Real-Time PCR Assay for the Detection of *Cyclospora cayetanensis* on Fresh Produce:

Blackberry Matrix Extension Study Results

March 6th, 2020

Sonia Almeria, Angela Assurian, Helen Murphy, and Alexandre J. da Silva

Center for Food Safety and Applied Nutrition

Office of Applied Research and Safety Assessment

8301 Muirkirk Road

Laurel, MD 20708

Email: maria.almeria@fda.hhs.gov

Phone: 240 402 3615

1. Background:

Cyclospora cayetanensis is a protozoan parasite causing an intestinal illness in humans called cyclosporiasis. The transmission of this parasite has been associated with the consumption of contaminated fresh produce or water (1). Human cyclosporiasis is a significant public health concern in the U.S. where large foodborne outbreaks, and several sporadic cases affecting hundreds of persons, have occurred since the mid-1990s. These cyclosporiasis outbreaks have been frequently associated with consumption of imported fresh produce including leafy greens and berries among others. The epidemiological investigations conducted during several multi-state outbreaks drew significant attention to the need for improved laboratory detection and characterization methodologies to identify and properly track sources of produce contamination (2).

Berries, particularly raspberries, have been historically linked to outbreaks of *C. cayetanensis* in the U.S. and Canada. In fact, *C. cayetanensis* first became a significant U.S. public health concern in 1996 and 1997 when multistate outbreaks attributed to raspberries imported from Guatemala occurred in both the U.S. and Canada with more than 1,000 reported illnesses in each of two outbreaks (3). Over the next few years, there were several outbreaks involving other berries as well; one example includes an outbreak in Ontario in 1999 which implicated a dessert that included fresh Guatemalan blackberries among other berries (3). In another outbreak at a wedding reception in Boston, a dessert containing strawberries (from California), blueberries (from Florida), blackberries (from Guatemala), and raspberries (from either Guatemala or Chile) was considered the vehicle of transmission (5). In this particular case it was not possible to link the outbreak to a specific berry type since multiple berries were involved. Other outbreaks including one in 2000 in Georgia and one in 2008 in California, were suspected to be caused by raspberries and/or blackberries as well (1).

The events reported above demonstrate the critical need for the FDA to be fully capable of testing commodities such as other berries, including blackberries, for the presence of *C. cayetanensis*; furthermore, these detection methods are critical for future assignments and/or outbreak investigations. A method for the detection of *C. cayetanensis* in produce was validated for cilantro and raspberries based on a multi-laboratory validation study and published in the FDA *Bacteriological Analytical Manual* (BAM) as Chapter 19B. Although the BAM Chapter 19B method has been validated for raspberries, it has not been validated for blackberries. The outcome of a matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on blackberries is described below.

2. Method:

The matrix extension was performed through a single laboratory validation study following guidelines for organisms posing unique isolation challenges found in the FDA Foods Program "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, Edition 3", published in 2019. The Microbiological Methods Validation Subcommittee (MMVS) previously specified that 10 replicates should be tested at the fractional level for matrix extension studies to BAM Chapter 19B. The matrix extension was performed by examination of 50 g samples of blackberries un-spiked or spiked with 5, 10 and 200 *C. cayetanensis* oocysts. The BAM Chapter 19B sample preparation and detection method was used with no modifications to wash produce, extract *C. cayetanensis* DNA, and perform molecular detection using qPCR analysis.

3. Results:

Table 1 shows a summary of the results obtained for the blackberry matrix extension study. The detection rate for the blackberry samples seeded with 5 oocysts and 10 oocysts was 70.0% and 90%, respectively. All blackberry samples seeded with 200 oocysts were positive and all unseeded blackberry samples were negative. No inhibited qPCR reactions were identified based on the performance of the internal amplification control (IAC). See Table 3 for detailed qPCR detection data for the matrix extension study including the number of positive qPCR replicates and C_T values for the *Cyclospora* and IAC targets for each sample. Following the data analysis protocol established for BAM Chapter 19B, reactions producing a C_T's greater than 38.0 were considered negative.

For comparison, a summary of the results obtained from the multi laboratory validation study (MLV) study on raspberries and cilantro is provided in Table 2. Results for detection of *C. cayetanensis* in blackberries using the validated method were similar to results obtained in the MLV study for cilantro and raspberries, with 5 *C. cayetanensis* oocysts identified as the limit of detection.

TABLE 1. *Summary of blackberry matrix extension results.*

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Blackberries (50 grams)	0	8	0	0.0%
	5	10	7	70.0%
	10	10	9	90%
	200	8	8	100.0%

TABLE 2. *MLV results for cilantro and raspberries.*

Matrix	Seeding Level	Positive samples (80 tested)	% positives
cilantro	0	0	0.0%
	5	25	31.3%
	10	64	80.0%
	200	80	100.0%
raspberries	0	0	0.0%
	5	40	50.0%
	10	72	90.0%
	200	80	100.0%

TABLE 3. *Blackberries matrix extension qPCR data.*

Number of oocysts spiked	No. positive qPCR reactions (out of 3 replicates)	*C. cayetanensis* rRNA 18S C_T value	IAC C_T value*
0	0	Und	24.4±0.1
0	0	Und	24.4±0.1
0	0	Und	23.5±0.1
0	0	Und	23.9±0.1
0	0	Und	24.4±0.1
0	0	Und	24.0±0.1
0	0	Und	24.3±0.1
0	0	Und	24.4±0.1
5	0	Und**	24.4±0.1
5	3	36.7±1.1	24.4±0.1
5	3	35.7±0.2	23.5±0.1
5	0	Und**	24.4±0.1
5	1	37.4	24.2±0.1
5	2	37.6±0.1	24.1±0.1
5	1	37.3	24.0±0.1
5	2	36.4±1.4	24.1±0.1
5	1	37.3	23.5±0.0
5	0	Und**	23.5±0.2
10	0	Und**	23.9±0.1
10	2	37.1±0.3	23.9±0.1
10	1	37.1	23.8±0.1
10	2	37.2±0.7	23.3±0.1
10	1	35.5	23.3±0.1

10	3	34.8±0.8	23.3±0.3
10	2	35.0±0.4	23.3±0.0
10	3	35.2±0.2	24.3±0.0
10	3	36.0±0.1	24.1±0.1
10	3	35.1±0.7	24.0±0.0
200	3	32.4±0.2	24.0±0.1
200	3	31.9±0.2	24.0±0.1
200	3	31.6±0.0	23.8±0.1
200	3	32.1±0.2	23.1±0.1
200	3	31.6±0.3	23.3±0.1
200	3	32.4±0.1	23.3±0.0
200	3	32.0±0.4	24.0±0.1
200	3	32.7±0.2	23.9±0.1

* All positive IAC qPCR reactions (out of 3 replicates)

** Undetermined when DNA diluted 1/4

4. **References:**

1. Centers for Disease Control, Parasites – U.S. Foodborne Outbreaks of Cyclosporiasis 2000-2014. Available at: https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html

2. Abanyie F, Harvey RR, Harris J, Wiegand R, Gaul L, desVignes-Kendrick M, Irvin K, Williams I, Hall R, Herwaldt B, Bosserman E, Qvarnstrom Y, Wise M, Cantu V, Cantey P, Bosch S, da Silva AJ, Hardin A, Bishop H, Wellman A, Beal J, Wilson N, Fiore A E, Tauxe R, Lance S, Slutsker L, Parise M, and the Multistate Cyclosporiasis Outbreak Investigation Team. 2013. Multistate Outbreaks of *Cyclospora cayetanensis* Infections Associated with Fresh Produce: Focus on the Texas Investigations. Epidemiology and Infection, 2015, Dec; 143 (16):3451-8. doi: 10.1017/S0950268815000370.

3. Herwaldt BL. *Cyclospora cayetanensis*: a review, focusing on the outbreaks of cyclosporiasis in the 1990s. Clin Infect Dis. 2000 Oct;31(4):1040-57.

5. Fleming CA, Caron D, Gunn JE, Barry MA. A foodborne outbreak of *Cyclospora cayetanensis* at a wedding: clinical features and risk factors for illness. Arch Intern Med. 1998 May 25;158(10):1121-5.