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

Bagged Shredded Green Cabbage Matrix Extension Study Results

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Sonia Almeria (Parasitology Team)

Helen Murphy (Parasitology Team)

Alexandre J. da Silva (SBRS Lead Parasitologist)

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 (cell:267 265 1502)

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, berries, carrots, and cabbage. The epidemiological investigations conducted during several multi-state outbreaks underscored the need for improved laboratory detection and characterization methodologies to identify and properly track sources of produce contamination (2). A method for the detection of *C. cayetanensis* in produce was validated for cilantro and raspberries in a multi-laboratory validation study (approved on 7/6/2016) and published in the FDA *Bacteriological Analytical Manual* (BAM) as Chapter 19b. Subsequently, the method was validated and approved by matrix extension studies for shredded carrots (2/7/2017), basil and parsley (6/16/2017), romaine lettuce (5/7/2019), and blackberries (4/24/2020).

In 2013, an investigation of cyclosporiasis cases in Iowa and Nebraska indicated that some restaurant associated illnesses may have been caused by a contaminated salad mix containing several types of lettuce, red and green cabbage and carrots (3). In 2016, a restaurant-associated sub-cluster of cyclosporiasis in Texas was epidemiologically linked to consumption of coleslaw containing shredded carrots and cabbage (4). Additionally, in June of 2020, a multistate outbreak was being investigated linked to consumption of bagged salad mixes containing iceberg lettuce, carrots and red cabbage (5).

The events reported above indicate a need to extend and validate the FDA detection method for *C. cayetanensis* to cabbage due to its potential links to *C. cayetanensis* outbreaks. The outcome of a matrix extension study performed to assess the BAM Chapter 19b method for detection of *C. cayetanensis* seeded on commercial cabbage (bagged shredded mix shredded green cabbage and shredded carrots, see Figure 1) is described below.

Figure 1. Twenty-five grams samples of bagged shredded cabbage mix.

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", published in 2019. The MMVS previously specified that 10 replicates should

be tested at the fractional level for matrix extension studies for BAM Chapter 19b. The matrix extension was performed by examination of 25 g samples of bagged shredded cabbage, either un-spiked or spiked with 5, 10 or 200 *C. cayetanensis* oocysts. The BAM Chapter 19b sample preparation and detection methods were used with no modifications to wash produce, extract DNA, and perform molecular detection using a qPCR analysis specific for *C. cayetanensis*.

3. Results:

Table 1 shows a summary of the results obtained for the shredded cabbage matrix extension study. The detection rates for samples seeded with 5 and 10 oocysts were 45.4% and 63.6%, respectively. All samples seeded with 200 oocysts were positive; all unseeded samples were negative. No inhibited qPCR reactions were identified based on the performance of the internal amplification control (IAC). See Table 4 for detailed qPCR detection data including the number of positive qPCR replicates, as well as C_T values for *C. cayetanensis* and IAC targets for each sample. Following the data analysis protocol established for BAM Chapter 19b, reactions producing C_T's greater than 38.0 were considered negative.

For comparison, summaries of the results obtained from the multi-laboratory validation study (MLV) and the carrots matrix extension study are provided in Tables 2 and 3, respectively. Results for detection of *C. cayetanensis* in the shredded cabbage, using the validated method, were similar to results obtained in the matrix extensions for carrots, and in the MLV study for cilantro and raspberries, with 5 *C. cayetanensis* oocysts identified as the limit of detection.

TABLE 1. *Summary of bagged, pre-cut, romaine salad matrix extension results.*

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Shredded cabbage (25 grams)	0	8	0	0%
	5	11	5	45.4%
	10	11	7	63.6%
	200	10	10	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. *Shredded carrots matrix extension results*

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Shredded carrots (25 grams)	0	8	0	0%
	5	10	5	50.0%
	10	7	9	70.0%
	200	8	8	100.0%

TABLE 4. *Shredded cabbage matrix extension qPCR data.*

# oocysts	18S No. positive qPCR reactions (out of 3 replicates)	18 S C_T value	IAC C_T value*
0	0	Und	26.1±0.2
0	0	Und	25.4±0.1
0	0	Und	25.2±0.1
0	0	Und	24.7±0.2
0	0	Und	25.1±0.1
0	0	Und	25.8±0.0
0	0	Und	27.0±0.1
0	0	Und	25.1±0.3
5	0	Und	25.9±0.1
5	0	Und	26.1±0.1
5	0	Und	25.8±0.1
5	0	Und	25.7±0.0
5	2	37.1±0.5	26.8±0.2
5	2	37.1±0.2	27.6±0.3
5	0	Und	26.3±0.1
5	3	37.0±0.6	26.5±0.1
5	1	37.6	25.3±0.2
5	0	Und	25.3±0.3
10	3	35.0±0.7	25.9±0.1
10	2	37.7±0.3	25.8±0.1
10	0	Und	25.6±0.1
10	1	35.4	25.8±0.1
10	3	35.6±0.5	26.5±0.2
10	0	Und	26.7±0.2
10	3	33.9±0.2	25.2±0.1
10	3	35.5±0.3	25.9±0.2
10	0	Und	25.1±0.1
10	1	37.2	25.1±0.2
10	0	Und	25.2±0.2
10	3	35.0±0.7	25.9±0.1
200	2	34.6±0.4	25.7±0.1
200	3	33.0±0.2	25.6±0.1
200	3	33.6±0.6	25.6±0.3
200	3	32.8±0.2	25.3±0.2
200	3	31.6±0.2	25.5±0.1
200	3	31.5±0.3	25.9±0.1
200	3	32.5±0.0	25.1±0.1
200	3	32.9±0.2	25.0±0.1
200	3	35.2±0.9	25.3±0.1
200	3	33.6±0.4	25.3±0.1

Und: Undetermined

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

** Undetermined when DNA diluted ¼ (as per protocol described in BAM Chapter 19b)

4. **References:**

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