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

Blueberry Matrix Extension Study Results

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1. Background:

Cyclospora cayetanensis is a protozoan parasite, which causes 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. 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 both 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. More than 1,000 illnesses were reported 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 (4). 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 case, it was not possible to link the outbreak to a specific berry type since multiple berries were involved.

The events reported above demonstrate the critical need for the FDA to be fully capable of testing commodities such as other berries, including blueberries, 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 and blackberries, it has not been validated for blueberries. The outcome of a matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on blueberries 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 commercial blueberries 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, extracting *C. cayetanensis* DNA, and performing molecular detection using qPCR analysis.

3. Results:

Table 1 shows a summary of the results obtained for the blueberry matrix extension study. The detection rate for the blueberry samples seeded with 5 oocysts and 10 oocysts was 80.0% and 90%, respectively. All blueberry 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).

For comparison, a summary of the results obtained from the matrix extension studies in blackberries is provided in Table 2, and the results from the multi laboratory validation study (MLV) study on raspberries and cilantro is provided in Table 3. See Table 4 for detailed qPCR detection data for the matrix extension study, including the number of positive qPCR replicates and C_T values for *C. cayetanensis* and IAC targets for each sample. Following the data analysis protocol established for BAM Chapter 19b, reactions producing a C_Ts greater than 38.0 were considered negative. Results for detection of *C. cayetanensis* in blueberries 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 blueberry matrix extension results.*

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Blueberries (50 grams)	0	8	0	0.0%
	5	10	8	80.0%
	10	10	9	90.0%
	200	8	8	100.0%

TABLE 2. *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	90.0%
	200	8	8	100.0%

TABLE 3. *MLV results for cilantro and raspberries.*

Matrix	Seeding Level	No. positive samples (80 tested)	% positive samples
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 4. *Blueberry matrix extension qPCR data.*

No. 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	24.9±0.3
0	0	Und	24.7±0.1
0	0	Und	25.3±0.1
0	0	Und	25.4±0.1
0	0	Und	24.6±0.3
0	0	Und	24.8±0.1
0	0	Und	25.4±0.1
0	0	Und	25.2±0.1
5	2	37.4±0.3	25.1±0.3
5	0	Und	25.1±0.2
5	1	37.4**	24.6±0.1
5	0	Und	24.7±0.1
5	3	37.1±0.6	24.8±0.3
5	1	36.3**	24.4±0.2
5	1	37.6**	27.0±0.1
5	2	36.7±1.1	27.0±0.2
5	1	36.4**	26.9±0.2
5	2	36.7±1.1	26.6±0.1
10	2	36.6 ±0.2	24.7±0.2
10	2	37.3±0.2	24.6±0.2
10	0	Und	24.7±0.2
10	3	36.7±1.1	24.6±0.1
10	2	36.1±1.8	24.4±0.3
10	3	36.6±0.7	24.6±0.2

10	2	37.5±0.1	26.2±0.1
10	1	36.2**	26.2±0.1
10	3	36.2±1.2	26.4±0.2
10	1	37.7**	26.3±0.1
200	3	32.2±0.3	24.4±0.2
200	3	33.0±0.4	24.3±0.0
200	3	33.0±0.1	24.2±0.1
200	3	32.4±0.2	24.4±0.0
200	3	32.7±0.0	24.4±0.1
200	3	32.9±0.1	24.4±0.1
200	3	32.4±0.2	26.2±0.1
200	3	32.9±0.2	26.1±0.1

Und=Undetermined (Not detected)

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

** Undetermined when DNA diluted ¼

4. **References:**

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