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Interlaboratory validation of an improved method for detection of *Cyclospora cayetanensis* in produce using a real-time PCR assay

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ABSTRACT

A collaborative validation study was performed to evaluate the performance of a new U.S. Food and Drug Administration method developed for detection of the protozoan parasite, *Cyclospora cayetanensis*, on cilantro and raspberries. The method includes a sample preparation step in which oocysts are recovered from produce using an enhanced produce washing solution containing 0.1% Alconox and a commercially available method to disrupt the *C. cayetanensis* oocysts and extract DNA. A real-time PCR assay targeting the *C. cayetanensis* 18S rDNA gene with an internal amplification control to monitor PCR inhibition provides species-specific identification. Five laboratories blindly analyzed a total of 319 samples consisting of 25 g of cilantro or 50 g of raspberries which were either uninoculated or artificially contaminated with *C. cayetanensis* oocysts. Detection rates for cilantro inoculated with 200, 10, and 5 oocysts, were 100%, 80%, and 31%, respectively. For raspberries, the detection rates for samples inoculated with 200, 10, and 5 oocysts were 100%, 90% and 50%, respectively. All uninoculated samples, DNA blank extracts, and no-template PCR controls were negative. Reproducibility between laboratories and analysts was high and the method was shown to be an effective analytical tool for detection of *C. cayetanensis* in produce.

1. Introduction

Cyclospora cayetanensis is a protozoan parasite that has emerged worldwide as an agent of a human diarrheal disease called cyclosporiasis. The disease can be transmitted by consumption of food or water contaminated with *C. cayetanensis* oocysts. In developed countries foodborne transmission of *C. cayetanensis* has become a major public health concern and is generally associated with contaminated fresh produce (Dixon, 2016). Foodborne outbreaks and sporadic cases of cyclosporiasis in the U.S have been documented since the mid-1990s and linked to imported fruit and vegetables, including basil, snow peas, berries, cilantro, and mixed salads (Abanyie et al., 2015; Centers for Disease Control and Prevention, 2016; Hall et al., 2012; Herwaldt, 2000; Herwaldt and

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Ackers, 1997). According to surveillance data accumulated by the Centers for Disease Control and Prevention since the mid 1990's, *C. cayetanensis* is second only to *Salmonella* sp. as the most common cause of diarrhea illness cases and outbreaks in the U.S. associated with imported food commodities that are regulated by the U.S. Food and Drug Administration (Crowe, 2016).

Robust validated methods for detection of *C. cayetanensis* in produce are essential to effectively address scientific gaps and support regulatory analytical needs during outbreak investigations. The epidemiological studies conducted during multi-state outbreaks in the last few years drew significant attention to the need for improved laboratory detection and characterization methodologies to identify and properly track sources of produce contamination (Abanyie et al., 2015). Most of the investigations conducted from year 2000–2016 did not identify the source or origin of contaminated produce that caused the cases of infection (Centers for Disease Control and Prevention, 2015).

An improved regulatory method for detection of *C. cayetanensis* in fresh produce was recently developed and assessed in a pre-collaborative study by the U.S. Food and Drug Administration (FDA) for regulatory use (Murphy et al., 2017a) to replace the method published in the FDA *Bacteriological Analytical Manual* (BAM) in 2004 (Orlandi et al., 2004). The new method employs an enhanced produce washing solution to recover *C. cayetanensis* oocysts from produce samples and a procedure to disrupt oocysts and purify DNA which is commercially available. Additionally, the conventional nested PCR (nPCR) assay employed in the original BAM method has been replaced with a species-specific hydrolysis probe-based real-time PCR (qPCR) assay targeting the *C. cayetanensis* 18S rRNA coding region which includes an internal amplification control (IAC). In the pre-collaborative trial, the improved method using the qPCR assay detected as few as 5 *C. cayetanensis* oocysts inoculated onto two matrices with historical ties to cyclosporiasis outbreaks (25 g cilantro or 50 g raspberry samples). The qPCR assay was not only sensitive and specific but also provided ease of execution, reduced matrix derived PCR inhibition, and the ability to monitor for PCR inhibition by multiplexing with an IAC to identify false-negative results.

In the present study, five independent contributing laboratories participated in a collaborative study led by FDA's Center for Food Safety and Applied Nutrition (CFSAN) to verify the efficacy and assess the reproducibility of the new sample preparation steps and the qPCR assay for detection of *C. cayetanensis* in produce. A total of 320 samples of fresh cilantro or raspberries, uninoculated or inoculated with *C. cayetanensis* oocysts, were sent to contributing laboratories for blind analysis. This study did not include a comparison to the produce washing and DNA extraction procedures described in the 2004 BAM protocol due to technical obstacles which prevented reasonable duplication of these steps. However, the conventional nPCR assay in the 2004 BAM was included as a reference molecular detection method. This collaborative study completes the validation process for implementation of the improved detection method for *C. cayetanensis* in fresh produce for regulatory use. The new method is available in the FDA BAM (Murphy et al., 2017b).

2. Materials and methods

2.1. Participating laboratories

Five laboratories from three U.S federal government agencies and one private laboratory contributed data to each phase of the collaborative study. Participating laboratories were designated as Labs 1–5.

2.2. Cyclospora oocysts

Individual human stool samples containing *C. cayetanensis* oocysts were supplied by Professor Jeevan Sherchand from Tribhuvan University Teaching Hospital, Microbiology and Public Health Hospital Research Laboratory in Kathmandu, Nepal and Dr. Ynes Ortega from the University of Georgia in Athens, Georgia, USA (this study was approved by the Institutional Review Board of FDA, RIHSC-ID#10-095F). Stool samples containing *C. cayetanensis* oocysts were preserved and stored at 4 °C in 2.5% potassium dichromate. *C. cayetanensis* oocysts were partially purified as previously described (Murphy et al., 2017a) to permit accurate enumeration. After purification, oocyst preparations were stored at 4 °C in 0.85% NaCl containing either 2.5% potassium dichromate or an antibiotic and antimycotic solution (No. A5955, Sigma-Aldrich, St. Louis, MO) using twice the manufacture's recommended concentration. Prior to enumeration, purified oocysts were washed and diluted in 0.85% saline to contain 300-600 oocysts/μl. Enumeration was performed by counting six replicates using a haemocytometer and a Zeiss Axio Imager D1 microscope (Zeiss, Oberkochen, Germany) with an HBO mercury short arc lamp (Osram, Munich, Germany) and a UV filter (350 nm excitation and 450 nm emission). Three oocyst inoculation solutions (20, 1, and 0.5 oocysts/μl) were prepared in sufficient volume to carry out the entire study by dilution of the enumerated oocyst stock in 0.85% NaCl. Before initiating the study, CFSAN verified that all three oocyst inoculation solutions produced C_q values in the expected ranges by qPCR analysis.

2.3. Experimental design

The originating laboratory (CFSAN) designed and conducted the study in two phases following guidelines of the FDA Office of Foods and Veterinary Medicine (U.S. Food and Drug Administration, 2015). Participating laboratories received standard operation procedures (SOPs) for review and participated in a conference call prior to each phase of the study to assure clear understanding of the study plan and procedures. All contributing laboratories participated in a trial analysis prior to each study phase to check reagents and assure that the provided SOPs were accurate.

Fig. 1 provides an overview of the collaborative study sample preparation and analysis procedures. Phase 1 of the collaborative study comprised eight rounds of analysis that occurred over a period of seven months. Each testing round took 5 consecutive days. In each round, CFSAN prepared and shipped to each participating laboratory a set of identically prepared coded samples. Each set consisted of eight produce samples; two were uninoculated, four were inoculated with 5 or 10 oocysts, and two were inoculated with 200 oocysts. Sample sets were also prepared for analysis at CFSAN, however, those results were not included as part of the collaborative study data because FDA guidelines for validation of microbial detection methods specify that the originating laboratory cannot contribute data to a collaborative validation study. As illustrated in Fig. 1, participating laboratories performed the produce washing procedure, DNA extraction procedure, and molecular analysis using nPCR to complete each study phase 1 testing round by day 5. Study phase 2 was initiated four months after completion of phase 1 and was carried out over a period of 13 days. After recoding to maintain sample anonymity, two analysts from each contributing laboratory performed independent and blinded qPCR analysis of the entire set of DNA samples generated in phase 1 to complete study phase 2 (Fig. 1).

2.4. Preparation of produce samples

The flow diagram in Fig. 1 summarizes the typical test sample

preparation and shipment timeline for each testing round of study phase 1. CFSAN obtained fresh cilantro or raspberries in good to fair condition from a local grocery store on the morning of day 1. Produce was imported and different brands were purchased depending on availability. Produce test samples were prepared by weighing 25 g of cilantro (with stems in excess of approximately 5 cm removed) or 50 g of raspberries in large weigh boats. Samples were inoculated with oocysts using a micro-pipet by applying 10 μl of the appropriate *C. cayetanensis* oocyst dilution solution in approximately 20 small droplets spread randomly over multiple surfaces of the sample. Samples were allowed to air dry uncovered at room temperature for 2 h, carefully transferred from weigh boats to BagPage +400 filter bags, sealed with BagClips (Interscience Lab Inc., Boston, MA), and held at 4 °C overnight. On day 2, filter bags containing samples were packed in insulated boxes on cold packs with bubble wrap to protect samples and prevent contents from shifting. Samples were then shipped for overnight delivery to participating laboratories and immediately unpacked and stored at 4 °C when received on day 3. Laboratories reported the condition of all produce samples to CFSAN upon receipt. Study phase 1 sample analysis took place on days 4 and 5.

2.5. The contributing laboratory protocols

The collaborative study SOPs followed the method for produce washing, DNA extraction, and PCR (including preparation of the synthetic *C. cayetanensis* positive control) as previously described for the pre-collaborative study (Murphy et al., 2017a) with a few modifications. The SOPs included detailed instructions to assure all steps of the analytical procedures were carried out in exactly the same manner by each contributing laboratory.

2.5.1. Reagents and supplies

CFSAN provided each collaborator with all reagents and critical supplies. Provided items for collaborative study phase 1 consisted of: Alconox; 25 ml serological pipets; 50 ml and 15 ml conical tubes; non-skirted 2 ml FastPrep tubes with caps and FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA); 500 μM nPCR primer stock solutions (F1E, R2B, CC719, and CRP999); 10 mM Tris pH 7.5, 0.1 mM EDTA (TE) for primer dilution; nonfat dry milk; 25 mM $MgCl_2$; synthetic *C. cayetanensis* positive control (500 copies/μl); and HotStarTaq Master Mix Kits (Qiagen, Valencia CA). Provided items for collaborative study phase 2 consisted of: Applied

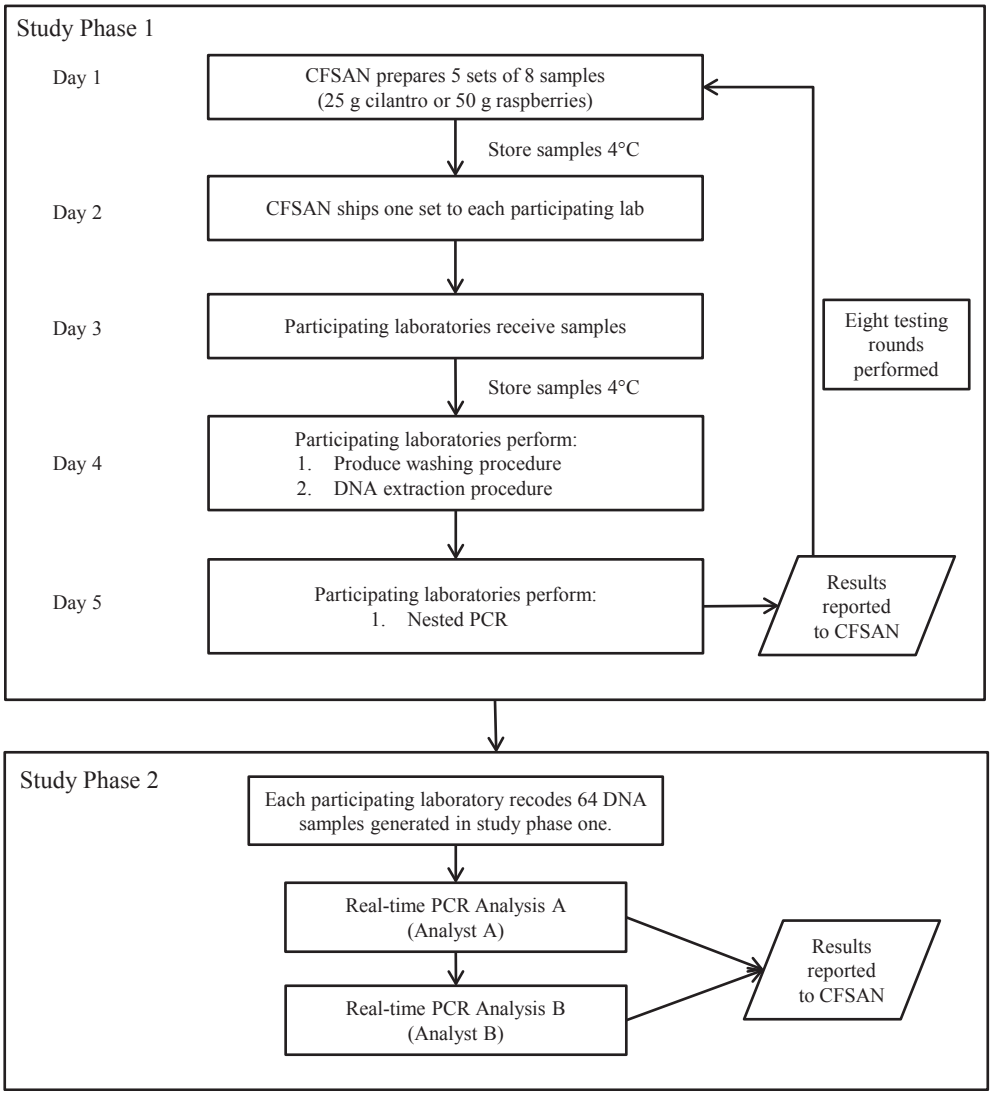

Fig. 1. Collaborative study flow diagram showing study phase 1 and 2 procedures. A total of eight testing rounds were performed in phase 1. Two independent analyses (A and B) were performed in phase 2.

Biosystems MicroAmp Fast Optical 96-Well Reaction Plates (0.1 ml) and Optical Adhesive Film (ThermoFisher Scientific, Waltham, MA); 500 μM primer stocks (Cyclo250F, Cyclo350R, dd-IAC-f, and dd-IAC-r); 100 μM *C. cayetanensis* and 50 μM IAC TaqMan® hydrolysis probes (Cyclo281T and dd-IAC-Cy5); TE for preparation of primer-probe stock mixes and sample dilution; synthetic IAC target ultramer (synIAC, 10^7 copies/μl); synthetic *C. cayetanensis* positive control (500 copies/μl); and QuantiFast Multiplex PCR Kits with ROX dye included in the master mix (Qiagen, Valencia CA). Analysts A and B in each laboratory were provided with separate complete sets of qPCR reagents.

2.5.2. Study phase 1: sample processing and analysis using nPCR

Oocysts were recovered from produce by washing samples twice with 100 mL of 0.1% Alconox and concentrating the washes by centrifugation. Samples producing unusually large wash pellets were divided into two lysing tubes prior to the DNA extraction bead-beating protocol and analyzed as two separate portions throughout the remainder of the study. DNA extraction was performed using a FastPrep®-24 (MP Biomedicals, Santa Ana, California) or comparable homogenizer to disrupt oocysts and the FastDNA SPIN Kit for Soil to purify DNA. A blank tube containing no sample was added as a control prior to the DNA extraction procedure in each testing round. The nPCR reactions were performed on the Applied Biosystems 9700 Thermal Cycler (ThermoFisher Scientific, Waltham, MA), the PCT-200 DNA Engine Cycler, or the MyCycler Thermal Cycler (Bio-Rad, Hercules, CA). Reaction mixes were prepared using 10 μM primer working solutions made by participants using the provided primer stock solutions. All nPCR experiments included study samples, a no-template control, and a positive control reaction containing 10^3 copies of target, all of which were analyzed in triplicate reactions. Participants were instructed to consider invalid and repeat any runs in which all three replicates of control reactions did not produce the expected result.

Procedures were performed exactly as reported for the pre-collaborative study (Murphy et al., 2017a) with two notable modifications. First, the produce washing procedure was initiated by participating laboratories within 48–72 h (versus 24–48 h) of sample preparation. Second, 4.0 μg/μl nonfat dry milk was included in all primary nPCRs to block inhibition, instead of repeating reactions in the presence of nonfat dry milk if they were initially negative without blocker.

2.5.3. Study phase 2: qPCR

Prior to study phase 2, samples were re-coded by individuals not performing sample analysis, according to a key provided by CFSAN, to assure the study was blinded for each participant performing the qPCR. The duplex qPCR assay targeting *C. cayetanensis* and the IAC was performed as described for the pre-collaborative study with one modification; the PCR kit provided included ROX dye in the master mix solution instead of as a separate component of the kit.

For execution of qPCRs, participants made two 20X primer-probe working solutions prior to preparation of the final reaction mix. The *C. cayetanensis* target 20X working solution contained 10 μM forward and reverse primers and 2.0 μM probe. The IAC target 20X working solution contained 2.0 μM forward and reverse primers, 4.0 μM probe, and 2×10^5 copies/μl IAC target ultramer. All laboratories performed qPCR analyses on an Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) in fast mode. Each experimental run consisted of study samples, a no-template control, and a positive control containing 10^3 copies of target; all were analyzed in triplicate reactions.

Participants analyzed qPCR results to verify successful runs using the Applied Biosystems 7500 Software (v.1.4 or v.2.3) with the baseline set from 6 to 15 cycles and a threshold line set at 0.010 for both target reactions. Participants considered the results of an experimental run valid only if all negative control reaction replicates were negative and all positive control reaction replicates were positive for amplification of the *C. cayetanensis* target, and all negative control and positive control reaction replicates were successful for amplification of the IAC target. If these conditions were not met, participants were instructed to repeat runs taking corrective actions after consultation with the originating laboratory if necessary.

Participants considered a study sample positive if one or more of the three qPCR *C. cayetanensis* target reaction replicates produced an amplification signal above the threshold line. A divided sample was considered positive if either portion of the sample was positive. A sample was considered negative when none of the three replicates produced an amplification signal above the threshold line. All samples initially negative were diluted fourfold in TE and tested a second time to reduce potential for false-negative results due to inhibition of the qPCR. Two analysts at each laboratory worked independently using the same instrument to perform two independent qPCR analyses (A and B) of the entire set of samples generated in study phase 1.

2.6. Analysis of collaborative study data

Study phase 1 data were reported to CFSAN upon completion of each testing round. Participating laboratories reported the number of positive nPCR replicates for each sample using a sample result reporting form. Samples were identified as positive if at least one of the three nPCR replicates produced a band of the expected size by agarose gel electrophoresis.

At the end of study phase 2, each analyst reported qPCR results for the entire set of samples to CFSAN by submitting the Applied Biosystems 7500 FAST Real-time PCR instrument run files. CFSAN reviewed all study data to determine optimal analysis settings and reanalyzed all run files after changing the *C. cayetanensis* target threshold line setting to 0.020. Reactions having an exponentially increasing fluorescence signal with a cycle threshold (C_q) value greater than 38.0 were considered negative. Samples were identified as positive if one or more replicates produced a *C. cayetanensis* target reaction with a C_q value less than or equal to 38.0. A sample that was negative for the *C. cayetanensis* target was considered inhibited when the IAC reaction failed or produced a mean C_q value more than three cycles higher compared to the negative control IAC reaction.

Collaborative study detection rates were calculated for each matrix at each inoculation level. Positive rates were calculated as the percentage of inoculated samples that gave a positive result. False-positive rates were calculated as the percentage of uninoculated samples identified as positive. Lower and upper 95% confidence intervals for detection rates were calculated by the Wald method using the online software Quickcalcs (GraphPad, San Diego; http://www.graphpad.com/quickcalcs/confInterval1/).

Mean qPCR C_q values at each matrix inoculation level were calculated using C_q values obtained by analysis of undiluted DNA samples. The mean *C. cayetanensis* target quantity (copies of 18S rDNA) detected with the qPCR assay at each matrix inoculation level was estimated by extrapolation of the mean qPCR C_q values on a standard curve. The standard curve was generated from qPCR analysis of 10-fold serial dilutions of the synthetic *C. cayetanensis* positive control target ranging from 10^5 to 1 copy per reaction. The estimated mean target quantity recovered (in copies) at each matrix inoculation level was calculated using the mean sample volume recovered after DNA extraction (67.1 μl). The amount of target anticipated in samples was estimated assuming 80% of oocysts were recovered during washing, DNA extraction was 50% efficient,

18 copies of the 18S rDNA gene per genome (estimated from whole genome sequencing of *C. cayetanensis*), and one or four genomes per oocyst (the oocyst preparation contained unsporulated and sporulated oocysts).

2.7. Statistical analysis of the collaborative study results

Two-tailed *P* values were calculated with Fisher's exact test using the software Quickcalcs (GraphPad, San Diego, CA; https://www.graphpad.com/quickcalcs/contingency1.cfm) to identify statistically significant differences between the number of positive results within or between laboratories and the detection rates for the nPCR and qPCR assays. A *P* value ≥ 0.05 indicates no statistical difference. Kappa values (κ) with 95% confidence intervals were calculated using the software Quickcalcs (GraphPad, San Diego, CA; https://www.graphpad.com/quickcalcs/kappa1.cfm) to determine the level of result agreement between qPCR analysis A and B for samples inoculated with 5 and 10 oocysts. Agreement can range from poor ($\kappa = 0.00$) to perfect ($\kappa = 1.00$).

3. Results

The collaborative study data consists of the nPCR and qPCR results from a total of 319 samples. Table S1 (Supplementary data) contains study data including the numbers of positive nPCR replicates and the qPCR C_q values for each sample reaction. The qPCR analysis was performed twice at each participating laboratory by two analysts working independently and blindly to obtain analysis A and B results. Each contributing laboratory received a total of eight cilantro samples and eight raspberry samples at each of four inoculation levels (0, 5, 10, and 200 oocysts) for analysis. One uninoculated cilantro sample sent to laboratory 1 was eliminated from the study as a result of a technical mishap during the produce washing step. Ten raspberry samples from laboratory 4 were each divided into two tubes prior to the DNA extraction procedure as described in section 2.5.2 because of large wash debris pellets. No invalid nPCR or qPCR experimental runs were reported by participating laboratories. CFSAN reviewed all data reported by the laboratories and scored samples as positive or negative by each detection method according to the criteria defined in section 2.6.

The number of cilantro and raspberry samples identified positive at each inoculation level by each contributing laboratory using the nPCR and qPCR assays are reported in Table 1. Each contributing laboratory identified all eight samples at the high inoculation level (200 oocysts) as positive using both detection methods. For samples inoculated with 10 oocysts, laboratories identified at least six replicates positive with the nPCR assay and at least five replicates positive with the qPCR assay. For samples inoculated with 5 oocysts, the number of positive replicates was between zero and seven with the nPCR assay and between one and six with the qPCR assay. Using the nPCR assay, laboratory 2 identified one uninoculated cilantro sample as positive and laboratory 3 identified two uninoculated raspberry samples as positive. Using the qPCR assay, all uninoculated samples were negative. At each matrix inoculation level, differences in the number of positive results found by nPCR, qPCR analysis A, and qPCR analysis B within each laboratory or between laboratories were not significant ($P \geq 0.1189$).

Close examination of the qPCR data found in Table S1 revealed additional information. A total of 78 and 73 inoculated samples were initially negative by analysis A and B, respectively. When these samples were re-tested using a fourfold dilution, five were positive by analysis A and three were positive by analysis B. Seven cilantro and six raspberry uninoculated samples produced one or more replicate reactions with late amplification signals by either analysis A or B or both. These uninoculated samples, scored negative because the C_q values were greater than the cut-off value of 38.0, did not appear randomly across contributing laboratories. Laboratory 3 had 10 of the 13 uninoculated samples producing high C_q amplification signals.

Partial inhibition of the IAC, identified by shifted IAC C_q values, was not observed for any study samples. Five study samples had complete failure of the IAC target reaction. All of these results were observed by laboratory 4 where analysts had three cilantro and two raspberry samples with negative IAC and *C. cayetanensis* target results. When the analysis of these samples was repeated at a fourfold dilution, all produced positive IAC target reactions. Three of these samples were inoculated; one inoculated with 5 oocysts remained negative for the *C. cayetanensis* target, and two inoculated with 200 oocysts were positive for the *C. cayetanensis* target when analyzed after dilution.

The nPCR and qPCR detection rates at each inoculation level for all cilantro and raspberry study samples are shown in Table 2. Significant increases in detection rates using the nPCR and the qPCR assays were observed for samples inoculated with 10 oocysts when compared to those inoculated with 5 oocysts (*P* values ≤ 0.0007). At the 5 and 10 oocyst inoculation levels, detection rates for cilantro samples were lower than for raspberry samples with one exception; qPCR analysis B produced identical rates for cilantro and raspberry samples inoculated with 10 oocysts (Table 2). Differences between detection rates for cilantro and raspberry samples were only significant for samples inoculated with 5 oocysts by nPCR ($P = 0.0001$), and for samples inoculated with 5 and 10 oocysts by qPCR analysis A ($P = 0.0411$ and 0.0367). Differences in detection rates between testing rounds were significantly variable by both nPCR and qPCR only for samples inoculated with 5 oocysts (rates ranged from 0% to 90%, data not shown).

Table 2 compares the performance of the nPCR and the qPCR assays for detection of *C. cayetanensis* in inoculated samples. Positive rates for qPCR analysis A and B were compared to rates obtained by nPCR at each matrix inoculation level. The only significant difference in the collaborative study detection rates for the two methods was an improved rate using the nPCR assay when compared to qPCR analysis A for cilantro samples inoculated with 5 oocysts ($P = 0.0367$). Analysis of uninoculated samples using the nPCR assay resulted in a false-positive rate of 2.6% for cilantro samples and 5.0% for raspberry samples. Analysis of uninoculated samples produced no false-positives using the qPCR assay.

There were no significant differences in detection rates between qPCR analysis A and analysis B (Table 2). Sample result agreement between analyses A and B at the lower inoculation levels was assessed by determining the number of samples producing the same result by analysis A and B (Table 3). Kappa values, determined for each matrix from the combined 5 and 10 oocyst inoculation level results (5 + 10), indicated moderate to good agreement for sample results between qPCR analysis A and B.

The mean *C. cayetanensis* 18S rDNA and IAC target C_q values obtained for analysis A and analysis B at each laboratory were calculated for cilantro and raspberry samples (Fig. 2). For each analyst, the mean of all cilantro and raspberry sample *C. cayetanensis* target C_q values obtained ranged from 34.0 ± 0.8 to 37.8 ± 0.2 when samples were inoculated with 5 oocysts, 34.2 ± 1.9 to 37.4 ± 0.5 when samples were inoculated with 10 oocysts, and 31.5 ± 1.7 to 34.1 ± 1.9 when samples were inoculated with 200 oocysts. The mean IAC target C_q values obtained by each analyst were more variable than the *C. cayetanensis* target values between laboratories and analyses A and B, ranging from 25.8 ± 0.8 to 31.2 ± 1.2 for all cilantro samples and 25.5 ± 0.4 to 30.4 ± 0.5 for all raspberry samples. Fig. 2 also shows the mean *C. cayetanensis* positive control C_q values for analysis A and analysis B at each laboratory which ranged from 28.0 ± 0.1 to 28.6 ± 0.4.

Table 1
Contributing laboratory detection results using nPCR and qPCR for analysis of cilantro and raspberry samples inoculated with *C. cayetanensis* oocysts.

Matrix	No. oocysts inoculated	No. positive samples[a]																	
		Lab 1			Lab 2			Lab 3			Lab 4			Lab 5			Total		
		nPCR	qPCR		nPCR	qPCR		nPCR	qPCR		nPCR	qPCR		nPCR	qPCR		nPCR	qPCR	
			A	B		A	B		A	B		A	B		A	B		A	B
Cilantro	0	0[b]	0[b]	0[b]	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	5	1	1	1	4	3	3	0	5	5	2	2	2	2	1	2	9	12	13
	10	8	7	8	8	5	7	6	6	6	8	5	6	7	6	8	37	29	35
	200	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	40	40	40
Raspberries	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	2	0	0
	5	3	6	4	7	6	4	5	4	4	7	3	3	5	3	3	27	22	18
	10	8	7	8	8	8	6	8	8	7	7	6	6	8	8	8	39	37	35
	200	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	40	40	40

[a] Each contributing laboratory processed 8 identically prepared food samples at each inoculation level for a total of 40 samples at each inoculation level. Molecular detection was carried out by performing nPCR and qPCR. Two independent qPCR analyses (A and B) of all samples were performed at each participating laboratory.
[b] Only seven uninoculated cilantro samples were analyzed by Lab 1. One uninoculated cilantro sample at Lab 1 was excluded from analysis due to a technical anomaly which occurred during the produce washing procedure.

The means of all *C. cayetanensis* target C_q values reported in the collaborative study were calculated for each matrix inoculation level (Table 4). These values were extrapolated on a standard curve (not shown), having a strong correlation coefficient ($R^2 = 0.987$) and an efficiency of 103%, to obtain a calculated estimate at each inoculation level of the mean predicted *C. cayetanensis* 18S rDNA target quantity recovered using the new method. The range of target quantity recovery predicted at each inoculation level is indicated (calculated as described in section 2.6).

4. Discussion

In this collaborative validation study, the efficacy of improved sample preparation methodology for recovery of *C. cayetanensis* oocysts from produce and preparation of DNA templates for detection by qPCR was assessed. Detection rates were determined using an optimized qPCR assay and compared to a reference nPCR assay. All five contributing laboratories successfully executed the study to completion. The new procedures using a 0.1% Alconox produce wash solution and a DNA extraction kit were successfully reproduced by multiple analysts from five laboratories to detect as few as 5 oocysts inoculated onto cilantro or raspberry samples.

Two minor anomalies occurred during the study sample processing steps. First, one sample from the initial testing round of study phase 1 had to be eliminated from the study because the laboratory reported that a bag leaked during the washing procedure. This was likely an operator error related to inexperience with the clips used to seal the bags and no further problems related to bag leakage occurred during the study. Second, laboratory 4 had to divide 31% of their raspberry samples prior to the DNA extraction bead-beating protocol because they produced unusually large wash debris pellets, however no other samples in the collaborative study

Table 2
Comparison of the collaborative study detection rates using nPCR and qPCR for analysis of cilantro and raspberry samples inoculated with *C. cayetanensis* oocysts.

Matrix	No. oocysts inoculated	No. samples analyzed[d]	Detection rate[a] (%)			False-positive rate[b] (%)			Fisher's exact test *P* values[c]		
			nPCR	qPCR A	qPCR B	nPCR	qPCR A	qPCR B	nPCR vs qPCR A	nPCR vs qPCR B	qPCR A vs qPCR B
Cilantro	0	39	—	—	—	2.6 (0.0, 11.5)	0	0	1.0000	1.0000	1.0000
	5	40	22.5 (12.1, 37.7)	30.0 (18.0, 45.5)	32.5 (20.0, 48.1)	—	—	—	0.6120	0.4531	1.0000
	10	40	92.5 (79.4, 98.1)	72.5 (57.0, 84.0)	87.5 (73.4, 95.0)	—	—	—	0.0367[e]	0.7119	0.1600
	200	40	100.0 (89.6, 100)	100.0 (89.6, 100)	100.0 (89.6, 100)	—	—	—	1.0000	1.0000	1.0000
Raspberries	0	40	—	—	—	5.0 (0.5, 17.4)	0	0	0.4937	0.4937	1.0000
	5	40	67.5 (51.9, 80.0)	55.5 (39.8, 69.3)	45.0 (30.7, 60.2)	—	—	—	0.3588	0.0707	0.5026
	10	40	97.5 (86.0, 100)	92.5 (79.4, 98.1)	87.5 (73.4, 95.0)	—	—	—	0.6153	0.2007	0.7119
	200	40	100.0 (89.6, 100)	100.0 (89.6, 100)	100.0 (89.6, 100)	—	—	—	1.0000	1.0000	1.0000

— Not applicable.
[a] Percentage of inoculated samples which gave a positive result by nPCR, qPCR analysis A (qPCR A), and qPCR analysis B (qPCR B). Different analysts at each participating laboratory performed qPCR A and qPCR B. Numbers in parentheses are the lower and upper 95% confidence intervals of detection rates.
[b] Percentage of uninoculated samples which gave a positive result with 95% confidence intervals in parentheses.
[c] *P* values calculated using the Fisher's exact test to identify statistical differences in results obtained between nPCR, and qPCR analysis A, and qPCR analysis B. $P \geq 0.05$ is not considered significant.
[d] A total of 40 produce samples were prepared by the originating laboratory at each level for each matrix. One uninoculated cilantro sample was excluded from the study due to a technical error which occurred during the washing procedure.
[e] Statistically significant difference between the two methods.

Table 3
Evaluation of sample result agreement for qPCR analyses A and B.

Matrix	No. oocysts inoculated	No. of samples[a]				
		PP	PN	NP	NN	κ[b]
Cilantro	5	12	0	1	27	–
	10	28	1	7	4	–
	5 + 10[c]	40	1	8	31	0.774 (0.637, 0.911)
Raspberries	5	16	6	2	16	–
	10	32	5	3	0	–
	5 + 10[c]	48	11	5	16	0.527 (0.327, 0.727)

— Not calculated.
[a] PP, positive by both analysis A and B; PN, positive by analysis A and negative by analysis B; NP, negative by analysis A and positive by analysis B; NN, negative by both analysis A and B.
[b] Kappa agreement (κ), 1.00 (perfect) to 0.00 (poor), with 95% confidence intervals (CI) shown in parentheses.
[c] Results from the 5 and 10 oocyst inoculation levels for each matrix were combined to calculate kappa agreement for the combined lower inoculation level results.

were divided. Divided samples were not unexpected, and pre-collaborative studies demonstrated that raspberry samples occasionally produced large debris pellets during the produce washing procedure. However, the disproportionate number of samples divided by laboratory 4 was unexpected. All laboratories reported that all of the samples were in good condition upon receipt. Therefore, it is likely that more rigorous handling procedures at laboratory 4 were responsible for the larger wash pellets, but the high incidence of divided samples did not adversely impact laboratory 4 results compared to other laboratories. Larger bead-beating tubes are available which could be used in the future for samples producing large wash debris pellets.

Laboratory 3 had an unusually high number of uninoculated samples producing qPCR C_q values above the cut-off value of 38.0 even though all qPCR no template controls and DNA extraction controls were negative. Laboratory 3 also had the highest rate of false-positive nPCR results. These results are most likely due to a very low level of laboratory environment contamination and highlight the importance of clean laboratory workflow for highly sensitive PCR applications.

One important objective of this study was to validate a duplex qPCR assay with an IAC which could replace the conventional nPCR assay currently used by FDA laboratories for detection of *C. cayetanensis*. Although detection rates at lower inoculation levels using the nPCR assay were slightly higher, the differences were only significant in one case. The credibility of the nPCR assay was

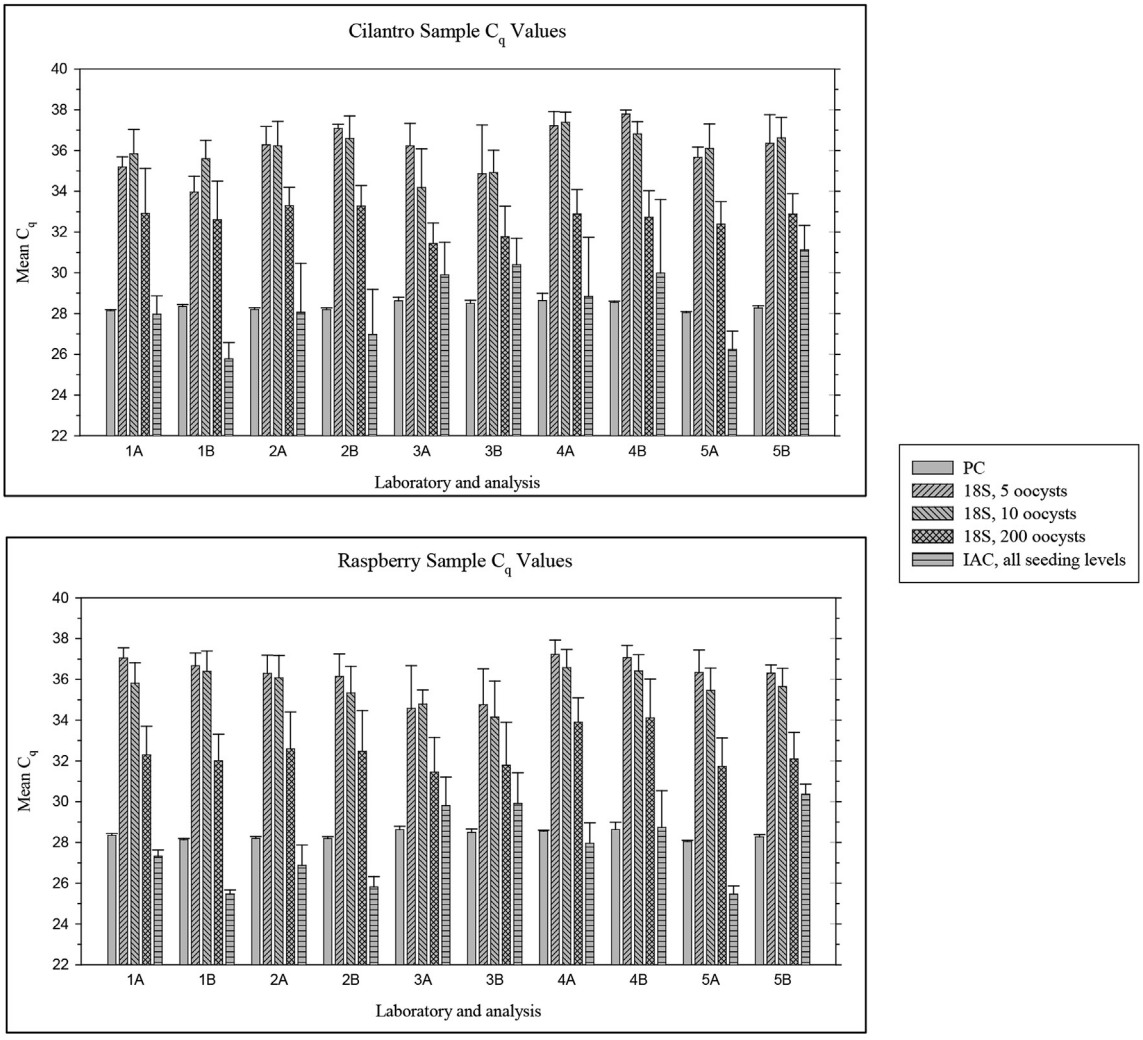

Fig. 2. Comparison of the mean qPCR C_q values produced by analyst (A or B) at each laboratory (1–5) for cilantro and raspberry samples. Bar shading designates the *C. cayetanensis* 18S rDNA (18S) values at each inoculation level and the internal amplification control (IAC) value calculated for all samples. The mean values for all *C. cayetanensis* positive control (PC) reactions performed by each analyst are shown on both panels. Standard deviation is represented by error bars.

Table 4
Cyclospora cayetanensis mean qPCR C_q values and calculated estimates of 18S rDNA target quantity detected in collaborative study samples at each matrix inoculation level.

Matrix	No. oocysts inoculated	C_T (Mean ± SD)[a]	Estimated 18S rDNA (copies)		
			Detected[b]	Total recovered[c]	Total range predicted[d]
Cilantro	5	36.0 ± 1.7	4.46	150	36–144
	10	35.9 ± 1.4	4.79	161	72–288
	200	32.6 ± 1.5	49.5	1660	1440–5760
Raspberries	5	36.1 ± 1.5	4.16	140	36–144
	10	35.6 ± 1.5	5.92	199	72–288
	200	32.5 ± 1.8	53.1	1782	1440–5760

[a] Mean C_q value for all positive qPCR samples in the collaborative study for each matrix and inoculation level.
[b] Mean target copy number detected in 2 μl of undiluted DNA samples.
[c] Mean target copy number in DNA samples calculated assuming a sample volume of 67.1 μl.
[d] Range of 18S rDNA target copies expected assumes from one to four genomes per oocyst, oocyst recovery was 80%, DNA extraction was 50% efficient, and 18 copies of 18S rDNA per genome.

diminished by false-positives which were observed only by nPCR in uninoculated samples at an overall rate of 3.8%. False-positives can result from amplicon contamination during PCR setup and are less problematic for qPCR which is performed in a closed system. Hydrolysis probe-based qPCR assays are preferred over conventional nPCR assays not only because they are highly sensitive and specific, but because they are technically simple to perform and produce rapid results that are clearly interpreted.

Evaluation of the collaborative study data demonstrates that the new method using the qPCR assay was robust and reproducible within laboratories and between laboratories. C_q values obtained for the synthetic positive control were very consistent. All analysts detected *C. cayetanensis* at the lowest inoculation level in a fraction of the replicates by qPCR. It was useful to compare the qPCR *C. cayetanensis* target mean C_q values obtained by each analysis at each inoculation level. Variability in C_q values for samples inoculated at the same level can be attributed to technical variations between laboratories and analysts in oocyst recovery, DNA extraction, and the qPCR assay. Although there were some significant differences between laboratories, mean *C. cayetanensis* target C_q values obtained were sufficiently consistent, resulting in comparable detection rates between laboratories.

A significant benefit of detection methods employing qPCR assays is that they provide useful quantitative information. The average quantity of *C. cayetanensis* 18S rDNA target isolated from inoculated samples using the new wash and DNA extraction method fell within predicted ranges at each inoculation level. This suggests that estimations of the efficiency of the sample preparation procedures are reasonable. Oocyst recovery was assumed to be 80% based on a previous study using an identical procedure to wash commercial spring mix (containing assorted lettuces and greens) inoculated with *C. cayetanensis* oocysts (Shields et al., 2012). Although a variety of DNA extraction methods have proven effective for molecular detection of *C. cayetanensis* oocysts isolated from food samples, the efficiency of DNA extraction was conservatively estimated at 50% because extraction from *C. cayetanensis* oocysts is generally viewed as an inefficient process (Lalonde and Gajadhar, 2008). Whole genome sequencing of *C. cayetanensis* may soon determine the exact 18S rRNA gene copy number per genome, further advancing the utility of the qPCR assay as a quantitative tool for assessment of *C. cayetanensis* contamination levels in food and water matrices.

The IAC in the qPCR assay provided an effective control for reaction inhibition which is essential for regulatory testing applications (Rodriguez-Lazaro et al., 2013). The results obtained with the IAC showed clearly that the qPCR assay used in this study was robust and not overly prone to inhibition. Matrix dependent variations in C_q values were not observed for the *C. cayetanensis* or the IAC targets. The IAC target C_q values obtained varied more than the *C. cayetanensis* target C_q values between analysts but always remained in a range that allowed observation of potential reaction inhibition. Differences in IAC C_q values may have resulted from inconsistencies between analysts in pipetting the IAC target stock solution during mastermix preparation.

5. Conclusions

Development of new or improved detection methods for *C. cayetanensis* is a priority for the FDA and other public health agencies. This collaborative study provides an improved detection method for *C. cayetanensis* which has been implemented into FDA's laboratories for regulatory testing and surveillance of fresh produce commodities implicated in outbreaks. The improved sample preparation steps and the optimized qPCR assay provide essential advancements to methodology in the FDA BAM for *C. cayetanensis* regulatory testing in produce. This streamlined molecular detection assay will facilitate larger scale surveillance studies needed to better understand the prevalence of *C. cayetanensis* in the US food supply. This study validates the method for use in cilantro and raspberries; additional studies are underway to extend the method to other food matrices. The DNA extraction procedure and the qPCR assay can be used in conjunction with methods under development for isolation of *C. cayetanensis* from water samples. The use of improved validated detection methods for food and water matrices will facilitate future studies to understand the prevalence of *C. cayetanensis* in fresh produce and the environment and to identify the necessary preventative control measures to reduce foodborne exposure to *C. cayetanensis*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2017.08.008.

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