

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

Carrot 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 mixed salads containing a number of leafy greens, berries, and cilantro. 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 2014 did not identify the specific source or origin of contaminated produce that caused the outbreaks

(<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 and approved by the MMVS for publication in the FDA *Bacteriological Analytical Manual*. Because additional produce commodities are also considered a risk for *C. cayetanensis* infection, CFSAN requested a matrix extension study of the new method for shredded carrots. The outcome of the matrix extension study performed to assess the previously validated method for detection of *C. cayetanensis* seeded on shredded carrots 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 shredded carrots un-spiked or spiked with 5, 10, 20 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 carrot matrix extension study. Detection rates for carrot samples seeded with 5 and 10 oocysts fell within the fractional positive target range and were 50.0% and 70.0%, respectively. All carrot samples seeded with 20 and 200 oocysts were positive and all unseeded carrot 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 numbers 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 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 shredded carrots 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.

TABLE 1. Summary of shredded carrot 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	10	7	70.0%
	20	10	10	100.0%
	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. Carrot 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	25.2±0.1

0	0	Und	24.7±0.2
0	0	Und	25.1±0.1
0	0	Und	25.0±0.2
0	0	Und	25.0±0.0
0	0	Und	24.8±0.2
0	0	Und	25.0±0.1
0	0	Und	25.2±0.0
5	2	36.3±1.3	24.2±0.1
5	2	37.0±1.1	25.8±0.06
5	2	36.9±0.5	26.1±0.2
5	1	35.5	25.9±0.3
5	0	Und	25.7±0.1
5	2	37.2±1.1	25.8±0.1
5	0	Und	25.9±0.1
5	0	Und	25.7±0.1
5	0	Und	25.3±0.2
5	0	Und	25.1±0.1
10	3	35.8±0.04	25.9±0.1
10	2	37.2±0.02	25.8±0.1
10	0	Und	25.7±0.3
10	3	36.1±1.23	24.5±0.05
10	3	35.1±0.2	24.5±0.05
10	0	Und	24.6±0.1
10	3	35.7±0.07	24.3±0.0
10	3	37.0±0.6	24.1±0.2
10	3	35.5±0.7	24.3±0.1
10	0	39.5**	24.2±0.1
20	2	37.0±0.8	25.4±0.0
20	1	37.7	25.6±0.2
20	3	36.5±0.8	25.5±0.1
20	3	37.4±0.4	24.2±0.2
20	3	35.8±0.6	24.4±0.4
20	3	35.8±0.6	24.4±0.0
20	3	35.3±0.2	24.2±0.1
20	3	36.3±0.6	24.0±0.1
20	1	36.3	24.2±0.1
20	3	34.1±0.2	24.2±0.1
200	3	31.5±0.2	25.5±0.1
200	3	32.2±0.4	25.8±0.6
200	3	33.3±0.3	25.4±0.0
200	3	32.1±0.2	24.2±0.1
200	3	31.9±0.2	24.2±0.0
200	3	32.8±0.8	24.1±0.2
200	3	32.7±0.9	24.2±0.2
200	3	33.3±0.4	24.1±0.1

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

** Undetermined when DNA diluted 1/4

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>

2. Crowe, S. 2016. Outbreaks Attributed to Foods Imported into the United States, 1996–2015. *In*, 6th Annual FDA Foods and Veterinary Medicine Science and Research Conference, Food Safety, Veterinary Medicine, Nutrition and Cosmetics Research: Meeting the Challenges of a Global Supply Chain. October 25-26, 2016, Silver Spring, MD.
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.