing sewage spills etc. Notably, because of the life cycle of the parasite, it's important that controls against human fecal contamination are implemented not only during the active growing and harvesting of produce, but also *before.* The persistence of *Cyclospora* in the environment is unknown, nor are the factors that trigger its transition into the infective state.

Surveillance studies to better understand where *Cyclospora* may be found are still underway. United Fresh always cautions against testing for lot acceptance because this can provide a false sense of security. In the case of *Cyclospor*a, conducting testing and appropriately interpreting results is even more complicated than for bacterial pathogens (e.g., is the organism alive? Is it infective?). For that reason, the focus on preventive measures will be the best use of industry resources.

[12] *Ortega and Sanchez. 2010. Update on Cyclospora cayetanensis, a food-borne and waterborne parasite. Clinical Microbiology Reviews. https://pubmed.ncbi.nlm.nih.gov/20065331/*