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Research Paper

Assessment of Commercial DNA Cleanup Kits for Elimination of Real-Time PCR Inhibitors in the Detection of *Cyclospora cayetanensis* in Cilantro

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ABSTRACT

Inhibited reactions have occasionally been observed when cilantro samples were processed for the detection of *Cyclospora cayetanensis* using quantitative real-time PCR (qPCR). Partial or total inhibition of PCR reactions, including qPCR, can occur, leading to decreased sensitivity or false-negative results. If inhibition occurs, this implies the need for additional purification or cleanup treatments of the extracted DNA to remove inhibitors prior to molecular detection. Our objective was to evaluate the performance of five commercial DNA cleanup kits (QIAquick purification kit from Qiagen [kit 1], OneStep PCR inhibitor removal by Zymo Research [kit 2], NucleoSpin genomic DNA cleanup XS from Macherey-Nagel [kit 3], DNA IQ system by Promega [kit 4], and DNeasy PowerPlant pro kit from Qiagen [5]) to minimize qPCR inhibition using the U.S. Food and Drug Administration–validated *Bacteriological Analytical Manual* (BAM) Chapter 19b method for detection of *C. cayetanensis* in cilantro samples containing soil. Each of the five commercial DNA cleanup kits evaluated was able to reduce the qPCR internal amplification control cycle threshold values to those considered to be normal for noninhibited samples, allowing unambiguous interpretation of results in cilantro samples seeded at both a high oocyst level (200 oocysts) and a low oocyst level (10 oocysts). Of the five kits compared, kits 1, 2, and 3 did not show significant differences in the detection of *C. cayetanensis,* while significantly higher cycle threshold values, indicating lower recovery of the target DNA, were observed from kits 4 and/or 5 in samples seeded with 200 and 10 oocysts ($P < 0.05$). This comparative study provides recommendations on the use of commercial cleanup kits which could be implemented when inhibition is observed in the detection of *C. cayetanensis* in cilantro samples using the BAM Chapter 19b method.

HIGHLIGHTS

- Inhibited detection of *C. cayetanensis* in cilantro samples can occur.
- Five commercial DNA cleanup kits were evaluated in inhibited cilantro samples.
- The kits reduced the inhibition levels of the internal amplification control.
- Results were conclusive at low and high levels of seeded oocysts.
- DNA cleanup minimized inhibition in cilantro with soil.

Key words: *Bacteriological Analytical Manual* Chapter 19b; Commercial cleanup kits; *Cyclospora cayetanensis*; PCR inhibitors

Cyclospora cayetanensis is an emerging foodborne protozoan parasite and the etiological agent of a gastrointestinal disease in humans called cyclosporiasis. Infected humans shed nonsporulated oocysts, which sporulate and become infective after approximately 7 to 15 days under ideal humidity and temperature conditions (23 to 27°C) in the environment *(27)*. Humans become infected with *C. cayetanensis* after consumption of food or water contaminated with these sporulated oocysts. In the United States, foodborne outbreaks and sporadic cases of cyclosporiasis have been reported since the mid-1990s, mainly linked to imported fruit and vegetables, including cilantro, berries, basil, snow peas, and mixed salads *(3)*. In 2019, there was a total of 2,408 laboratory-confirmed domestically acquired cases from 37 states in the United States, with 144 of the people affected needing hospitalization *(8)*. Cilantro is a high-risk fresh produce commodity historically linked to several outbreaks of *C. cayetanensis* in North America *(1, 3, 7, 10)*. Consumption of fresh cilantro imported from the state of Puebla in Mexico was linked to clusters of cases

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during 2013 to 2015 *(1, 38)*. In the most recent years, 2018 and 2019, there have also been confirmed cases associated with consumption of cilantro, mainly in Mexican-style restaurants *(7, 8)*.

C. cayetanensis cannot be propagated in vivo or in vitro. Therefore, improved molecular methods for detection and characterization of the parasite are essential to identify and track sources of produce contamination and to strengthen surveillance *(1)*. Quantitative real-time PCR (qPCR) is a high-throughput tool, useful for routine surveillance of fresh produce for protozoan oocysts. However, any PCR, including qPCR, can be prone to inhibitors of these enzymatic reactions, which may result in decreased sensitivity and even produce false-negative results *(29)*.

The U.S. Food and Drug Administration (FDA) developed and validated a qPCR method for *C. cayetanensis* detection in cilantro and raspberries, and the method is now publicly available in the *Bacteriological Analytical Manual* (BAM) under Chapter 19b *(23)*. The performance of the BAM method has additionally been evaluated in other high-risk fresh produce linked to outbreaks (i.e., basil, parsley, shredded carrots, and shredded cabbage with carrot mix) *(4),* in different types of berries *(5),* and in romaine lettuce *(23)*. The method includes 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 duplex TaqMan qPCR targeting the species-specific *C. cayetanensis* 18S rRNA gene and an exogenous noncompetitive internal amplification control (IAC). The IAC is a versatile internal control for use in DNA in real-time PCR *(9)* which monitors for reaction failures due to matrix-derived PCR inhibition. Molecular detection of *C. cayetanensis* in produce following BAM Chapter 19b using this TaqMan real-time PCR assay provides a robust, streamlined, and rapid analytical procedure for surveillance, outbreak response, and regulatory testing of foods. However, as for any molecular method based on PCR, there is a potential for partial or complete inhibition of amplification of the *C. cayetanensis* 18S rRNA gene and IAC targets if inhibitors are present in the DNA samples.

Inhibition of PCR by plant components has been described and could derive from various substances, including polysaccharides, polyphenols, pectin, and xylan *(19, 29, 31, 39)*. In one study, resinous plant components released during washing and DNA extraction of rosemary were observed to completely inhibit qPCR melting curve analysis in a study of detection of DNA from *Eimeria* oocysts *(20)*. In berries, polyphenol inhibitors are common, including flavonoids (anthocyanins, flavonols, flavones, flavanols, flavanones, and isoflavonoids), stilbenes, tannins, and phenolic acids *(37),* although berries of different types did not inhibit qPCR when following the BAM Chapter 19B method *(5)*. In addition to the inhibitory substance type or class, the concentration of the inhibitor is important for its inhibitory effect *(31)*. A simple and widely applied method to reduce inhibitors in a sample is by dilution of the sample of the extracted nucleic acid, thereby diluting the PCR inhibitors *(11, 22, 32, 33)*. In the BAM Chapter 19b method, dilution is included in the protocol, where samples are analyzed undiluted and at 1:4 dilution. Therefore, the cleanup of DNA would be a secondary step and, thus, could be an effective approach to reduce qPCR inhibition in samples when needed.

Cilantro may be contaminated with soil, and in fact, in our laboratory experiments, we found that cilantro samples with added soil inhibited the PCR reaction when using the BAM Chapter 19b method. The inhibition did not seem directly related to the cilantro itself, but rather to the small amounts of soil in the cilantro samples. Soil and environmental samples with dead biomass may contain humic and fulminic acids, which inhibit PCR even at low concentrations *(17, 18)*. Humic acid is one of the most common inhibitors existing in environmental soil, as it can be coextracted with the DNA samples and can inhibit PCR at the amplification steps or have adverse effects on the DNA extraction via sequestration *(6, 13, 19, 30, 34–36)*. Additional PCR inhibitors found in soil were clay and magnesium *(21)*.

Removal of the PCR inhibitors from the DNA extracts can be performed using commercial kits *(12)*. Commercially available DNA cleanup methods are rapid and could help lead to the success of highly sensitive assays like the *C. cayetanensis* qPCR. Commercial DNA cleanup methods for nucleic acid purification and PCR use a variety of strategies for removing PCR inhibitors and increasing the robustness of PCR enzymes (use of phenol-chloroform extraction, silica columns, magnetic silica beads for nucleic acid isolation, and immunocapture methods). However, the efficiency of each kit is strongly dependent on the type of food being tested *(31)*.

The objectives of the present study were to evaluate the performance of five commonly used commercial DNA cleanup kits regarding their inhibitor removal abilities when extracting DNA from cilantro samples containing soil and to assess the impact, if any, on detection of *C. cayetanensis* in cilantro.

MATERIALS AND METHODS

Oocyst and cilantro sample preparation. A preparation of *C. cayetanensis* oocysts originating from an Indonesian patient, purified as previously indicated *(4, 5, 25),* with an estimated 50% sporulated oocysts was used for these investigations. The use of the clinical stool samples positive for *C. cayetanensis* oocysts was approved by the institutional review board of the FDA (protocol no. 15-039F). Six replicates of the partially purified oocysts were counted by two different analysts using a hemocytometer to estimate the concentration and the sporulation rate of oocysts in the preparation. Oocysts were then diluted in 0.85% NaCl to estimated concentrations of 20 oocysts per μL and 1 oocyst per μL for seeding experiments *(5)*. The same diluted preparations of 200 oocysts and 10 oocysts were used for all cilantro seeding experiments.

Commercial fresh cilantro was obtained from a local grocery store and processed within 24 h. Individual cilantro test samples (25 g) were prepared as described previously *(25)*. Cilantro samples were weighed and then sprinkled with small amounts of soil (0.3 g) rich in clay, collected from a farm in Georgia. The original soil was autoclaved and determined to be free of *C. cayetanensis* by qPCR. Cilantro samples without added soil were

TABLE 1. *Characteristics of five commercial DNA cleanup protocols used in this study to minimize the effects of PCR inhibitors in the BAM Chapter 19b* C. cayetanensis *qPCR assay*

Characteristic	QIAquick PCR purification kit (kit 1)	OneStep PCR inhibitor removal kit (kit 2)	NucleoSpin gDNA cleanup XS (kit 3)[a]	Promega DNA IQ (kit 4)	DNeasy PowerPlant pro kit (kit 5)[b]
Time duration (min)	Around 30	Around 7	20	Around 30	Around 30
Column based?	Yes	Yes	Yes	No	Yes
Incubation (temp and time)	No	No	Yes (90°C for 5–8 min)	Yes (room temp 2 times for 5 min; 65°C for 5 min)	Yes (2–8°C for 5 min)
Extra equipment or reagents not provided	Ethanol	All provided	Tris-EDTA, ethanol, heat block or bath	Dithiothreitol, magnetic stand, ethanol, isopropyl alcohol, heat block	All provided from step 5 onward
Special needs		Needs to start with minimum 50 µl DNA	Removal of residual ethanol with open lid at 90°C for 5–8 min		
Cost per reaction ($)	2.30	2.20	2.70	3.20	4.00
No. of steps according to protocols in kits	8	2	6	6	9

[a] Recommended final elution volume in this kit protocol is 10 µL (once) or 10 µL plus 10 µL (twice).
[b] The protocol was started at step 5 of the manufacturer's protocol, allowing the same DNA extraction protocol to be used for all samples and all kits.

prepared concurrently for comparison. Subsequently, all cilantro samples were seeded with 200 *C. cayetanensis* oocysts ($n = 9$) or 10 *C. cayetanensis* oocysts ($n = 8$), using 10 µL of the 20 oocysts per µL dilution to seed 200 oocysts and 10 µL of the 1 oocyst per µL dilution to seed 10 oocysts in a dropwise fashion using a micropipet; approximately 10 to 20 droplets were spread randomly over multiple surfaces of the sample *(25)*. After seeding, samples were air dried for approximately 2 h and held at 4°C for 48 to 72 h in BagPage filter bags (Interscience Laboratories, Inc., Boston, MA) sealed with binder clips.

Oocyst recovery and molecular detection of *C. cayetanensis* in cilantro samples with and without added soil. The washing, DNA extraction, and molecular detection steps for cilantro followed the FDA's BAM Chapter 19b method *(23, 25)*. The processing of samples included three steps: (i) washing of produce to recover *C. cayetanensis* oocysts, (ii) DNA extraction of wash pellets containing concentrated oocysts, and (iii) qPCR analysis using a dual TaqMan method targeting the *C. cayetanensis* 18S rRNA genes, together with amplification of the IAC to identify potential false-negative results and monitor for reaction failure due to PCR inhibitors.

Briefly, two wash steps were performed with 0.1% Alconox detergent, followed by sequential centrifugations to recover, pool, and concentrate the wash debris. Produce wash debris pellets were stored at 4°C for up to 24 h or frozen at –20°C 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, CA). The qPCR was performed on an Applied Biosystems 7500 fast real-time PCR system (ThermoFisher Scientific, Waltham, MA). A commercially prepared synthetic gBlocks gene fragment (HMgBlock135m, Integrated DNA Technologies, Coralville, CA) was used as a positive control for amplification of the *C. cayetanensis* 18S rRNA gene *(23)*.

Each experimental qPCR run consisted of study samples, a nontemplate control (NTC), and positive controls containing 10-fold serial dilutions from 10^3 to 10 copies of the synthetic positive control analyzed in triplicate reactions for 45 cycles. 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) of 38.0 or lower. According to this method, reactions were considered inconclusive for samples seeded with 200 oocysts if the IAC reaction failed or produced an average C_T value more than three cycles higher than that of the NTC for the same assay. Samples showing partial inhibition of the IAC reaction, producing an average C_T value more than two cycles higher than that of the NTC, were used for samples seeded with 10 oocysts.

All DNA extracts for each seeding level (200 and 10 oocysts) from seeded cilantro samples with added soil showing qPCR inhibition based on their IAC C_T values were then pooled, and pooled DNA samples were tested by qPCR before going through cleanup.

The mean *C. cayetanensis* target quantity (copies of 18S rDNA) per reaction mixture volume detected with the qPCR assay at each matrix inoculation level was calculated by extrapolation of the qPCR C_T values on a standard curve, generated from 10-fold serial dilutions of the synthetic positive-control target and ranging from 10^3 to 10 copies per reaction mixture volume.

Comparison of commercial cleanup kits for reduction of qPCR inhibition in cilantro samples with added soil. Three 25-µL aliquots of each DNA pool from seeded (200 and 10 oocysts) cilantro samples with soil that showed IAC inhibition by qPCR were cleaned up using each of the five DNA cleanup kits.

The commercial kits evaluated were the QIAquick purification kit from Qiagen (Germantown, MD) (kit 1), OneStep PCR inhibitor removal by Zymo Research (Irvine, CA) (kit 2), NucleoSpin genomic DNA cleanup XS from Macherey-Nagel (Bethlehem, PA) (kit 3), DNA IQ system by Promega (Madison,

TABLE 2. C. cayetanensis *qPCR results for cilantro samples with or without soil, seeded with known numbers of* C. cayetanensis *oocysts*

		Mean C_T value ± SD in cilantro samples[a]:			
		With soil		With no soil	
Seeding level	Sample	18S rRNA	IAC	18S rRNA	IAC
200 oocysts	1	32.6 ± 0.3	30.8 ± 1.64	32.3 ± 0.0	24.6 ± 0.1
	2	36.7 ± 0.7	41.9 ± 0.7	32.0 ± 0.4	24.7 ± 0.2
	3	39.2 ± 1.0	Undetermined	32.5 ± 0.2	24.3 ± 0.1
	4	37.2 ± 0.3	Undetermined	32.3 ± 0.3	23.9 ± 0.2
	5	37.1 ± 3.6	36.2	31.8 ± 0.2	23.8 ± 0.0
	6	36.0 ± 0.9	35.8 ± 1.5	31.5 ± 0.2	24.5 ± 0.1
	7	33.7 ± 0.5	28.5 ± 0.1	31.1 ± 0.0	24.0 ± 0.1
	8	33.4 ± 0.5	28.8 ± 0.7	31.9 ± 0.2	23.9 ± 0.2
	9	34.4 ± 0.5	29.6 ± 0.3	31.8 ± 0.2	23.8 ± 0.1
	Avg of 1–9	35.4 ± 0.6	33.2 ± 1.3	31.9 ± 0.5	24.4 ± 0.8
	Pooled DNA[b]	33.3 ± 0.4	33.2 ± 0.1		
10 oocysts	1	34.5 ± 0.3	25.4 ± 0.8	36.9 ± 0.6	24.5 ± 0.1
	2	35.1 ± 0.5	26.27 ± 0.7	36.7 ± 1.1	24.3 ± 0.0
	3	36.1 ± 0.8	26.35 ± 0.3	36.9 ± 0.4	24.8 ± 0.2
	4	35.1 ± 0.6	25.32 ± 0.1	35.5 ± 1.0	25.2 ± 0.1
	5	36.2 ± 1.6	25.47 ± 0.1	35.6 ± 1.6	24.7 ± 0.2
	6	36.6 ± 0.8	25.66 ± 0.0	36.0 ± 0.0	24.7 ± 0.1
	7	35.5 ± 0.6	25.7 ± 0.5	34.7 ± 0.4	24.9 ± 0.1
	8	36.2 ± 0.9	27.9 ± 0.6	34.6 ± 0.8	24.8 ± 0.2
	Avg of 1–8	35.5 ± 0.8	26.4 ± 0.4	35.9 ± 0.7	24.7 ± 0.1
	Pooled DNA[b]	35.5 ± 0.2	26.7 ± 0.5		

[a] The internal amplification control (IAC) C_T values for the nontemplate control (NTC) were 24.5 ± 0.1 for the assay with 200 oocysts and 24.3 ± 0.1 for the assay with 10 oocysts.
[b] Pooled DNA from cilantro samples with soil showing inhibition of qPCR based on IAC C_T values at seeding levels of 200 or 10 oocysts.

WI) (kit 4), and DNeasy PowerPlant pro kit from Qiagen (kit 5) (Table 1). In the present study, all DNA extractions prior to the cleanup step were performed with homogenization using the FastPrep-24 kit as described above. The commercial DNA cleanup kit protocols were then performed following the manufacturer's recommendations, with small modifications. For kit 2, the recommended minimum initial volume of DNA is 50 µL, and therefore, 25 µL of molecular-grade water was added to the initial 25 µL of DNA. For kit 3, after two sequential elution steps using 10 µL as recommended in the kit, 30 µL of molecular-grade water was added to achieve a final volume of 50 µL, as for all the other kits, to facilitate comparison of the kits. The procedure for kit 5 began at step 5 of the protocol because the manufacturer's instructions specified a bead-beating protocol for DNA extraction that was different from that used in the present study.

To compare kit performance, "cleaned" DNA was analyzed by qPCR reactions run in triplicate for each sample as previously described.

Statistical analysis. Statistically significant differences between C_T values obtained after cleanup of DNA with the five kits were analyzed by nonparametric one-way analysis of variance (Kruskal Wallis test) and multiple comparisons by Dunn's multiple comparison test, using GraphPad version 8.3 (GraphPad, San Diego, CA), with a P value of ≤ 0.05 indicating statistical differences.

RESULTS

The initial step before evaluating the comparison of DNA cleanup methods for the removal of matrix-associated PCR inhibitors was to obtain enough DNA from cilantro samples seeded with *C. cayetanensis* oocysts with added soil that showed IAC inhibition in the BAM Chapter 19b qPCR method for each pool.

Previous experiments to obtain inhibited *C. cayetanensis* qPCR were performed using (i) fresh cilantro in poor condition, when cilantro started to age, losing its natural consistency and becoming softer and slimy with the presence of fluid (2 samples), or (ii) using cilantro that was vigorously processed using a stomacher and glass beads (6 samples). No interference in the qPCR reaction was observed (data not shown) in either scenario. On the other hand, cilantro samples with a small amount of added soil consistently inhibited the IAC in the *C. cayetanensis* qPCR and were therefore used for comparison of DNA cleanup kits.

Results in cilantro samples with added soil. DNAs from nine individual 25-g seeded (200 oocysts) cilantro samples with added soil shown to have inhibited the IAC C_T value by qPCR (Table 2) were pooled. The pooled DNA showed an average 18S rRNA C_T value of 33.3 ± 0.4 and average IAC C_T value of 33.2 ± 0.1 (Table 2), while the average IAC C_T value for the NTC in the same qPCR experimental run was 24.5 ± 0.1. The pooled DNA was therefore considered inhibitory because the IAC C_T value was more than three C_T values higher than the IAC C_T value for the NTC. Similarly, a second set of DNAs from eight individual 25-g cilantro samples with added soil, each seeded with 10 oocysts, which were shown to have partially

TABLE 3. *Comparison of commercial DNA cleanup kits for minimizing the effects of inhibitors on the qPCR assay of* C. cayetanensis *from cilantro samples with added soil and seeded with known numbers of* C. cayetanensis *oocysts*

		Mean ± SD		
Seeding level	Kit	18S rRNA C_T value[a]	18S rRNA copy no./reaction	IAC C_T value
200 oocysts	1, QIAquick	33.4 ± 0.1 A	38.2 ± 0.5	24.9 ± 0.2
	2, OneStep	33.05 ± 0.2 A	47.8 ± 6.5	24.7 ± 0.0
	3, NucleoSpin	33.2 ± 0.4 A	43.1 ± 10.2	24.4 ± 0.3
	4, DNA IQ	35.5 ± 0.3 B	10.2 ± 2.4	23.9 ± 0.1
	5, DNAse PowerPlant pro	34.1 ± 0.9 AB	26.9 ± 16.1	24.1 ± 0.1
10 oocysts	1, QIAquick	37.3 ± 0.2 AB	2.4 ± 0.2	25.0 ± 0.0
	2, OneStep	36.6 ± 0.4 AB	4.6 ± 1.3	24.5 ± 0.2
	3, NucleoSpin	36.0 ± 0.6 A	7.2 ± 3.2	24.6 ± 0.4
	4, DNA IQ	38.0 ± NA[b]	1.4 ± NA	24.0 ± 0.1
	5, DNeasy PowerPlant pro	37.4 ± 0.7 B	2.6 ± 1.6	24.1 ± 1.2

[a] Different letters within the corresponding oocyst level show statistically significant differences among kits.
[b] NA, not applicable—only a single replicate in one sample was positive.

inhibited IACs by qPCR were pooled. This second set showed a mean 18S rRNA C_T value of 35.5 ± 0.2 and a mean IAC C_T of 26.7 ± 0.5 (Table 2), while the NTC mean IAC C_T was 24.3 ± 0.1. This pool was considered partially inhibited because the mean IAC C_T value was only 2.4 C_T values higher than the IAC C_T value for the NTC in the same run.

For cilantro samples without soil, processed at the same time as the cilantro samples with residual soil, the average IAC target C_T values were 24.4 ± 0.8 and 24.7 ± 0.1 for samples seeded with 200 oocysts (9 samples) and samples seeded with 10 oocysts (8 samples), respectively (Table 2).

Comparison of commercial DNA cleanup kits in reduction of qPCR inhibition by cilantro samples. The protocols varied among the kits. Table 1 presents some of the main characteristics of the kits used. Four of the kits were column-based methods for DNA cleanup, while one kit (kit 4) was based on magnetic beads. The number of steps, based on the commercial protocols, and the time needed in the procedure varied among kits. Kit 2, having only one step, was the shortest procedure.

A clear reduction in the IAC C_T values after DNA cleanup of both DNA pools was observed for all the kits. IAC C_T values returned values very similar to those found for the IAC C_T values for the NTCs and to those values observed in samples of cilantro without soil (Table 2). In cleaned DNA from the samples seeded with 200 oocysts, the IAC values decreased from the original 33.2 ± 0.1 to C_T values ranging from 23.9 to 24.9 in the five kits, and in samples seeded with 10 oocysts, the average IAC C_T values decreased from an average of 26.7 ± 0.5 to values ranging from 24.0 to 25.0 in the five kits analyzed (Table 3). The lowest IAC C_T values after cleanup were observed using kit 4 and kit 5 for samples seeded with both 200 and 10 oocysts, while the highest IAC C_T values after cleanup were observed using kit 1, also at both seeding levels (Table 3).

Every cleaned DNA sample extracted from cilantro seeded with 200 oocysts was positive for the *C. cayetanensis* 18S rRNA independent of the cleanup kit used (Table 3). The average 18S rRNA C_T value for the 200-oocyst-level pooled DNA was 33.3 ± 0.4 before the cleanup step, while the average 18S rRNA C_T value for the cleaned-up DNA samples ranged from 33.05 ± 0.2 in kit 2 to 35.5 ± 0.3 in kit 4 (Table 3). The kits ranked from lowest to highest mean C_T values in the detection of the *C. cayetanensis* 18S rRNA gene in the following order: kit 2, kit 3, kit 1, kit 5, and finally, kit 4. The average fold increases of the amplifiability of the 18S rRNA target for the kits (normalized to the value for kit 4, which showed the lowest

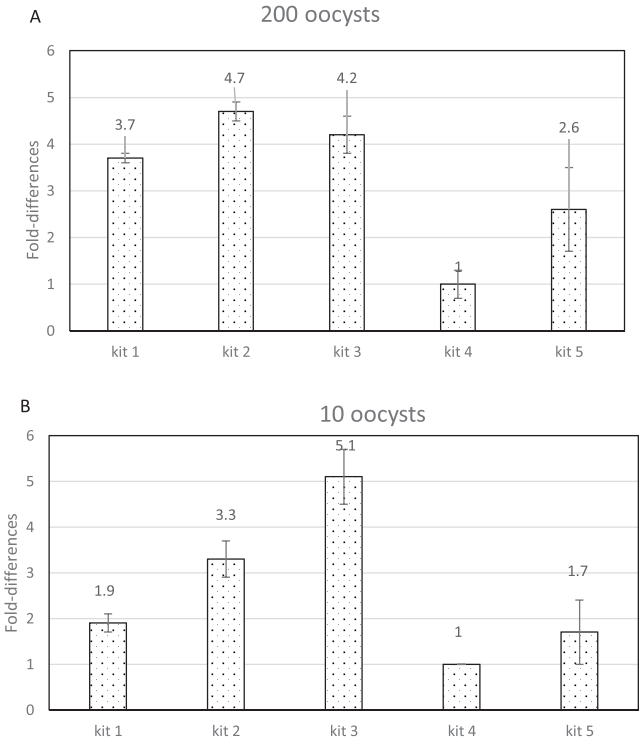

FIGURE 1. *Relative fold differences in 18S rRNA copy numbers detected in cilantro samples with added soil using five commercial DNA cleanup kits. Samples were seeded with 200 oocysts (A) or 10 oocysts (B). Fold differences were calculated by normalization of 18S rRNA copy numbers detected to those found using kit 4 (lowest value). Error bars indicate standard errors.*

amplification) were 4.7, 4.2, 3.7, and 2.6 for kits 2, 3, 1, and 5, respectively (Fig. 1A).

High variability in 18S rRNA gene C_T values was observed in samples seeded with 10 oocysts; a portion of replicates were negative for the 18S rRNA gene after DNA cleanup using all the kits with the exception of kit 3, which showed positive results in all replicates and samples. Only one replicate in one sample was found to be positive using kit 4 at the low seeding level (data not shown). A similar sensitivity ranking was observed for the kits when testing samples seeded with 10 oocysts as found for samples seeded with 200 oocysts, with samples treated with kit 5 and kit 4 showing the lowest sensitivity. The ranking from lowest to highest *C. cayetanensis* 18S rRNA target mean C_T values was kit 3, kit 2, kit 1, kit 5, and kit 4. Average fold increases in the amplifiability of the 18S rRNA target in each kit, normalized to the value for kit 4, were 5.1-, 3.3-, 1.9-, and 1.7-fold for kits 3, 2, 1, and 5, respectively, in samples seeded with 10 oocysts (Fig. 1B).

No statistically significant differences were observed in the 18S rRNA mean C_T values for cleaned DNA samples originally seeded with 200 and 10 oocysts among kit 1, kit 2, and kit 3 ($P > 0.05$). Significant differences in mean 18S rRNA C_T values were observed in cleaned DNA samples seeded with 200 oocysts using kit 4 versus kit 1, kit 2, and kit 3 ($P = 0.01$, $P < 0.001$, and $P < 0.001$, respectively), but not when compared with kit 5 ($P = 0.42$). When samples seeded with 10 oocysts were analyzed, the only significant differences in 18S rRNA C_T values observed were between kit 3 (lowest C_T value, best detection) versus both kit 4 and kit 5 ($P = 0.0004$ and $P = 0.03$, respectively). No statistically significant differences were observed between kit 1 and kit 2 ($P > 0.05$), and no significant differences were observed between kit 4 and kit 5 ($P > 0.05$).

DISCUSSION

In the present study, the efficacy of five commercial DNA cleanup kits for reduction of IAC inhibition and for detection of *C. cayetanensis* oocysts in cilantro samples that previously showed the presence of qPCR inhibitors was assessed. The final objective of this study was to reduce qPCR inhibition caused by cilantro samples when executing the BAM Chapter 19b qPCR method for *C. cayetanensis* detection. The qPCR method is a duplex TaqMan real-time PCR assay with an IAC which provides an effective control for reaction inhibition and is essential for regulatory testing applications *(24, 28)*. Inhibition of the IAC is rarely found in cilantro samples without added soil when analyzed following the BAM Chapter 19b method. In a total of 80 cilantro samples analyzed in a multilaboratory validation study, only three cilantro samples were found to be inhibited *(24)*. The robustness of the method in cilantro was demonstrated in the present study in preliminary experiments when fresh cilantro in poor condition or vigorously processed failed to inhibit qPCR reactions. On the other hand, cilantro samples with a small amount of added soil (0.3 g) consistently inhibited the *C. cayetanensis* qPCR. Such samples containing soil inhibitors were selected for comparison of the effectiveness of commercial DNA cleanup kits in reduction of qPCR inhibition.

Although there are some studies comparing DNA cleanup methodology, to our knowledge, no previous studies have been performed in fresh produce samples. For example, kit 3, the NucleoSpin DNA cleanup XS kit, was considered an alternative for the concentration, purification, and removal of PCR inhibitors from existing DNA extracts *(15, 16)* and removed commonly encountered PCR inhibitors in forensic samples *(12)*. Similarly, kit 4, the DNA IQ system, was very effective for the removal of inhibitors found in forensic samples *(14)*. The present study showed that the five commercial cleanup kits successfully eliminated soil inhibitors of the qPCR for detection of *C. cayetanensis* in DNA extracts from cilantro samples. The mechanism of action for many inhibitory substances is unknown. PCR inhibitors might cause amplification failure by directly interfering with the DNA polymerase *(2, 34)* and by binding to template DNA *(26)*. For real-time PCR assays, the interference with fluorescent probes or increased background fluorescence represents an additional mechanism for which PCR inhibitors may decrease sensitivity *(31)*. In a previous study using a 6-FAM (6-carboxyfluorescein) probe, as in the qPCR in the present study, the reaction was not affected by fluorescence quenching *(34)*.

Based on the results of the present study, each of the five commercial kits analyzed successfully returned IAC C_T values to those considered normal in the cleaned DNA in cilantro samples seeded at both a high oocyst level (200 oocysts) and a low oocyst level (10 oocysts). At the higher seeding level (200 oocysts), all samples were also positive for detection of *C. cayetanensis* independent of the kit used for DNA cleanup. However, some differences were observed when quantitative data were compared. Of the five kits compared, kits 1, 2, and 3 did not show significant differences in the detection of the *C. cayetanensis* 18S rRNA gene in samples seeded with 200 oocysts, while the other two kits (kits 4 and 5) showed significantly lower detection. A very similar trend in *C. cayetanensis* detection was also observed when the kits were compared using samples seeded with the low level of oocysts (10 oocysts), with the least sensitivity (lowest detection) being observed using the same two kits (kits 4 and 5). In one of those kits (kit 5), we did not follow the first part of the protocol that involved a different DNA extraction, as DNA extraction was performed following the BAM Chapter 19b method, although we would not expect the different DNA extraction method to account for these differences. The second kit (kit 4) was based on a different platform using immunomagnetic beads, instead of being column based like the other four kits analyzed.

Although some decrease of sensitivity due to DNA loss was expected during the cleanup process, kits 1, 2, and 3 did not display any major detection differences based on the 18S rRNA gene mean C_T values observed in the initial pooled DNA and previously observed in noninhibited samples *(24)*. The mean C_T values for the *C. cayetanensis* 18S rRNA gene target found for cilantro samples which were not inhibitory in the multilaboratory validation study of the BAM Chapter 19b method were 32.6 ± 1.5 for 40

samples seeded with 200 oocysts and 35.9 ± 1.4 for 40 samples seeded with 10 oocysts *(24)*. The mean C_T values found for cleaned DNA using kits 1, 2, and 3 in the present study ranged from 33.0 to 33.4 in samples seeded with 200 oocysts and from 36.0 to 37.3 in samples seeded with 10 oocysts. Kit 2 had additional advantages due to its technically simple protocol, short completion time, and lower cost per reaction. Kits 1, 2, and 3 also showed the best results in diminishing qPCR inhibition engendered by the addition of contaminating soil in cilantro, as shown by the fold increases in the amplifiability of their templates compared to the values for the other kits.

In conclusion, the use of commercial kits for cleanup of DNA extracts from cilantro samples with soil, which inhibits qPCR, was effective in the elimination of those inhibitors and reduced the potential for inconclusive results. Therefore, the use of these DNA cleanup kits may provide an effective means to reduce inhibition of qPCR stemming from inhibitors in cilantro samples when executing the BAM Chapter 19b method. These results are of critical importance when the method is applied during surveys or traceback investigations of outbreaks linked to the parasite in cilantro and in fresh produce in general. Improved detection of the parasite will facilitate future studies to understand the prevalence of *C. cayetanensis* in fresh produce and in the environment and to identify the necessary preventative control measures to reduce foodborne exposure to *C. cayetanensis*.

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