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

Basil Matrix Extension Study Results

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1. Background:

Cyclospora cayetanensis is a protozoan parasite causing human diarrheal disease associated with the consumption of fresh produce or water contaminated with the parasite's oocysts (*1*). According to surveillance data accumulated by the Centers for Disease Control and Prevention since the mid 1990's, *C. cayetanensis* is second to *Salmonella* sp. as the most common cause of diarrhea illness and outbreaks in the U.S. associated with imported food commodities that are regulated by the U.S. FDA (*2*). During this period, large outbreaks and sporadic cases affecting hundreds of persons have continued to occur annually associated with consumption of imported fresh produce including berries and a variety of leafy greens.
In the last few years the epidemiological investigations conducted during 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 (*3*). This is crucial to support epidemiological investigations and regulatory actions since a number of those investigations conducted from year 2000 to 2016 did not identify the specific source or origin of contaminated produce that caused the cases of infection. (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html). A method for detection of *C. cayetanensis* in produce was recently validated for cilantro and raspberries. A matrix extension was performed for carrots and these commodities were approved by the MMVS for publication in the FDA *Bacteriological Analytical Manual.*
The FDA is planning an assignment for May 2017 aiming at determining the prevalence of *C. cayetanensis, Salmonella* and *E. coli* in cilantro, basil and parsley. However, the *C. cayetanensis* regulatory method has not yet been validated for regulatory detection of *C. cayetanensis* on basil and parsley. Basil was possibly linked to a number of outbreaks in the U.S., but it was definitely linked to the outbreaks that took places in various counties of Missouri in 1999 (4). The outbreaks in Missouri were associated with consumption of a chicken pasta salad and a tomato basil salad, which contained other herbs in addition to basil. These outbreaks were also important because for the first time, *C. cayetanensis* was detected molecularly and microscopically in leftovers of the implicated foods confirming the epidemiological findings (4).

In this study, a matrix extension of the *C. cayetanensis* validated method was performed to permit use of the method in a new regulatory assignment including basil. The outcome of the matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on basil is described below.

2. Method:

MMVS provided directives to perform the matrix extension through a single laboratory validation study following guidelines for organisms posing unique isolation challenges found in the FDA OFVM "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds" published in 2015. The MMVS further specified that 10 replicates should be tested at the fractional level. The matrix extension was performed by examination of 25 gram samples of basil un-spiked or spiked with 5, 10 and 200 *C. cayetanensis* oocysts. The validated sample preparation and detection method was used 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 basil matrix extension study. Detection rate for basil samples seeded with 5 oocysts fell within the fractional positive target range and was 70.0%. All basil samples seeded with 10 oocysts and 200 oocysts were positive and all

unseeded basil samples were negative. No inhibited qPCR reactions were identified based on the performance of the internal amplification control. 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 internal amplification control (IAC) targets for each sample. Following the analysis protocol established for the MLV study, reactions producing a C_T's greater than 38.0 were considered negative.

For comparison, a summary of the results obtained from the MLV study is provided in Table 2. Results for detection of *C. cayetanensis* in basil using the validated method were similar to results obtained in MLV study for cilantro and raspberries with 5 *C. cayetanensis* oocysts identified as the limit of detection based on the fractional results. In all cases the fractional range (25-75%) was found at 5 oocysts.

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

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Basil (25grams)	0	8	0	0%
	5	10	7	70.0%
	10	10	10	100.0%
	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. *Basil 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	25.0±0.2
0	0	Und	25.4±0.1
0	0	Und	25.2±0.1
0	0	Und	25.3±0.1
0	0	Und	25.0±0.2
0	0	Und	25.5±0.1
0	0	Und	24.7±0.2
5	**3**	**36.0±1.2**	**25.9±0.4**
5	**1**	**37.4**	**26.0±0.3**
5	**0**	**Und**	**25.8±0.1**
5	**3**	**36.2±1.3**	**25.6±0.2**
5	**0**	**39.2****	**25.5±0.8**
5	**3**	**35.6±0.6**	**25.8±0.1**
5	**2**	**37.04±0.7**	**25.2±0.2**
5	**2**	**35.4±0.1**	**25.2±0.2**
5	**2**	**35.9±0.6**	**25.6±0.6**
5	**0**	**Und**	**25.6±0.9**
10	**3**	**36.1±0.6**	**25.8±0.1**
10	**3**	**32.9±1.9**	**25.6±0.2**
10	**3**	**35.0±0.8**	**25.6±0.2**
10	**3**	**34.1±0.2**	**25.4±0.2**
10	**3**	**35.9±1.3**	**25.1±0.3**
10	**3**	**35.2±0.2**	**26.4±0.4**
10	**3**	**36.13±1.2**	**25.8±0.9**
10	**3**	**35.6±0.7**	**26.4±0.8**
10	**3**	**35.1±0.4**	**25.4±0.1**
10	**3**	**34.1±0.5**	**25.5±0.2**
200	**3**	**30.7±0.1**	**25.4±0.2**
200	**3**	**30.8±0.0**	**25.9±0.3**
200	**3**	**30.9±0.1**	**25.7±0.2**
200	**3**	**30.5±0.2**	**27.1±0.2**
200	**3**	**32.7±0.2**	**27.2±0.5**
200	**3**	**30.0±0.1**	**24.9±0.1**
200	**3**	**32.0±0.4**	**26.2±0.1**
200	**3**	**31.0±0.12**	**26.2±0.2**
200	**3**	**30.1±0.1**	**25.8±1.1**
200	**3**	**35.1±0.5**	**24.7±0.3**

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

** This sample was Undetermined when diluted 1/4

4. References:

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