

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

**Parsley 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. Recently, the method was also validated for basil, but the *C. cayetanensis* regulatory method has not yet been validated for regulatory detection of *C. cayetanensis* on parsley. 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 parsley. The outcome of the matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on parsley 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 parsley 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 parsley matrix extension study. Detection rate for parsley samples seeded with 5 and 10 oocysts fell were 80.0% and 90.0%, respectively. All parsley samples seeded with 200 oocysts were positive and all unseeded parsley samples were negative. No inhibited qPCR reactions were identified based on the performance of the internal amplification control. 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 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 matrix extension in basil is provided in Table 2, and from the MLV study is provided in Table 3. Results for detection of *C. cayetanensis* in parsley using the validated method were similar to results obtained in the matrix extension for basil and in MLV study for cilantro and raspberries, with 5 *C. cayetanensis* oocysts identified as the limit of detection.

TABLE 1. Summary of parsley matrix extension results.

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Parsley (25grams)	0	8	0	0%
	5	10	8	80%
	10	10	9	90%
	200	9	9	100.0%

TABLE 2. 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 3. *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. Parsley matrix extension qPCR data.

# oocysts	18S No. positive qPCR reactions (out of 3 replicates)	18 S C <sub>T</sub> value	IAC C <sub>T</sub> value*
0	0	Und	24.8±0.15
0	0	Und	25.3±0.2
0	0	Und	24.5±0.04
0	0	Und	24.8±0.1
0	0	Und	24.7±0.1
0	0	Und	24.7±0.1
0	0	Und	24.7±0.3
0	0	Und	24.4±0.03
5	3	37.3±1.3	25.4±0.2
5	3	36.6±0.7	25.9±0.2
5	2	37.0±0.7	25.3±0.1
5	3	36.6±1.7	25.1±0.2
5	2	35.8±1.1	24.8±0.2
5	2	37.3±0.2	25.4±0.2
5	3	36.9±1.3	25.0±0.05
5	0	Und	25.6±0.3
5	0	Und	25.4±0.2
5	1	37.5	26.5±0.1
10	3	33.9±0.5	25.3±0.02
10	3	35.8±0.9	25.1±0.03
10	3	34.8±0.2	24.7±0.1
10	3	34.3±0.4	25.1±0.3
10	3	36.7±0.9	24.7±0.15
10	3	35.2.3	24.7±0.3
10	1	37.0	24.6±0.4
10	3	34.5±0.1	25.2±0.1
10	0	Und	25.0±0.15
10	3	35.5±1.6	25.3±0.2
200	3	30.9±0.1	25.1±0.3
200	3	30.4±0.1	25.0±0.2
200	3	30.7±0.4	24.6±0.2
200	3	30.3±0.1	24.5±0.1
200	3	30.4±0.2	24.6±0.2
200	3	30.3±0.3	24.8±0.2
200	3	30.1±0.2	24.5±0.02
200	3	30.3±0.2	24.5±0.2
200	3	31.0±1.5	24.1±0.3

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

#### 4. References:

- Centers for Disease Control, Parasites – U.S. Foodborne Outbreaks of Cyclosporiasis 2000-2014. Available at: <https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html>
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3. 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. Epub 2015 Apr 13.