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Optimization and validation of methods for isolation and real-time PCR identification of protozoan oocysts on leafy green vegetables and berry fruits

Laura F. Lalonde *, Alvin A. Gajadhar

Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, 116 Veterinary Road, Saskatoon, Saskatchewan S7N 2R3, Canada

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ABSTRACT

Leafy green vegetables and berry fruits have vastly different physical and biochemical characteristics, are typically consumed raw with minimal washing, and are potential transmission vehicles for food-borne disease caused by protozoan parasites such as *Cryptosporidium*, *Cyclospora* and *Toxoplasma*. Validation of a generic oocyst isolation and detection method applicable to each leafy green and berry type is required to provide reliable laboratory support for surveillance programs and as necessary for disease outbreak investigations. The objectives of the current study were to optimize and validate the performance of these methods for the isolation of protozoan oocysts from several types of leafy greens and berries. *Eimeria papillata* oocysts were used as a surrogate for coccidia of public health concern to spike produce samples. An artificial stomacher or orbital shaker was used, followed by centrifugation, to isolate and concentrate oocysts, respectively, and a qPCR melt curve analysis (qPCR MCA) was used for detection and identification. Processing methods, wash buffers and storage conditions were evaluated and optimized for five types of berries (blackberries, blueberries, cranberries, raspberries and strawberries), five types of herb (cilantro, dill, mint, parsley, thyme) and green onions. Blackberries, cranberries, raspberries and strawberries were most effectively washed by orbital shaking with an elution solution, while glycine buffer was more effective for blueberries. Stomaching with a glycine buffer was optimal for oocyst recovery in leafy herbs with soft stems, while aromatic woody-stemmed herbs such as thyme required orbital shaking to minimize the release of PCR inhibitors. Oocyst recovery from green onions was highest when processed by orbital shaking with elution solution. Oocyst recovery rates ranged from 4.1–12% for berries and 5.1–15.5% for herbs and green onions. As few as 3 oocysts per gram of fruit, or 5 oocysts per gram of herbs or green onions could reliably be detected using the optimized isolation methods and qPCR MCA.

1. Introduction

The importation of leafy green vegetables (leafy greens) and berry fruits from Central and South America is steadily increasing (Anonymous, 2010). Much of this produce is minimally processed and consumed raw, leading to an increasing risk of exposure to parasites that would normally be controlled by food processing temperatures (Gajadhar, 2015a; Gamble, 2015). Outbreaks of gastrointestinal illness related to the consumption of imported leafy green herbs, vegetables, or berries contaminated with *Cyclospora*

* Corresponding author. Tel.: +1 306 385 7815; fax: +1 306 385 7866.
E-mail address: laura.lalonde@inspection.gc.ca (L.F. Lalonde).

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cayetanensis have occurred sporadically in North America and Europe since 1996 (Dixon, 2015; Doller et al., 2002; Gibbs et al., 2013; Herwaldt and Ackers, 1997; Hoang et al., 2005; Insulander et al., 2010; Lopez et al., 2007). *Cryptosporidium* has been detected in ready-to-eat vegetables and irrigation water in both developing and developed countries (Amoros et al., 2010; Rzeżutka et al., 2010), and *Toxoplasma* has also recently been detected in leafy greens in Poland (Lass et al., 2012). Since they are generally consumed raw and with minimal washing, a wide variety of leafy greens and berries with vastly different physical and chemical characteristics are potential vehicles for food-borne disease caused by *Toxoplasma*, *Cryptosporidium* or *Cyclospora*, therefore a broad-specificity oocyst isolation and detection screening method should be validated as needed for each type.

Several methods have been developed for the microscopic or molecular detection of *Cyclospora* (Shields et al., 2012; Steele et al., 2003) or *Cryptosporidium* (Cook et al., 2006a, 2006b, 2007; Robertson and Gjerde, 2000) oocysts on fruits and vegetables, however most target only a single protozoan oocyst species, and have not been validated for use in multiple types of produce. In order to deliver reliable survey results, to establish accurate baseline data, and to facilitate timely and effective food-borne disease outbreak investigations and response, a validated screening method for the detection of oocysts of multiple species of protozoan parasites is required. Past method development and validation studies have focused primarily on single produce types (such as lettuce or raspberries) or protozoan species (mainly *Cryptosporidium* or *Giardia*) using microscopy to detect oocysts (Cook et al., 2006a, 2006b, 2007; Shields et al., 2012, 2013). Microscopic methods for detection and identification of *Cyclospora* and other protozoan of human health concern are limited due to inconsistent staining of oocysts, time required for oocysts to be sporulated for taxonomic identification, and the need for parasitology experience to distinguish oocysts from fecal or other debris (Gajadhar et al., 2015; Mansfield and Gajadhar, 2004). Although several commercial immunomagnetic separation (IMS) and immunofluorescent antibody (IFA) kits are available for *Cryptosporidium* and *Giardia*, none are currently available for *Cyclospora* or *Toxoplasma*. A real time PCR assay with melt curve analysis (qPCR MCA) has been developed for the simultaneous detection and differentiation of several protozoan oocyst species of public health importance (*Cryptosporidium*, *Cyclospora*, *Sarcocystis*, *Cystoisospora* and *Toxoplasma*) (Lalonde and Gajadhar, 2011). It is capable of detecting and identifying a wide variety of coccidia present in food, clinical, or environmental matrices. The qPCR MCA approach is well suited for high-throughput screening, can differentiate *Cyclospora* from other coccidia, including *Eimeria* (Lalonde and Gajadhar, 2011), and has been successfully applied to detecting zoonotic protozoan species in human fecal samples (Lalonde et al., 2013); however it has not been validated for use in leafy greens or berries.

Processing consists of isolation and detection of oocysts from the food matrix. Adequate validation of the oocyst isolation method is necessary for each type of leafy greens or berries tested, and the processes performance characteristics such as sensitivity and accuracy for these products should be determined. Certain fresh, ready to eat leafy greens and berries have inherent physical or biochemical properties which make removing and detecting protozoan oocysts difficult. Leafy greens and berries often contain phenols, polysaccharides, pectin and other compounds that inhibit PCR (Schrader et al., 2012) and may reduce the sensitivity of molecular assays. Raspberries and blackberries are incredibly delicate and temperature sensitive, resulting in tissue breakdown during processing which can lead to debris that that physically interferes with the recovery of oocysts. They also have fine hair-like projections that may trap oocysts (Kniel et al., 2002), making isolation a challenge. To overcome these physical and chemical challenges and maintain a suitable level of assay sensitivity, method modifications specific for each type of leafy green and berry may be necessary and require validation to ensure that tests used are fit for purpose. The objectives of the current study were to 1) optimize and determine the performance characteristics of methods for isolation of protozoan oocysts from several types of leafy greens and berries, and 2) validate the qPCR MCA assay for use to detect protozoan oocysts in these matrices.

2. Materials and methods

2.1. Oocysts and produce samples

Oocysts of *Eimeria papillata* were used as a surrogate for those of *C. cayetanensis* and other coccidia of public health concern in all recovery experiments, because they are non-pathogenic and fresh oocysts were available. The *E. papillata* oocysts were propagated by passage in mice, and isolated from feces by washing and straining with water and by Sheather's flotation (Levine, 1985). The oocysts were sporulated (72%) and stored at 4 °C in 2.5% potassium dichromate until used. Stocks of *E. papillata* used for spiking produce samples were prepared by triplicate enumeration with a hemocytometer and diluted in an antibiotic–antimycotic solution (Streptomycin, Penicillin, Amphotericin B, Life Technologies) for use within 30 days. For low concentration stocks, triplicate aliquots of oocyst dilutions were dried on microscope slides with concave wells and enumerated using a fluorescent microscope to detect autofluorescence.

Produce samples used for spiking and recovery experiments were purchased at local retail grocery stores and included five types of berries (blackberries, blueberries, cranberries, raspberries and strawberries), five types of herb (cilantro, dill, mint, parsley, thyme) and green onions. Unspiked samples of produce were tested alongside spiked samples from each batch to ensure they had not been naturally contaminated with oocysts. Additionally, samples containing wash buffer only (no produce) were spiked and processed with each batch as positive controls to verify the performance of the processing and flotation procedures. Produce samples were spiked with 5000 oocysts (unless otherwise indicated) ~18 h prior to processing to most closely mimic field contamination. Enumerated aliquots of oocysts were pipetted onto the surface of pre-weighed produce samples in several 5–10 μl droplets, covered and stored at 4 °C or room temperature overnight.

2.2. Wash solutions and processing methods

Three wash solutions were evaluated for effectiveness during the method optimization phase of this study: 1 M glycine buffer pH 5.5, 1 M glycine buffer pH 3.5 (Cook et al., 2006a, 2006b), and an elution solution (disodium pyrophosphate 0.563 mM $H_2Na_2P_2O_7$, 42.8 mM NaCl) (Riner et al., 2007; Shields et al., 2012). The buffers selected for evaluation with each berry or leafy green type were based on published results of previous studies (Cook et al., 2006a, 2006b; Riner et al., 2007; Shields et al., 2012), and preliminary evaluations in our laboratory. Processing methods evaluated for berries were orbital shaking in bottles for 1 min or 60 min (Cook et al., 2006a). For aromatic or woody-stemmed herbs and green onions that contain high levels of PCR inhibitors, horizontal orbital shaking in a stomacher filter bag with wash buffer (30 min per side) and stomaching (as for herbs) were compared to determine the method with the highest recovery of oocysts. Storage of produce samples at room temperature or at 4 °C post-inoculation was also evaluated. Unless otherwise indicated, 16 samples per treatment were evaluated over a two day period for all method optimization experiments.

2.3. Isolation of oocysts from herbs

Representative samples (35 ± 0.5 g) of intact herb stems and leaves were selected and placed in a strainer stomacher bag (Seward). Samples were processed by stomacher (Seward) for 1 min at 200 rpm with 200 ml wash buffer. The strainer bags were then supported in a large beaker and the sample, filter bag and outer plastic bag were all rinsed with approximately 30–40 ml of 1 M glycine buffer. The inner filter bag containing the herbs was detached and gently compressed by hand through the outer plastic bag to remove excess liquid and discarded. Collected wash buffer was poured in 250-ml conical bottom disposable centrifuge bottles and concentrated by centrifugation at 2000 ×*g* for 15 min. Supernatant was removed by vacuum aspiration leaving twice the volume of the remaining pellet or a minimum volume of 2.5 ml. Oocysts were isolated from the produce wash using a Sheather's flotation method (Levine, 1985). The pellet was re-suspended by vortexing and transferred to a 16 × 100 mm glass tube and mixed with Sheather's solution by gentle inversion to avoid introducing air bubbles. Sheather's solution was added to the tube until a slight convex meniscus was visible, overlaid with a 22 × 22 mm glass coverslip and centrifuged with a swing-out rotor at 450 ×*g* for 10 min. The coverslip was lifted off and oocysts were rinsed from the coverslips with 1 ml of wash buffer with 0.1% Tween 20 into a 2.0-ml tube. In addition, the top 2 ml of Sheather's solution in the flotation tube which may also contain oocysts was collected using a wide bore pipette, transferred to a 15-ml plastic centrifuge tube, mixed with H_2O and washed once by centrifugation for 15 min at 2000 ×*g*. After similarly removing the supernatant, the remaining concentrated 0.5 ml pellet was combined with the coverslip rinses in the 2.0 ml tube and concentrated by centrifugation at 20,000 ×*g* for 15 min. The final pellet was washed 2× with water by centrifugation as above to remove any remaining Sheather's solution, which could inhibit PCR.

2.4. Isolation of oocysts from berries

Samples (60 ± 1 g) of fresh berries were selected and placed in a 1 l wide mouth plastic bottle and shaken on an orbital shaker for 1 min or 60 min at 100 rpm (for raspberries and blackberries) or 130 rpm (for all other berries) with 200 ml of wash buffer. The samples were transferred to a strainer stomacher bag supported in a beaker and the berry surfaces, mesh bag, and outer plastic bag were rinsed with wash buffer containing 0.1% Tween 20. The mesh inner bag was detached, shaken gently to release excess wash buffer and discarded. Remaining wash buffer was transferred to a 250 ml conical bottom centrifuge tube and concentrated by centrifugation and oocysts isolated by Sheather's flotation as described above for herbs.

2.5. Isolation of oocysts from green onions

Samples (35 ± 3 g) of green onions and 200 ml wash buffer were placed in a stomacher bag with a strainer, sealed with clips, and shaken on an orbital shaker at 130 rpm for 30 min on each side to ensure adequate buffer–sample contact. Samples were rinsed as for herbs, and wash buffer was concentrated and oocysts isolated by flotation as described above for berries and herbs.

2.6. DNA extraction and qPCR melting curve analysis

DNA was extracted from the final produce wash oocyst suspension (~100 μl) using the QIAamp DNA Mini Kit (Qiagen) with modifications to the manufacturer's protocol as described previously, except that the final elution volume in the supplied AE buffer was 40 μl (Lalonde and Gajadhar, 2008). DNA was stored at 4 °C if used within 7 days or at −20 °C for longer periods. A no-template negative control of only reagents and a positive control containing a known number (5000 unless otherwise indicated) of *E. papillata* oocysts spiked on the produce were included in each batch of samples for DNA extraction. For the qPCR standard curve, DNA was extracted from 10^5 purified *E. papillata* oocysts which were enumerated by triplicate readings using a hemocytometer, and 10-fold serial dilutions were prepared in PCR quality H_2O to be equivalent to 10^5–10^1 oocysts.

Detection of spiked oocyst DNA was performed using reaction mix contents, qPCR amplification conditions, and melt curve analysis as described previously (Lalonde et al., 2013). The standard curve and a reagent-only no-template control were included on each plate. Following the amplification and melting curve protocols, collected fluorescence data and Cq values were analyzed using the CFX Manager Software (version 3.0, Bio-Rad) to generate melt curves and provide an estimate of oocyst number for

each sample as described previously (Lalonde and Gajadhar, 2011). Percent recovery was calculated as follows: (oocyst quantity estimated from qPCR / oocyst number spiked on produce) × 100.

2.7. Statistical analysis

Oocyst numbers recovered from the different experimental treatments examined during the method optimization phase were compared using two-tailed, non-parametric, Mann–Whitney t-tests (GraphPad Prism software, version 4.03). Significance was reported when $P \leq 0.05$.

3. Results

3.1. Optimization of wash buffers

The effectiveness of the elution solution versus 1 M glycine buffer (pH 5.5) was compared for raspberries, blueberries, blackberries and strawberries. The elution solution improved the recovery of *Eimeria* oocysts in raspberries, blackberries and strawberries by 72, 39, and 70% respectively while 1 M glycine buffer (pH 5.5) was 78% more effective for washing blueberries. The elution solution and a lower pH glycine buffer (pH 3.5) were similarly evaluated for cranberries and raspberries; the elution solution improved recovery of *Eimeria* oocysts by 132 and 68% for these berries, respectively.

The effectiveness of elution solution and 1 M glycine buffer (pH 5.5) were compared for green onions, basil, cilantro and mint. Glycine buffer improved recovery of *Eimeria* oocysts in basil, cilantro and mint by 17, 52, and 24%, respectively while the elution solution was 34% more effective for washing green onions.

3.2. Optimization of storage and processing methods

Post-spiking storage at room temperature and 4 °C was evaluated for raspberries, blueberries, blackberries, strawberries to determine optimal conditions for transport of samples to the laboratory for testing. Refrigeration of the berries after spiking with *Eimeria* oocysts improved recovery by 174% (±7) on average ($P < 0.0001$). Shaking times of 60 min and 1 min with elution solution were evaluated for raspberries (n = 8 samples per treatment) and no significant difference ($P = 0.7984$) was observed in the recovery of *Eimeria* oocysts following these two treatments. For green onions, stomaching and shaking were compared to determine the most suitable method; 74% more oocysts were recovered from samples processed by horizontal shaking in a stomacher bag with elution solution ($P = 0.004$).

To determine the processing method which releases the lowest amount of PCR inhibitors from aromatic woody-stemmed herbs such as sage, thyme and rosemary, un-spiked samples of these herbs were processed either by horizontal shaking or stomaching, and the resulting wash spiked with *Eimeria* oocysts prior to DNA extraction and qPCR MCA. For sage, thyme, and rosemary, detection of *Eimeria* oocyst DNA in the wash extracts was 100, 51, and 80% higher for orbital shaking compared to stomaching. However, for rosemary, the percent recovery was very low for both methods; DNA from only 2.3 and 1% of spiked *Eimeria* oocysts could be detected by qPCR. Removing the rosemary leaves from the woody stems improved the detection of *Eimeria* DNA slightly to 4.3%.

3.3. Detection limit

The optimized processing methods were utilized to determine the lowest level of *Eimeria* oocysts that could be reliably detected for berries, herbs, and green onions. For berries, 625, 312, or 156 oocysts (equivalent to approximately 10, 5, or 3 oocysts per gram of fruit) were spiked onto 6 samples per treatment and processed as above using the conditions outlined in Table 1. For herbs, 1250, 625, 312, or 156 oocysts (equivalent to approximately 36, 18, 9, or 5 oocysts per gram of leafy greens) were spiked onto 6 samples per treatment and processed using the optimized conditions shown in Table 1.

Table 1
Summary of most effective wash buffers and processing treatments evaluated for berries, herbs and green onions.

Produce type	Processing treatment			
	Sodium pyrophosphate	Glycine pH 5.5	Shaking (min)	Stomaching
Blackberries	Yes	No	Yes (1)	No
Blueberries	No	Yes	Yes (1)	No
Cranberries	Yes	No	Yes (1)	No
Raspberries	Yes	No	Yes (1)	No
Strawberries	Yes	No	Yes (1)	No
Leafy herbs	No	Yes	No (0)	Yes
Thyme/woody	No	Yes	Yes (60)	No
Green onions	Yes	No	Yes (60)	No

Table 2
Number of spiked produce samples and the number of *Eimeria* oocysts subsequently detected by qPCR MCA and the average percent recovery ± SEM.

Produce type	Oocyst number spiked (% recovery ± SEM)			
	1250	625	312	156
Blackberries	ND	6/6 (3.4 ± 0.9)	6/6 (3.6 ± 1.0)	6/6 (5.4 ± 1.3)
Blueberries	ND	6/6 (11.8 ± 3.8)	6/6 (14.3 ± 2.6)	6/6 (9.8 ± 1.5)
Cranberries	ND	6/6 (8.3 ± 1.2)	6/6 (5.8 ± 1.3)	6/6 (3.0 ± 0.6)
Raspberries	ND	6/6 (12.2 ± 1.5)	6/6 (12.5 ± 2.4)	6/6 (8.9 ± 3.9)
Strawberries	ND	6/6 (9.4 ± 1.0)	6/6 (15.3 ± 4.1)	6/6 (4.7 ± 1.0)
Cilantro	ND	6/6 (14.2 ± 2.0)	6/6 (11.2 ± 1.8)	6/6 (16.8 ± 1.8)
Dill	6/6 (17.6 ± 2.0)	4/6 (5.8 ± 1.9)	5/6 (6.5 ± 3.0)	6/6 (8.5 ± 2.5)
Mint	ND	6/6 (5.3 ± 0.5)	6/6 (5.0 ± 0.2)	6/6 (6.3 ± 0.9)
Parsley	6/6 (15.8 ± 2.4)	6/6 (15.6 ± 2.8)	5/6 (12.9 ± 3.4)	5/6 (17.7 ± 7.3)
Thyme	6/6 (3.2 ± 0.3)	6/6 (6.2 ± 0.8)	6/6 (12.7 ± 1.7)	ND
Green onions	ND	4/6 (2.7 ± 1.1)	6/6 (8.8 ± 3.1)	4/6 (3.7 ± 2.3)

ND = not determined.

For all berry types, 3 oocysts per gram of sample could consistently be detected using qPCR MCA (Table 2). Overall recovery rates were lowest for blackberries and cranberries — 4.2 and 5.7% respectively, while higher oocyst recovery was observed for strawberries (9.8%), raspberries (11.2%) and blueberries (12%). For herbs and green onions, 5–9 oocysts per gram could consistently be detected by qPCR MCA. Overall recovery rates were highest for parsley (15.5%), cilantro (14.1%) and dill (9.6%), while lower recovery rates were observed for mint (5.5%), thyme (7.4%), and green onions (5.1%).

4. Discussion

This study demonstrated that some washing buffers and processing methods are more effective than others for recovery and detection of oocysts by qPCR MCA in some leafy green and berry varieties. For instance, oocysts were best removed from smooth-skinned blueberries using a glycine buffer, but the irregular surfaces of raspberries, strawberries, blackberries were more effectively washed using the elution solution which contains a surfactant (sodium pyrophosphate) that allows for more complete wetting of the fruit (Shields et al., 2012). Shield et al. (2012) also noted that *Cyclospora* recovery was improved when raspberries were washed with a commercial detergent containing the surfactant tetrasodium pyrophosphate. For most leafy greens, where previous studies have shown stomaching to be the most effective processing method (Cook et al., 2006a), a glycine wash buffer was more effective. In preliminary studies, we observed that buffers containing a surfactant or detergent were unsuitable for produce types that were processed by stomaching, since the excessive bubbles produced seemed to interfere with oocyst recovery.

The oocyst detection method employed, whether by IMS–IFA and microscopy, or a molecular method such as used in this study, should be considered when selecting a produce processing method. Certain processing methods, such as stomaching, damage plant tissues and release more PCR inhibitors than less vigorous approaches such as orbital shaking. Thus, even though stomaching may be suitable for processing leafy greens for the detection of oocysts by microscopy, it is generally not appropriate for use in combination with molecular assays. In the current study, oocyst detection by qPCR MCA was higher when aromatic, woody-stemmed herbs such as thyme, oregano, chives, and green onions were processed by orbital shaking compared to stomaching. Molecular detection methods such as qPCR MCA are more strongly impacted by the presence of PCR inhibitors originating from plants or chemicals. In this study, steps were employed to eliminate inhibitors, such as washing of oocysts prior to DNA extraction to remove processing buffers, using column-based DNA extraction, and the addition of BSA to the qPCR reaction mix. Due to the varying amounts of PCR inhibitors that may be released by different processing methods (stomaching vs shaking for instance), oocyst numbers detected by qPCR may differ from those detected by microscopy. Therefore, the processing recommendations presented here are most appropriate for use with a molecular detection method.

In some cases, high levels of inhibitors in the plant material made effective detection of oocysts by molecular methods impossible. This was the case for rosemary in the current study where the resinous plant components released during processing completely inhibited qPCR. Therefore, certain herbs high in polyphenolic and polysaccharide compounds may require further treatment during DNA extraction to remove these and other inhibitors. High quality DNA has been extracted from sage using a lysis buffer containing activated charcoal, cetyltrimethylammonium bromide (CTAB), and polyvinylpyrrolidone (PVP) (Abu-Romman, 2011). Commercial immunomagnetic separation (IMS) kits (available for *Cryptosporidium* oocysts) and DNA extraction kits specifically designed for use in plants, soil or water may also improve detection. Employment of such treatments were not undertaken here, but should be considered, alongside less vigorous processing methods such as orbital shaking or reducing the speed of stomaching treatment, for oocyst detection in highly aromatic herbs by molecular methods.

A significant improvement in oocyst recovery was observed in this study when berries were refrigerated post-spiking compared to room temperature storage prior to processing and testing. Temperature is clearly a critical factor in maintaining the integrity of the plant tissue to ensure minimal deterioration during processing, and may also affect the integrity of the oocysts as well. Leafy greens and berries are typically refrigerated during transportation, at retail, and after purchase, so storage at 4 °C after spiking may more closely mimic conditions occurring after natural contamination with oocysts. Excessive plant debris likely interferes with the efficiency of Sheather's flotation reducing oocyst recovery. In addition, improper storage may damage plant

tissues and release PCR inhibitors which also reduce oocyst recovery. Laboratories performing testing for protozoan oocysts in produce should carefully consider sample fitness criteria and reject samples that are in poor condition (rotting, wilted, frozen, moldy, juicy, etc.) and decomposing. Such conditions may be due to improper handling during transport, extended shelf life, etc.

The recovery rates reported for this study were calculated based on a standard curve consisting of serially diluted gDNA from purified *Eimeria* oocysts. This could result in an underestimation of recovery of spiked oocysts in the produce samples since Cq values are likely higher in the presence of plant inhibitors co-extracted in the produce washes. Ideally, a standard curve consisting of enumerated surrogate oocysts such as *Eimeria* spp. in the appropriate produce wash could be used to improve quantification accuracy as it would more closely mimic the conditions of the test samples. In addition, no correction was made for the inevitable inconsistency in the exact number of oocysts spiked per test sample due to pipetting variability; which may have contributed to the high variation (SEM) sometimes observed in recovery rates. These factors contributed to a lower percent recovery of oocysts in the present study than rates reported for similar studies that used microscopic detection methods (Cook et al., 2006a; Shields et al., 2012). Despite these limitations, the qPCR MCA assay was useful for determining the relative differences between the various elution buffers and processing methods tested in this study, thus allowing for determination of the most efficient method for each type of leafy green and berry when a molecular detection method is used. In addition, knowledge of the low efficiency of recovery of protozoan oocysts from various produce types will be useful information for risk assessment when the methods are applied to surveys or trace back investigations. Further improvements to the produce wash methods to enhance oocyst recovery and increase the sensitivity of oocyst detection may be necessary since current recovery rates are low and would likely result in false negative results in a survey or disease outbreak investigation.

The qPCR MCA is a screening assay designed to simultaneously detect the presence or absence of multiple protozoan oocyst species of human health importance in several matrices (Lalonde and Gajadhar, 2011; Lalonde et al., 2013), including leafy greens and berries, and therefore has several advantages over traditional species-specific microscopy methods. Definitive identification of oocysts by microscopy alone requires significant parasitological expertise and follow-up sporulation or molecular identification, and debris from produce washes may resemble oocysts and lead to false-positive results. The qPCR products can be sequenced directly to verify the species detected and provide further information on the zoonotic potential of the contaminated sample (Lalonde et al., 2013), which is information not always available from oocysts detected by microscopy alone. The universal primer set used with the assay may also detect unknown or emerging protozoan oocysts present in produce samples which may or may not be pathogenic for humans, but could indicate fecal contamination or poor hygienic practices during processing. Thus, when combined with the specific processing methods developed here, the qPCR MCA is a useful non-subjective, high-throughput tool for routine surveillance of berries and leafy greens for protozoan oocysts. The processing procedures optimized in this study could also be used together with a variety of other DNA-based protozoan oocyst detection methods depending on the purpose of the testing program.

Conflict of interest

There is no conflict of interest.

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