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Evaluation of the U.S. Food and Drug Administration validated method for detection of *Cyclospora cayetanensis* in high-risk fresh produce matrices and a method modification for a prepared dish

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ABSTRACT

The performance of the U.S. Food and Drug Administration (FDA) validated method for regulatory detection of *Cyclospora cayetanensis* in leafy greens and berries was evaluated in additional high-risk fresh produce items and in a dish prepared with these produce commodities. The method was robust and reproducible in basil, parsley, shredded carrots, shredded cabbage and carrot mix, and could detect as few as 5 oocysts in 25 g samples. Some differences in *C. cayetanensis* detection were found among the fresh produce analyzed. Significantly lower target gene copy numbers per reaction were obtained with shredded carrots, and shredded cabbage and carrot mix compared to leafy greens, which highlights the importance of evaluating the performance characteristics of validated methods in different food matrices. In the prepared dish, coleslaw with dressing, the method was optimized to detect 5 oocysts in a 25 g sample by using 1.0% Alconox® in the washing solution instead of 0.1% as originally described. These data are important to assess the prevalence of *C. cayetanensis* in different produce items and to support outbreak investigations.

1. Introduction

Cyclospora cayetanensis is an intestinal protozoan parasite that causes a diarrheal illness in humans called cyclosporiasis. Symptoms of cyclosporiasis include explosive watery diarrhea, nausea, fatigue, increased gas, weight loss, bloating, and loss of appetite, with symptoms typically beginning an average of 7 days after infection (Herwaldt, 2000). Humans become infected with *C. cayetanensis* after consuming food or water contaminated with the parasite's sporulated oocysts. Infected humans shed non-sporulated oocysts, which require 7–15 days under ideal conditions (23–27 °C) in the environment, to sporulate and become infective (Ortega and Sanchez, 2010).

Cyclosporiasis is an emerging infectious disease in developing and developed countries such as the U.S. and Canada (Dixon, 2016). Global trade of foods may play a significant role in the transmission of *C. cayetanensis* in the U.S., considering that some of the outbreak cases have been traced back to fresh produce imported from developing regions. The food items implicated in these outbreaks included dishes prepared with fresh produce, such as basil, snow peas, berries, cilantro and bagged mixed greens (Abanyie et al., 2015; CDC, 2016; Dixon, 2016; Hall et al., 2012; Herwaldt, 2000; Ho et al., 2002; Kozak et al., 2013; Ortega and Sanchez, 2010). In 2017, there were a total of 1065 cases reported from 40 states in the U.S. with 52 hospitalizations, and at least 597 (56%) of those were domestically acquired (CDC, 2017).

The identification of food items that serve as vehicles in cyclosporiasis outbreaks represents a major challenge. The long incubation period for *C. cayetanensis* infection, the short shelf life of implicated commodities (i.e., fresh produce), and the complex epidemiological investigations required to identify the contaminated produce item present in a dish with multiple ingredients, are among the factors that hamper these investigations. Produce can become contaminated with *C. cayetanensis* in the field and during harvest, storage and transportation. Factors including poor worker hygiene practices and contaminated soil and agricultural water could also play a role in this process (Chacin-Bonilla, 2017). (Chacin-Bonilla, 2017). Recent studies have shown *C. cayetanensis* contamination in ready to eat and pre-packaged/bulked vegetable products in Canada and Europe (Caradonna et al., 2017; Dixon et al., 2013; Lalonde and Gajadhar, 2016a) serving as an indication that the current sanitation processes do not guarantee food safety when dealing with certain parasites of fecal origin (Caradonna et al., 2017).

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Improved methods for detection and characterization of the parasite are essential to identify and track sources of produce contamination and to strengthen surveillance (Abanyie et al., 2015). *Cyclospora cayetanensis* cannot be propagated *in vivo* or *in vitro,* currently making the use of enrichment methods to confirm its presence in foods or environmental samples impossible. The U.S. Food and Drug Administration (FDA) developed and validated a new regulatory method for detection of *C. cayetanensis* in produce (Murphy et al., 2017a, 2018). The method employs an enhanced washing solution to recover *C. cayetanensis* oocysts from produce, a commercially available procedure to disrupt the oocysts and purify their DNA, and a species-specific TaqMan™ real-time PCR assay targeting the *C. cayetanensis* 18S rRNA gene for molecular detection. This FDA method was originally validated for the detection of *C. cayetanensis* in cilantro and raspberries, two of the matrices historically linked to cyclosporiasis outbreaks in North America (Abanyie et al., 2015; Herwaldt, 2000; Ho et al., 2002; Murphy et al., 2017a). However, a variety of other fresh produce have been implicated in outbreaks. For example, basil, in some instances in prepared dishes (CDC, 1997; Kozak et al., 2013; Lopez et al., 2001), was linked to multiple outbreaks in Canada and in the U.S. (Chacin-Bonilla, 2017; Herwaldt, 2000; Kozak et al., 2013; Ortega and Sanchez, 2010). In fact, the first time *C. cayetanensis* was molecularly and microscopically detected in an implicated food was in leftovers from a chicken pasta salad containing basil linked to outbreaks in Missouri in 1999, confirming the original epidemiological data (Lopez et al., 2001). *Cyclospora cayetanensis* contamination has also been reported in basil in fresh produce surveillance studies in Vietnam and Nepal, and parsley was positive for *C. cayetanensis* in samples tested in Egypt (reviewed by Dixon, 2016). In Germany in 2000–2001, butterhead lettuce, mixed lettuce, dill, parsley and green onions were associated with an outbreak involving 34 people who ate salads (Döller et al., 2002). 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) (FDA, 2013). Additionally, in 2016, a restaurant-associated sub-cluster of cyclosporiasis in Texas was epidemiologically linked to consumption of coleslaw containing shredded carrots and cabbage (Fox, 2017).

It is important to assess the efficacy of the new validated FDA method for detection of *C. cayetanensis* in additional produce matrices and in prepared dishes to identify potential improvements for use during future outbreak investigations or surveillance activities. Method modifications may be needed to strengthen perfomance in various types of fresh produce and, in particular, in prepared dishes which may include multiple fresh produce items and other ingredients. The objective of the present study was to evaluate the performance of the FDA method for detection of *C. cayetanensis* in fresh produce items previously linked to outbreaks, i.e. shredded carrots, shredded cabbage with carrot mix, basil, and parsley, and to evaluate specific modifications developed for optimal use in a prepared dish, coleslaw.

2. Material and methods

2.1. Preparation of oocysts and initial seeding studies

Purified *C. cayetanensis* oocysts originating from a patient from Indonesia, and stored in 2.5% potassium dichromate, were used in these experiments; approximately 50% of the oocysts were sporulated in this preparation. Sporulated oocysts should have higher gene copy numbers than non-sporulated oocysts, but to our knowledge there are no published comparison data on this regard. The use of the oocysts was approved by the institutional review board of the FDA (protocol number 15–039F). The oocysts were washed with 0.85% NaCl and concentrated prior to enumeration. Six replicates of the purified oocysts were counted using a hemocytometer on an Olympus BX51 microscope (Optical Elements Corporation, Dulles, VA, U.S.). Oocysts were then diluted in 0.85% NaCl to contain 20 oocysts/μL and 1 oocysts/μL for seeding experiments.

The oocysts were initially seeded in the validated produce matrices (cilantro and raspberries); the analysis demonstrated that the previously established performance standards for the detection method (Murphy et al., 2017a) were achieved (data not shown). Subsequently, the same preparation of oocysts was used for all seeding experiments in shredded carrots, cabbage and carrot mix, basil, parsley, and prepared coleslaw described in the present study.

2.2. Sample preparation and seeding in fresh produce

The fresh produce analyzed consisted of bagged shredded carrots, bagged shredded green cabbage with carrot mix (commercial classic coleslaw all natural, approximately 95% shredded green cabbage and 5% shredded carrots), sweet basil, and Italian parsley. All produce was fresh, showing no signs of deterioration, obtained from local grocery stores, and stored at 4 °C for no longer than 24–48 h prior to seeding. Individual fresh produce test samples (25 g for each of the commodities) were prepared as described previously (Murphy et al., 2017a).

The samples were seeded with 200, 10, or 5 oocysts by dropwise application of 10 μL or 5 μL of the appropriate oocyst dilution using a micro-pipet to spread the oocysts randomly over multiple surfaces of the sample. Unseeded samples were also included as negative controls and processed together with the seeded samples. Unseeded and seeded samples were allowed to air dry uncovered at room temperature for approximately 2 h. Afterwards, samples were carefully transferred to BagPage +400 filter bags (Interscience Lab Inc., Boston, MA), sealed by securing the folded openings with small binder clips, and held at 4 °C for 48–72 h prior to initiating the produce wash step. No more than 12 samples were processed per experiment which included at least one unseeded sample plus samples with all three seeding levels. Between eight to eleven sample replicates for each matrix were examined unseeded and at each seeded level.

A total of 141 samples of fresh produce (36 shredded carrot samples, 40 shredded green cabbage and carrot mix samples, 38 sweet basil samples, and 37 Italian parsley samples) were analyzed in this study.

2.3. FDA BAM chapter 19B method for detection of C. cayetanensis in fresh produce

The wash protocol to recover the oocysts from fresh produce, the DNA extraction of concentrated oocysts, and the qPCR analysis using a TaqMan™ method targeting the *C. cayetanensis* 18SrRNA gene were performed as described in the FDA's BAM Chapter 19B (Murphy et al., 2017a, 2017b). The wash protocol to recover the oocysts from fresh produce was performed using 0.1% Alconox® detergent and sequential centrifugations to recover, pool, and concentrate the wash debris. After this step, the produce wash debris pellets were stored at 4 °C for up to 24 h or frozen at −20 °C for longer periods prior to DNA isolation. The DNA extraction procedure was performed using the FastDNA SPIN Kit for Soil in conjunction with a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, California) and extracted DNA samples were stored at 4 °C for up to 2 days prior to performing PCR or at −20 °C for longer term storage.

A TaqMan™ dual real-time PCR assay targeting both the *C. cayetanensis* 18S rRNA gene and an exogenous internal amplification control (IAC) was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA). The IAC reaction was used to monitor for reaction failure due to matrix derived PCR inhibition. The commercially prepared synthetic gBlocks gene fragment (Integrated DNA Technologies, Coralville, CA) was used as a positive control for amplification of the *C. cayetanensis* 18S rRNA gene (Cyc18SrDNA control). Serial dilutions of the positive control target, covering six orders of magnitude ranging from 5×10^4 to 0.5 copies/μL, were prepared. For positive control reactions and standard curve experiments, 2.0 μL of the appropriate positive control dilutions were used as template in real-time PCR reactions to achieve the desired

target concentrations ranging from 10^5 to one copy per reaction. Real-time PCR standard curve reactions were performed in the absence and presence of 2.0 μL of DNA extracts from unseeded matrix samples for each fresh produce commodity to verify the robustness of the qPCR reactions in the presence of potential matrix derived qPCR inhibitors (Murphy et al., 2017a, 2017b).

Each experimental real-time PCR run consisted of study samples, a non-template control (NTC), and positive controls containing 10-fold serial dilutions from 10^3 to 10 copies of the Cyc18SrDNA control analyzed in triplicate reactions. Runs were only considered valid if all three replicates of the positive control reactions produced the expected positive result with a cycle threshold (C_T) 38.0 or lower. Samples were considered positive if one or more replicates produced a *C. cayetanensis* target reaction with a C_T value less than or equal to 38.0. Reactions with C_T values greater than 38.0 were considered negative, and considered inconclusive if the IAC reaction failed or produced an average C_T value more than three cycles higher when compared to the NTC. All DNA extracts from seeded produce samples which initially tested negative by real-time PCR were re-tested in triplicate using 2.0 μL of a four-fold dilution of the original template DNA and were considered positive for the presence of *C. cayetanensis* if at least one replicate produced a positive result.

2.4. Optimization and evaluation of the FDA BAM chapter 19B method for detection of C. cayetanensis in prepared coleslaw with dressing

The performance of the FDA BAM Chapter 19B method in recovery of *C. cayetanensis* was evaluated in prepared coleslaw: Commercial classic coleslaw all natural, (approximately 95% shredded green cabbage and 5% shredded carrots) with added commercial dressing. The commercial dressing (commercial dressing coleslaw original) contained as ingredients soybean oil, sugar, apple cider, vinegar, water, egg yolks, less than 2.0% of salt, natural flavor, dried torula yeast, celery seed, spice, xanthan gum, dehydrated onion and dehydrated garlic. Samples of 25 g shredded cabbage and carrot mix were seeded with 5, 10 or 200 C. *cayetanensis* oocysts and allowed to air dry uncovered at room temperature for approximately 2 h. Unseeded samples were also included and processed with the seeded samples. Subsequently, one tablespoon of commercial coleslaw dressing was added to each sample, mixed, and samples were carefully transferred to BagPage +400 filter bags, sealed by securing the folded openings with small binder clips, and held at 4 °C for 48 h.

Two independent experiments were conducted to establish the optimal washing conditions for the recovery of the oocysts from the prepared coleslaw dish. In each experiment, samples seeded with 5, 10 and 200 oocysts were washed with 0.1% Alconox® or 1.0% Alconox®. Careful aspiration of the top fat layer on the centrifuge tubes was performed after each centrifugation. A total of 20 prepared coleslaw samples were analyzed in these comparative experiments using the two different concentrations of Alconox®. In subsequent experiments a total of 38 prepared coleslaw samples were analyzed following the FDA protocol (section 2.3) with 1.0% Alconox® used in the washing step.

2.5. Statistical analysis

Detection rates were calculated for each matrix at each inoculation level. Positive rates were calculated as the percentage of inoculated samples which gave a positive result. Statistically significant differences between positive rates of detection for each seeding level in the different fresh produce matrices and among seeding levels in each matrix were analyzed by Fisher's exact or Chi-square tests using GraphPad (GraphPad, San Diego, CA). The number of 18S rRNA gene copies/reaction was automatically calculated by the 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) by extrapolation of the mean C_T value of each sample on a standard curve generated for the synthetic *C. cayetanensis* positive control run in each plate (from 10^3 to 10 copies per reaction). Differences in 18S rRNA gene copies per reaction (2 μL of DNA in each reaction) at each seeding level among commodities were analyzed by one-way analysis of variance (ANOVA), Kruskal-Wallis test and Dunn's and/or Tukey's multiple comparison test using GraphPad Software (GraphPad, San Diego, CA). A P value < 0.05 indicates statistical difference.

3. Results

3.1. Cyclospora cayetanensis detection in fresh produce

The FDA BAM Chapter 19B method for produce washing, DNA extraction, and qPCR performed well without modification for detection of *C. cayetanensis* in the fresh produce matrices (bagged shredded carrots, bagged shredded cabbage with carrot mix, basil, and parsley) evaluated in this study. The performance of the qPCR assay was assessed by analysis of serial dilutions of the *C. cayetanensis* positive control in the presence of DNA extracts from unseeded produce samples. The standard curves showed no significant decline in qPCR reaction efficiency or sensitivity in the presence of DNA extracts for the matrices examined in this study (data not shown).

Table 1 shows TaqMan™ real-time PCR detection results for the *C. cayetanensis* 18S rRNA gene and IAC targets for each fresh produce sample analyzed in the study. As few as five seeded *C. cayetanensis* oocysts were identified in all four fresh produce matrices examined using the FDA BAM method. All produce samples seeded with 200 oocysts were positive and all unseeded samples from each fresh produce were negative (Table 1). All samples found negative when analyzed undiluted were also negative when analyzed at four-fold dilutions, including one shredded carrot sample seeded with 10 oocysts and one basil sample seeded with 5 oocysts, each producing one real-time PCR replicate with C_T values above 38.0 (Table 1). Amplification of the IAC was successful for all the samples tested in this study with increases in C_T values of no more than approximately one cycle when compared to the NTC suggesting that inhibition was low or insignificant.

Detection results are summarized for each seeding level and each matrix analyzed in Table 2. Detection rates for samples seeded with 5 oocysts were 50%, 45.4%, 70% and 80% in carrots, cabbage and carrot mix, basil, and parsley, respectively. The mean *C. cayetanensis* C_T values for positive samples at this seeding level ranged from 36.1 ± 0.9 in basil to 37.1 ± 0.4 in cabbage and carrot mix. The calculated mean number of 18S rRNA gene target copies per reaction when samples were seeded with 5 oocysts was lower for shredded cabbage and carrot mix samples compared to basil samples ($P = 0.05$).

Detection rates for samples seeded with 10 oocysts were 70%, 63.6%, 100% and 90% for shredded carrots, shredded cabbage and carrot mix, basil, and parsley samples, respectively. Detection rates for samples seeded with 10 oocysts were higher than rates for samples seeded with 5 oocysts; however, the differences were not statistically significant for any of the fresh produce analyzed ($P > 0.05$). All three real-time PCR replicates for each basil sample seeded with 10 oocysts were positive (Table 1). Cabbage and carrot mix samples had the lowest mean *C. cayetanensis* 18S rRNA gene copies per reaction (Table 2), but differences in the mean copy number per reaction in samples seeded with 10 oocysts were not statistically significant among any of the fresh produce analyzed at this seeding level ($P > 0.05$). Additionally, differences in the mean copy number per reaction for samples seeded with ten oocysts compared to those seeded with five oocysts were also not statistically significant ($P > 0.05$).

At the 200 oocysts seeding level, all three real-time PCR replicates for each sample analyzed were positive except for one replicate in one shredded cabbage and carrot mix sample (Table 1). Statistically significant differences in 18S rRNA gene copy numbers per reaction were observed among the fresh produce matrices at the 200 oocysts seeding level ($P < 0.0001$, Fig. 1). Parsley showed significantly higher mean copy numbers per reaction compared to all other matrices at this

Table 1
Real-time PCR results for fresh produce samples unseeded and seeded with *Cyclospora cayetanensis* oocysts.

No. of oocysts seeded	Carrots			Basil			Parsley			Cabbage and carrot mix		
	18S rRNA		IAC	18S rRNA		IAC	18S rRNA		IAC	18S rRNA		IAC
	No. positive replicates[a]	Mean CT value	Mean CT value	No. positive replicates[a]	Mean CT value	Mean CT value	No. positive replicates[a]	Mean CT value	Mean CT value	No. positive replicates[a]	Mean CT value	Mean CT value
0	0	Und	25.2 ± 0.1	0	Und	26.1 ± 0.2	0	Und	24.8 ± 0.1	0	Und	25.8 ± 0.1
0	0	Und	24.7 ± 0.2	0	Und	25.0 ± 0.2	0	Und	25.3 ± 0.2	0	Und	27.0 ± 0.1
0	0	Und	25.1 ± 0.1	0	Und	25.4 ± 0.1	0	Und	24.5 ± 0.0	0	Und	25.1 ± 0.3
0	0	Und	25.0 ± 0.2	0	Und	25.2 ± 0.1	0	Und	24.8 ± 0.1	0	Und	24.9 ± 0.5
0	0	Und	25.0 ± 0.0	0	Und	25.3 ± 0.1	0	Und	24.7 ± 0.1	0	Und	25.8 ± 0.1
0	0	Und	24.8 ± 0.2	0	Und	25.0 ± 0.2	0	Und	24.7 ± 0.1	0	Und	25.5 ± 0.1
0	0	Und	25.0 ± 0.1	0	Und	25.5 ± 0.1	0	Und	24.7 ± 0.3	0	Und	25.0 ± 0.1
0	0	Und	25.2 ± 0.0	0	Und	24.7 ± 0.2	0	Und	24.4 ± 0.0	0	Und	25.2 ± 0.0
5	2	36.3 ± 1.3	24.2 ± 0.1	3	36.0 ± 1.2	25.9 ± 0.4	3	37.3 ± 1.3	25.4 ± 0.2	0	Und	25.9 ± 0.2
5	2	37.0 ± 1.1	25.8 ± 0.1	1	37.4	26.0 ± 0.3	3	36.6 ± 0.7	25.9 ± 0.2	0	Und	26.1 ± 0.1
5	2	36.9 ± 0.5	26.1 ± 0.2	0	Und	25.8 ± 0.1	2	37.0 ± 0.7	25.3 ± 0.1	0	Und	25.8 ± 0.1
5	1	35.5	25.9 ± 0.3	3	36.5 ± 1.3	25.6 ± 0.2	3	36.6 ± 1.7	25.1 ± 0.2	0	Und	25.7 ± 0.1
5	0	Und	25.7 ± 0.1	0	39.2**	25.5 ± 0.8	2	35.8 ± 1.1	24.8 ± 0.2	2	37.1 ± 0.5	26.8 ± 0.2
5	2	37.2 ± 1.1	25.8 ± 0.1	3	35.6 ± 0.6	25.8 ± 0.1	2	37.3 ± 0.2	25.4 ± 0.2	2	37.1 ± 0.2	27.6 ± 0.3
5	0	Und	25.9 ± 0.1	2	37.04 ± 0.7	25.2 ± 0.2	3	36.9 ± 1.3	25.0 ± 0.0	0	Und	26.3 ± 0.1
5	0	Und	25.7 ± 0.1	2	35.4 ± 0.1	25.2 ± 0.2	0	Und	25.6 ± 0.3	3	37 ± 0.6	26.5 ± 0.1
5	0	Und	25.3 ± 0.2	2	35.9 ± 0.6	25.6 ± 0.6	0	Und	25.4 ± 0.2	1	37.6	25.3 ± 0.2
5	0	Und	25.1 ± 0.1	0	Und	25.6 ± 0.9	1	37.5	26.5 ± 0.1	0	Und	25.3 ± 0.3
5										2	36.6 ± 1.3	25.1 ± 0.1
10	3	35.8 ± 0.1	25.9 ± 0.1	3	36.1 ± 0.6	25.8 ± 0.1	3	33.9 ± 0.5	25.3 ± 0.0	3	35.0 ± 0.7	25.9 ± 0.1
10	2	37.2 ± 0.0	25.8 ± 0.1	3	32.9 ± 1.9	25.6 ± 0.2	3	35.8 ± 0.9	25.1 ± 0.0	2	37.7 ± 0.3	25.8 ± 0.2
10	0	Und	25.7 ± 0.3	3	35.0 ± 0.8	25.6 ± 0.2	3	34.8 ± 0.2	24.7 ± 0.1	0	Und	25.6 ± 0.1
10	3	36.1 ± 1.2	24.5 ± 0.05	3	34.1 ± 0.2	25.4 ± 0.2	3	34.3 ± 0.4	25.1 ± 0.3	1	35.4	25.8 ± 0.1
10	3	35.1 ± 0.2	24.5 ± 0.05	3	35.9 ± 1.3	25.1 ± 0.3	3	36.7 ± 0.9	24.7 ± 0.1	3	35.6 ± 0.5	26.5 ± 0.2
10	0	Und	24.6 ± 0.1	3	35.2 ± 0.2	26.4 ± 0.4	3	35.2 ± 0.3	24.7 ± 0.3	0	Und	26.7 ± 0.2
10	3	35.7 ± 0.1	24.3 ± 0.0	3	36.1 ± 1.2	25.8 ± 0.9	1	37.0	24.6 ± 0.4	3	33.9 ± 0.2	25.2 ± 0.1
10	3	37.0 ± 0.6	24.1 ± 0.2	3	35.6 ± 0.7	26.4 ± 0.8	3	34.5 ± 0.1	25.2 ± 0.1	3	35.5 ± 0.3	25.9 ± 0.2
10	3	35.5 ± 0.7	24.3 ± 0.1	3	35.1 ± 0.4	25.4 ± 0.1	0	Und	25.0 ± 0.1	0	Und	25.1 ± 0.1
10	0	39.5**	24.2 ± 0.1	3	34.1 ± 0.5	25.5 ± 0.2	3	35.5 ± 1.6	25.3 ± 0.2	0	Und	25.2 ± 0.2
10										1	37.22	25.1 ± 0.2
200	3	31.5 ± 0.2	25.5 ± 0.1	3	30.7 ± 0.1	25.4 ± 0.2	3	30.9 ± 0.1	25.1 ± 0.3	2	34.6 ± 0.4	25.7 ± 0.1
200	3	32.2 ± 0.4	25.8 ± 0.6	3	30.8 ± 0.0	25.9 ± 0.3	3	30.4 ± 0.1	25.0 ± 0.2	3	33.0 ± 0.2	25.6 ± 0.1
200	3	33.3 ± 0.3	25.4 ± 0.0	3	30.9 ± 0.1	25.7 ± 0.2	3	30.7 ± 0.4	24.6 ± 0.2	3	33.6 ± 0.6	25.6 ± 0.3
200	3	32.1 ± 0.2	24.2 ± 0.1	3	30.5 ± 0.2	27.1 ± 0.2	3	30.3 ± 0.1	24.5 ± 0.1	3	32.8 ± 0.2	25.3 ± 0.2
200	3	31.9 ± 0.2	24.2 ± 0.0	3	32.7 ± 0.2	27.2 ± 0.5	3	30.4 ± 0.2	24.6 ± 0.2	3	31.6 ± 0.2	25.5 ± 0.1
200	3	32.8 ± 0.8	24.1 ± 0.2	3	30.0 ± 0.1	24.9 ± 0.1	3	30.3 ± 0.3	24.8 ± 0.2	3	31.5 ± 0.3	25.9 ± 0.1
200	3	32.7 ± 0.9	24.2 ± 0.2	3	32.0 ± 0.4	26.2 ± 0.1	3	30.1 ± 0.2	24.5 ± 0.0	3	32.5 ± 0.1	25.1 ± 0.1
200	3	33.3 ± 0.4	24.1 ± 0.1	3	31.0 ± 0.1	26.2 ± 0.2	3	30.3 ± 0.2	24.5 ± 0.2	3	32.9 ± 0.2	25.1 ± 0.1
200				3	30.1 ± 0.1	25.8 ± 1.1	3	31.0 ± 1.5	24.1 ± 0.3	3	35.2 ± 0.9	25.2 ± 0.2
200				3	35.1 ± 0.5	24.7 ± 0.3				3	33.6 ± 0.4	25.1 ± 0.1

Und: Undetermined after 45 reaction cycles.
**the samples were negative when analyzed at four-fold dilution of DNA.
[a] Out of three replicates/reaction.
C_T values are expressed as the mean ± standard deviation of the positive replicate real-time PCR reactions for each sample. No standard deviation is shown when only one replicate produced a positive result.

Table 2
Cyclospora cayetanensis detection results for fresh produce 25 g samples by matrix and seeding level.

	No. oocysts inoculated	No. samples analyzed	No. samples positive	% positive samples[a]	Mean C_T[b]	Mean 18S rRNA (copies/reaction[b,c])
Shredded carrots	0	8	0	0	Und	0
	5	10	5	50	36.6 ± 0.7	1.5 ± 1.9
	10	10	7	70	36.0 ± 0.8	4.5 ± 4.3
	200	8	8	100	32.5 ± 0.6	61.4 ± 35.7
Cabbage and carrot mix	0	8	0	0	Und	0
	5	11	5	45.4	37.1 ± 0.4	0.6 ± 0.8
	10	11	7	63.6	35.8 ± 1.4	2.4 ± 2.8
	200	10	10	100	33.1 ± 1.2	26.1 ± 18.3
Basil	0	8	0	0	Und	0
	5	10	7	70	36.1 ± 0.9	2.8 ± 2.2
	10	10	10	100	35.0 ± 1.0	10.0 ± 14.2
	200	10	10	100	31.4 ± 1.5	85.2 ± 44.2
Parsley	0	8	0	0	Und	0
	5	10	8	80	36.9 ± 0.5	1.9 ± 1.2
	10	10	9	90	35.3 ± 1.1	6.8 ± 5.0
	200	9	9	100	30.5 ± 0.3	146.1 ± 22.5

Und: Undetermined after 45 qPCR reaction cycles.
[a] Percentage of seeded samples which gave a positive result by qPCR analysis.
[b] Mean ± SD. From three replicates per reaction/sample analyzed.
[c] 18S rRNA copies gene/reaction were calculated from the software based on standards run on each plate.

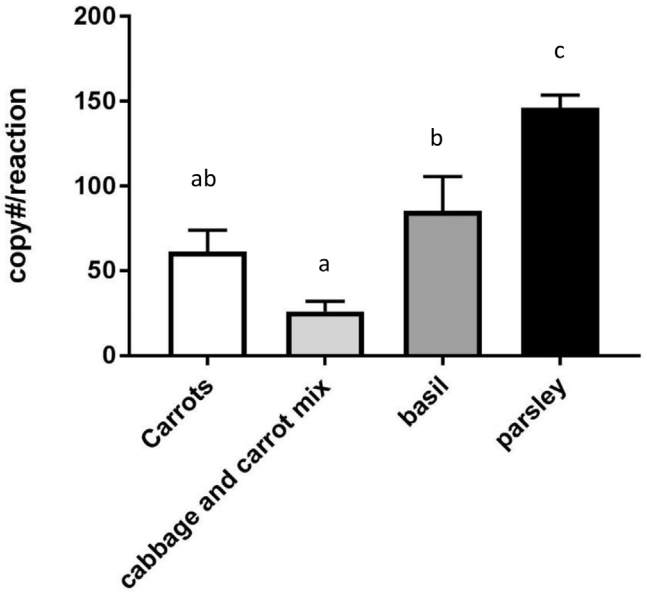

Fig. 1. Comparison of mean copy number of the 18S rRNA gene determined per qPCR reaction (2 µL of DNA/reaction) in carrots, cabbage and carrots mix, parsley, and basil after seeding samples with 200 *Cyclospora cayetanensis* oocysts. Arbitrary letters a, b, and c, were indicated over columns. Different letters over the columns indicate statistically significant differences among matrices ($P < 0.05$). Significant differences were observed between cabbage and carrots mix samples compared to both basil and parsley samples, and in parsley compared to all other matrices. No significant differences were observed between carrots and cabbage and carrots mix or between carrots and basil. The standard error is represented by error bars.

seeding level. Mean copy numbers per reaction were significantly lower in cabbage and carrot mix samples compared to both basil and parsley samples (Fig. 1). No significant differences in copy number per reaction were observed between carrots and cabbage and carrot mix or between carrots and basil.

At each seeding level (5, 10 and 200 oocysts), no significant differences were observed in any qPCR detection results (rate of positivity or mean copy numbers per reaction) between carrots and cabbage and carrot mix samples.

3.2. Cyclospora cayetanensis detection in prepared coleslaw (shredded cabbage and carrot mix with added dressing)

Two preliminary experiments demonstrated that a higher concentration of Alconox® in the wash solution was needed to emulsify the apparent amount of fat in prepared coleslaw samples and substantially improved detection (Table 3A). The number of samples seeded with 5 and 10 oocysts found positive was higher when washing was performed with a 1.0% Alconox® solution instead of a 0.1% Alconox® solution (Table 3A). All samples seeded at 200 oocysts level were positive using both wash concentrations, but substantially higher mean copy numbers per reaction were observed in samples seeded with 200 oocysts when washed with 1.0% Alconox® (Table 3A).

The modified washing protocol using 1.0% Alconox was subsequently used for the analysis of all prepared coleslaw samples reported in Table 3B. As few as five *C. cayetanensis* oocysts seeded on 25 g of prepared coleslaw were detected using this modification. Positive rates obtained by real-time PCR were 100%, 90%, and 80% for prepared coleslaw samples seeded with 200, 10, and 5 oocysts, respectively (Table 3B). All unseeded samples were negative. The 18S rDNA gene copy numbers per reaction in prepared coleslaw were statistically higher compared to those in shredded cabbage and carrot mix for samples seeded with 5 oocysts ($P = 0.01$), 10 oocysts ($P = 0.01$) and 200 oocysts ($P = 0.03$).

4. Discussion

To better understand the impact of *C. cayetanensis* in food safety, more studies using reliable laboratory detection methods are needed to estimate the prevalence of this parasite in a variety of produce commodities and complex dishes. Some studies have detected *C. cayetanensis* in the U.S. in domestic produce and in effluent samples from water treatment plants (Dixon et al., 2013; Kitajima et al., 2014). The significance of these findings is unclear, and it is not known if any domestically acquired cases of cyclosporiasis in the U.S. were associated with food that was contaminated in the U.S. or they occurred as a result of the consumption of contaminated imported produce. Furthermore, due to the complexity of *C. cayetanensis* epidemiologic investigations, it is possible that produce commodities historically associated with cyclosporiasis outbreaks represent only a fraction of those

Table 3A
Detection of *Cyclospora cayetanensis* in 25 g prepared coleslaw (dressing added) samples using either 0.1% or 1.0% Alconox® detergent in the produce wash solution. Two independent experiments were conducted (experiment 1 and experiment 2). In each experiment samples seeded with 5, 10 and 200 oocysts were washed with 0.1% Alconox® or 1.0% Alconox®.

Coleslaw	0.1% Alconox				1% Alconox		
	No. oocysts inoculated	No. samples analyzed (# positive)	Mean C_T[a]	Mean 18S rRNA (copies/reaction)	No. samples analyzed (# positive)	Mean C_T[a]	Mean 18S rRNA (copies/reaction)
Experiment 1	5	2 (0)	Und*	0	2 (2)	37.0 ± 0.3	1.5
	10	2 (0)	Und	0	2 (2)	34.0 ± 0.4	11.2
	200	1 (1)	35.6 ± 0.4	3.8	1 (1)	29.7 ± 0.2	215
Experiment 2	5	2 (0)	Und	0	2 (0)	Und	0
	10	2 (1)	35.8 ± 0.1	6.8	2 (2)	35.8 ± 0.7	7.4
	200	1 (1)	34.2 ± 0.2	21.1	1 (1)	32.5 ± 0.3	75.2

Table 3B
Detection of *Cyclospora cayetanensis* in prepared coleslaw samples seeded with different levels of oocysts.

Matrix	No. oocysts inoculated	No. samples analyzed	No. of positive samples (%)	Mean C_T[a]	Mean 18S rRNA (copies/reaction[a])
Prepared coleslaw 1% Alconox	0	8	0 (0)	Und	0
	5	10	8 (80)	36.6 ± 0.9	4.5 ± 1.4
	10	10	9 (90)	35.8 ± 1.5	7.4 ± 1.6
	200	10	10 (100)	31.2 ± 1.3	119.0 ± 26.2

[a]Mean ± SD. From three replicates per reaction/sample analyzed.
[b]Percentage of seeded samples which gave a positive result by qPCR analysis.
Und = Undetermined after 45 qPCR reaction cycles.

serving as vehicles for infection with the parasite. In this study, the validated U.S. FDA regulatory method for detection of *C. cayetanensis* in cilantro and raspberries was shown to be very effective for the detection of *C. cayetanensis* in additional high-risk fresh produce matrices as well as in prepared coleslaw.

Molecular detection methods in food matrices that contain high levels of background DNA, and possibly PCR inhibitors, need to be highly sensitive, specific and robust. The validated FDA method using a duplex TaqMan™ real-time PCR assay was robust and reproducible in all fresh produce analyzed in this study and no matrix derived qPCR inhibition was observed. As in the previous study analyzing fresh cilantro and raspberries (Murphy et al., 2017a, 2018), the method was sensitive with as few as five *C. cayetanensis* oocysts detected in 25 g samples (0.2 oocysts per gram) when seeded on carrots, cabbage and carrot mix, basil, and parsley samples. These studies validate the use of the FDA method to detect *C. cayetanensis* on these additional commodities.

To our knowledge, there are no previously published studies of *C. cayetanensis* detection in carrots or in cabbage and carrot mix. Recent unpublished epidemiological data have linked *C. cayetanensis* infection to carrots and coleslaw, and carrots were also linked to outbreaks caused by *Cryptosporidium parvum*, another important foodborne coccidian parasite (Ethelberg et al., 2009; Rimšelienė et al., 2011). Other methods have been previously published for detection of *C. cayetanensis* oocysts spiked in basil and parsley. In basil, Steele et al. (2003) detected 10 oocysts in 100 g of basil by nested PCR; Lalonde and Gajadhar. (2008), reported detection of 10 oocysts and 1 oocyst in 9/15 and 2/15 samples, respectively, when spiked onto wash sediment from 30 g basil samples using ITS-2 rDNA conventional PCR, and Chandra et al. (2014), detected 100 oocysts inoculated per 25 g of basil in 27.8%–51.9% of the samples analyzed using 6 different washing solutions after 24 h post-inoculation, by 18S nested PCR. In addition, a recent study showed a detection limit of 5 oocysts of *Eimeria papillata* (as a surrogate for *C. cayetanensis*) per gram of parsley by SYBR Green real-time PCR followed by melting curve analysis (Lalonde and Gajadhar, 2016b). However, it is not possible to directly compare the detection limit of the method evaluated in this study with other methods described in the literature due to different methodologies, sample sizes and/or gene targets.

Some studies focusing on the evaluation of methods to detect *C. cayetanensis* in produce commodities have reported clear differences in detection among certain fresh produce items. Steele et al. (2003) reported detection of 10 oocysts in 100 g of raspberries and basil, but only 1000 oocysts per 100 g in mesclun lettuce by nested PCR. Lalonde and Gajadhar (2016b) observed differences in the overall recovery rates in parsley, cilantro, and dill (highest recovery rates) compared to mint, thyme, and green onions (lower recovery rates), while the presence of high levels of inhibitors made detection of oocysts in rosemary impossible. In our study, different levels of *C. cayetanensis* 18S rRNA target gene copy numbers per reaction were detected among the fresh produce analyzed, with lower detection in carrots and cabbage and carrot mix compared to basil and parsley. The differences in *C. cayetanensis* detection among the analyzed fresh produce highlights the importance of evaluating the performance characteristics of validated methods in a variety of different food matrices. A possible explanation for the lower detection of *C. cayetanensis* in carrots and cabbage and carrot mix could be the fact that both were shredded fresh-cut produce. Processing operations such as cutting, shredding and slicing may alter the surface structure of fruit and vegetable tissues, and irregular surfaces after shredding of carrots and coleslaw (cabbage and carrot mix) could potentially harbor oocysts and hinder recovery during washings.

Variations in calculated *C. cayetanensis* 18S rRNA gene target copy numbers per reaction were observed among samples within each matrix at each seeding level, as previously reported by Murphy et al. (2017a) using the same protocol, or in other studies using different techniques (Lalonde and Gajadhar, 2016b). In spiking experiments there is always some inevitable inconsistency in the exact number of oocysts seeded per sample, due to pipetting variability, particularly with low oocyst counts, which may contribute to high variation (SD) observed in the number of copies of 18S rRNA gene in samples seeded at the same oocyst level. Additionally, minor variations in efficiency at each step of the procedure for each sample replicate are likely to contribute to small variations in the outcomes. This variation (high SD) does limit accuracy of quantification, and further studies would be required to identify and resolve issues related to quantification. However, even with variations, the comparison of gene target copy numbers by real time PCR was useful for identifying significant differences among matrices, which will be useful information for risk assessment when the method is applied to surveys or traceback investigations in a particular matrix.

Interestingly, significantly higher *C. cayetanensis* 18S rRNA gene copy numbers were detected in parsley compared to basil in samples seeded with 200 oocysts. Little is known about how *C. cayetanensis* interacts with different types of plant surfaces. Differences in efficiency of the DNA extraction among matrices may impact detection, although these differences should have been minimized by using the same

commercial kit for all the samples in the study.

One goal of this study was to optimize the FDA method to detect low numbers of oocysts in prepared coleslaw, which has a high fat content due to the presence of dressing. The use of a higher concentration of detergent (1.0% Alconox®) in the washing solution improved the detection of *C. cayetanensis* oocysts in prepared coleslaw samples seeded with 5 and 10 oocysts. The 18S rRNA gene copy numbers per reaction in prepared coleslaw were compared to those in shredded cabbage and carrot mix (no dressing) to assure that the method was appropriate and to identify any limitations for its use during future outbreak or surveillance investigations. In fact, in this prepared dish, the improved washing step with 1.0% Alconox® provided a very high detection rate, with statistically significantly higher 18S rDNA gene copy numbers detected at the 5, 10 and 200 oocysts seeding levels, compared to the copy numbers obtained for coleslaw without dressing added which was washed with 0.1% Alconox®. The use of a higher percentage of Alconox® detergent likely facilitated the washing process for prepared coleslaw by emulsifying the fat in the dressing. To our knowledge, no previous studies have attempted to detect *C. cayetanensis* in dishes with dressing. However, *C. parvum* outbreaks were epidemiologically linked to contaminated garnish on dressings such as béarnaise sauce containing chopped fresh parsley (Insulander et al., 2008) or salad garnish on chanterelle sauce (Gherasim et al., 2010).

Further development and evaluation of effective methods to identify *C. cayetanensis* in high risk fresh produce matrices and prepared dishes is critically important for support of *C. cayetanensis* prevalence studies and for outbreak investigations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.07.013.

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