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Simultaneous detection of four protozoan parasites on leafy greens using a novel multiplex PCR assay☆

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ABSTRACT

Pathogen contamination of fresh produce presents a health risk for consumers; however, the produce industry still lacks adequate tools for simultaneous detection of protozoan parasites. Here, a simple multiplex PCR (mPCR) assay was developed for detection of protozoan (oo)cysts and compared with previously published real-time PCR assays and microscopy methods. The assay was evaluated for simultaneous detection of *Cryptosporidium*, *Giardia*, *Cyclospora cayetanensis,* and *Toxoplasma gondii* followed by parasite differentiation via either a nested specific PCR or a restriction fragment length polymorphism (RFLP) assay. Spiking experiments using spinach as a model leafy green were performed for assay validation. Leaf-washing yielded higher recoveries and more consistent detection of parasites as compared with stomacher processing. Lowest limits of detection using the nested mPCR assay were 1–10 (oo)cysts/g spinach (in 10 g samples processed), and this method proved more sensitive than qPCR for parasite detection. Microscopy methods were more reliable for visual detection of parasites in lower spiking concentrations, but are more costly and laborious, require additional expertise, and lack molecular confirmation essential for accurate risk assessment. Overall, the nested mPCR assay provides a rapid (< 24 h), inexpensive ($10 USD/sample), and simple approach for simultaneous detection of protozoan pathogens on fresh produce.

1. Introduction

Consumer demand for “ready to eat” fresh produce has been consistently rising as people modify dietary practices to eat more healthy foods without investing time in preparation. This economic reality has consequences for food safety for the simple reason that the shelf life of fresh foods like leafy vegetables is short, implying time from field to fork can facilitate transmission of pathogenic organisms present in soil or water that has contaminated crops. Water and foodborne pathogens currently represent the most important cause of enteric illness, with diarrhea representing the most prevalent infectious illness worldwide (Neira and Pruss-Ustun, 2016). Pathogens can be introduced on fresh produce during cultivation through contact with contaminated irrigation water (Amoros et al., 2010; Mota et al., 2009; Thurston-Enriquez et al., 2002), soil or fertilizer, as well as via handling and downstream processing and packaging steps. Current industry practices for monitoring microbial quality either focus on bacterial indicators that are poorly correlated with the presence of diverse classes of pathogens, or rely on the detection of specific bacterial pathogens (e.g., *Salmonella* and *Listeria*) after cultivation to ascertain food safety. Protozoan pathogens, on the other hand, are rarely tested for in fresh produce. Yet protozoan contamination of fresh produce is of growing importance – in both advanced and emerging market economies (Caradonna et al., 2017; Dixon, 2016; Lalonde and Gajadhar, 2016).

Four key foodborne protozoan pathogens, including *Cryptosporidium*, *Giardia*, *Cyclospora cayetanensis,* and *Toxoplasma gondii,* were targeted in this study due to their current or projected capacity to cause significant illness in fresh produce consumers. *Cyclospora cayetanensis* and *Cryptosporidium* spp. are currently considered the most important protozoan pathogens, based on the number of disease outbreaks in people that have been associated with contaminated fresh produce (Ramos et al., 2013). A multistate outbreak of

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☆ Author Note: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

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cyclosporiasis (with 511 laboratory-confirmed cases) recently reported in the United States (2018) was linked to ready-to-eat salad mix sold at fast-food restaurants (CDC, 2018). Also, an outbreak of *C. cayetanensis* in 2017 was implicated in more than 1000 cases in the United States (CDC, 2017). While a common food source was not implicated in the 2017 outbreak, previous outbreaks were associated with consumption of salad mix and cilantro from Mexico (Buss et al., 2016). Scores of previous outbreaks have been associated with raspberries, basil, snow peas, and lettuce imported from central and South America (Ramos et al., 2013). This pathogen has been previously associated with tropic and subtropical climates, but the rising occurrence of cyclosporiasis in North America demonstrates how increasing demands for imported produce can lead to emergence of pathogens in new regions. Unlike *C. cayetanensis* which is a human pathogen, zoonotic transmission of *Cryptosporidium* can occur with oocysts (environmental state of the parasite) originating from either human or animal feces. Numerous gastrointestinal disease outbreaks have been attributed to produce contaminated with *Cryptosporidium* spp., most notably associated with consumption of apple cider, lettuce, onion and green onion (Dixon, 2016). Like *Cryptosporidium*, *Giardia* spp. can be shed by many animal hosts as well as humans. This protozoan can also cause gastrointestinal disease in infected people, and contamination of produce has been documented widely, including on leafy vegetables (Dixon et al., 2013).

The zoonotic protozoan *Toxoplasma gondii* is unique among foodborne parasites because its only known definitive hosts are felids (Hutchison et al., 1969). Infection with this protozoan is widespread in humans worldwide, and consumption of raw fruits and vegetables is a significant risk factor for acquiring *T. gondii* (Jones and Dubey, 2012). An outbreak of toxoplasmosis in Brazil was associated with the consumption of green vegetables in 2009 (Ekman et al., 2012), and more recently with the consumption of Acai juice in 2016 (Morais et al., 2016). Recent reports note that the oocyst stage of this parasite poses a larger than previously recognized health risk to humans (Boyer et al., 2011; Mangiavacchi et al., 2016), with a single oocyst sufficient to cause infection in susceptible hosts (Dubey et al., 1996; Torrey and Yolken, 2013). An additional unique feature of *T. gondii* is the parasite's lifelong persistence in muscle and brain tissues, with the potential for causing severe disseminated disease and even death later in life (Dubey and Beattie, 1988). Non-fatal but serious sequelae due to toxoplasmosis include ocular disease and severe birth defects, and an association with psychiatric disorders has also been reported (Bahia-Oliveira et al., 2017). Thus, although reports of foodborne outbreaks are uncommon (likely due to the lack of acute symptoms in most infected individuals (Dubey and Beattie, 1988)), the serious and life-long health implications of *T. gondii* infection warrant that sensitive and efficient methods for detection of this pathogen on produce be developed.

Despite the risk of exposure to protozoan parasites through consumption of fresh produce, standardized methods are only currently available for *Cryptosporidium* and *Giardia* and require time-consuming techniques that are conducted by specialized laboratories. The overall objective of this study was to develop a new multiplex polymerase chain reaction (mPCR) assay that can simultaneously detect and differentiate the presence of four protozoan pathogens in a rapid, accurate and affordable manner. The detection limits of the newly developed multiplex PCR assay were assessed in systematic laboratory spiking experiments using spinach as a model leafy green vegetable. Spinach was selected as a model leafy green based on a report from the World Health Organization recognizing this product as one of several fresh produce commodities requiring ‘highest priority’ for addressing food safety issues on a global scale (FAO/WHO, 2008). Results of the mPCR assay performance were further compared with commercially available immunoassays and microscopy-based methods, as well as with previously reported real-time quantitative PCR (qPCR) assays.

2. Materials and methods

2.1. Parasite preparations

Live *Cryptosporidium parvum* (*C. parvum*) oocysts (Iowa isolate) were purchased from Sterling Parasitology Laboratory at University of Arizona (Tucson, AZ, USA). Live *Giardia enterica* cysts (H3 isolate) were purchased from Waterborne™ Inc. (New Orleans, LA, USA). The nomenclature for *G. enterica* has been proposed specifically for *Giardia duodenalis* (synonyms *intestinalis* or *lamblia*) Assemblage B (Thompson and Monis, 2012), and therefor this species name will be referred to herein. *Toxoplasma gondii* (*T. gondii)* oocysts (Type II) and *Cyclospora cayetanensis* (*C. cayentanesis*) oocysts were generously provided by David Arranz Solís at University of California, Davis, USA, and Michael Arrowood at the Centers for Disease Control and Prevention (CDC), USA, respectively. Collectively, when referring to oocysts of *Toxoplasma*, *Cyclospora*, and *Cryptosporidium,* and cysts from *Giardia*, the term (oo)cysts is used herein. To inactivate (oo)cysts for reducing exposure risk to laboratory personnel, viable (oo)cysts suspended in 1 mL PBS in microcentrifuge tubes were placed in a dry heating block at 80 °C for 3 min (*C. parvum*), 70 °C for 15 min (*G. enterica*), or 80 °C for 20 min (*T. gondii*). The selected inactivation protocol for each parasite followed published reports for loss of parasite infectivity in vivo (Travaille et al., 2016) combined with observation of intact parasite walls conducted in our laboratory. Due to lack of heat inactivation studies reported for *C. cayetanensis,* combined with an observed loss of oocyst wall integrity following heat treatments, *C. cayetanensis* oocysts were applied with no inactivation procedure.

2.2. Multiplex PCR (mPCR) assay development and validation

2.2.1. Nucleic acid extraction

Ten-fold serial parasite dilutions (10-10,000 (oo)cysts) were prepared after enumerating stock solutions using a hemocytometer chamber and visualization under light microscopy. DNA was extracted from 100 μL (oo)cyst aliquots using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) with slight modifications (Adell et al., 2014; Shapiro et al., 2010). In brief, (oo)cysts were mixed with 180 μL of ATL buffer and subjected to one freeze-thaw cycle (4 min in liquid nitrogen and 4 min in boiling water). Proteinase K (40 μL) was then added and samples were incubated overnight at 56 °C. The nucleic acids were eluted from membrane columns with 50 μL (mPCR), or 100 μL (qPCR) of AE buffer (10%).

2.2.2. Multiplex PCR

A nested multiplex PCR (mPCR) assay was developed by designing a new primer set (forward and reverse) to simultaneously amplify a target region of the 18S ribosomal RNA (rRNA) gene of *C. parvum, T. gondii,* and *C. cayentanesis* (Table 1). The m18S primer set was then multiplexed with a previously published primer set targeting the glutamate dehydrogenase (GDH) gene of *G. enterica* (Read et al., 2004) in one external PCR reaction. The GDH assay was selected after initial attempts to multiplex primers targeting the 18S rRNA region of *G. enterica* were not successful, due to the significantly higher GC content in this protozoan. The GDH primers developed by Read et al. (2004) have been previously shown to amplify several different assemblages of *Giardia*, including Assemblages A and B that are most relevant for human infections (Read et al., 2004). To discriminate the four parasites via internal nested reaction, three internal primer sets specifically targeting *C. parvum, T. gondii,* and *C. cayentanesis* were designed within the external m18S amplicon (Table 1), while nested GDH primers were used for *Giardia enterica* (Read et al., 2004).

Several PCR assay conditions including primer concentrations (20, 30, 40, and 50 pmol/μL of reaction), annealing temperature (56 °C, 58 °C, 59 °C, 60 °C, and 62 °C), cycle numbers (20, 25, 30, and 35) and template DNA volume (2 and 5 μL) were evaluated for assay

Table 1
Primer sets used in nested multiplex PCR (mPCR) for simultaneous detection of *Cryptosporidium parvum*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Giardia enterica*.

Protozoa	Target gene	Primer	Direction	Nucleotide sequence (5' – 3')	Amplicon size (bp)	Reference
External reaction						
Cryptosporidium *T. gondii* *C. cayetanensis*	18S[a]	m18SeF	Forward	CGGGTAACGGGGAATTAGGG	751–779	This study
		m18SeR	Reverse	TCAGCCTTGCGACCATACTC		
Giardia	GDH[b]	GDHeF	Forward	TCAACGTYAAYCGYGGYTTCCGT	455	Read et al., (2004)
		GDHiR	Reverse	GTTRTCCTTGCACATCTCC		
Internal reaction						
Cryptosporidium	18S[a]	m18ScryF	Forward	TGGAATGAGTTAAGTATAAACCCCT	543	This study
		m18ScryR	Reverse	GCTGAAGGAGTAAGGAACAACC		
T. gondii	18S[a]	m18StoxF	Forward	GGTGTGCACTTGGTGAATTCTA	405	This study
		m18StoxR	Reverse	TGCAGGAGAAGTCAAGCATGA		
C. cayetanensis	18S[a]	m18ScycF	Forward	TCGTGGTCATCCGGCCTT	359	This study
		m18ScycR	Reverse	TCGTCTTCAAACCCCCTACTG		
Giardia	GDH[b]	GDHiF	Forward	CAGTACAACTCYGCTCTCGG	432	Read et al., (2004)
		GDHiR	Reverse	GTTRTCCTTGCACATCTCC		

[a] 18S small subunit (ssu) ribosomal RNA (rRNA) gene.
[b] GDH: glutamate dehydrogenase gene.

optimization. Optimal conditions were obtained for detecting the lowest parasite concentrations using the following settings: internal and external PCR reactions (final volume 50 µL) contained 1x PCR buffer 1 with $MgCl_2$ (15 mM), 1.5 U of AmpliTaq® DNA polymerase (Applied Biosystems™, CA, USA), 0.2 µM (external) or 0.5 µM (internal) of each primer, 0.2 mM of deoxynucleotide (dNTP) (Invitrogen, CA, USA), 3.2 µg/µL of bovine serum albumin (BSA) (Calbiochem, CA USA), and 5 µL of genomic DNA for external reaction or 2 µL of external amplicon for internal reaction as DNA template. PCR conditions for the external reactions consisted of an initial denaturation at 94 °C for 3 min followed by 35 cycles at 95 °C for 40 s (denaturation), 58 °C for 40 s (annealing), and 72 °C for 90 s (extension), with a final extension step of 72 °C for 4 min. PCR conditions for the internal reactions were similar to those of the external reaction except for the annealing temperature, which was increased to 59 °C for *G. enterica, T. gondii* and *C. cayetanensis* and to 60 °C for *C. parvum.* The nested amplification products were visualized in a 2% agarose gel containing ethidium bromide using an ultraviolet (UV) transilluminator.

The sensitivity (limit of detection (LOD)) of the mPCR assay was tested first on individual parasite stocks, followed by testing a mixture of all four protozoan parasites prepared in 10-fold serial dilutions ranging from 10 to 1,000 (oo)cysts (Table 2). The identity of parasite amplicons of the expected sizes on agarose gels was confirmed via sequence analysis. Briefly, amplified products were purified using a QIAquick gel extraction kit (Qiagen, CA, USA) and the samples were submitted to the UC Davis core DNA Sequencing Facility. The forward and reverse sequences were aligned using a multiple sequence comparison by log-expectation (GENEIOUS software, Auckland, New Zealand), and the consensus sequence was then compared with GenBank reference sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The specificity of each set of internal primers was tested on the three other target protozoa using individual parasite DNA as template. Virtual alignment of all primer sets was also tested for specificity against additional protozoan parasites using the Primer Testing Tool in Geneious R11 (Biomatters Ltd., NJ, USA). Specific parasites and corresponding GenBank accession numbers tested were as follows: *Neospora caninum* (GQ899206), *Hammondia heyroni* (KT184370), *Sarcocystis neurona* (U07812), *Eimeria bovis* (AB769578), and *Cystoisospora canis* (KT184368). Specificity of all primers was also tested for alignment with the selected leafy green used in spiking experiments – spinach (L24420) – as well as with *Candida albicans* (AF114470), a yeast commonly present in animal (including human) feces.

2.2.3. *Restriction fragment length polymorphism (RFLP)*

A second approach for simultaneous differentiation of the four selected protozoans was evaluated using a newly developed RFLP assay. The first step of this approach was performing the external mPCR assay described above, simultaneously amplifying DNA from all four targets. In the second step, amplicons were subjected to digestion using the BsaBI restriction enzyme (New England BioLabs Inc., MA, USA). The resulting digestion patterns allowed for the differentiation of each protozoan parasite due to unique cleaving patterns, even in the case of multiple parasites being present in a single contaminated batch of leafy greens (Table S1). Briefly, 5 µL of external amplicon DNA was combined with 0.5 µL BsaBI restriction enzyme, 2 µL 10X CutSmart® Buffer (New England BioLabs Inc., MA, USA) and 12.5 µL sterile PCR grade water. Samples were digested at 60 °C for 1 h followed by 20 min of incubation at 80 °C for enzyme inactivation. Digested DNA products were visualized in a 2% agarose gel containing ethidium bromide using a UV transilluminator.

Table 2
Amplification of parasites by conventional multiplex PCR (mPCR) assay performed as a simplex nested assay on individual parasites or as a multiplex assay on a mixture of four target protozoan parasites. Assay limits of detection (ALODs) are indicated in bold.

(Oo)cyst/extract	(Oo)cyst/reaction	Successful amplification/replicates tested							
		Cryptosporidium parvum		*Toxoplasma gondii*		*Cyclospora cayetanensis*		*Giardia enterica*	
		Simplex	Multiplex	Simplex	Multiplex	Simplex	Multiplex	Simplex	Multiplex
1000	100	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
100	10	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10	1	3/3	**1/3**	3/3	**1/3**	**3/3**	**1/3**	**2/3**	**3/3**
1	0.1	**1/3**	0/3	**3/3**	0/3	0/3	0/3	0/3	0/3
0	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

2.2.4. Quantitative PCR (qPCR)

Quantitative PCR (qPCR) assays for each parasite were selected from previously published literature (Guy et al., 2003; Hill et al., 2007; Opsteegh et al., 2010; Shields et al., 2013). The oligonucleotides of primers and probes of each qPCR assay and their amplicon sizes are indicated in Table S2. Each 25-μL qPCR reaction mixture contained 10 μL of template DNA, final concentration of 1x TaqMan® Environmental Master Mix 2.0 (Applied Biosystems™, CA, USA), and final concentrations of each primer and probe as previously reported (Table S2). The thermal cycling conditions included a pre-incubation step of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles (45 cycles for *Cyclospora*) of 95 °C for 15 s and 55 °C (*Cryptosporidium*) or 60 °C (all the other protozoa) for 1 min. To prepare standard curves, serial dilutions of parasite DNA extracted from known numbers of (oo)cysts were used, and the assay limit of quantification (ALOQ) was determined on the standard curve using the lowest concentration where at least 4 of 8 replicates were amplified (Table S3) (Schriewer et al., 2013). For sample calculation, concentrations detected in the total thermal cycles that were less than the ALOQ were defined as detected but not quantifiable (DNQ).

2.3. Microscopy-based methods: immunomagnetic separation (IMS) - direct fluorescent antibody test (DFA) and membrane filtration

IMS was performed using Dynabeads™ GC-Combo (Applied Biosystems, CA, USA) to separate *C. parvum* and *G. enterica* (oo)cysts from sample pellets following manufacturer's recommendations. The final suspensions of 100 μL were transferred to 3-Well SuperStick™ Slides (Waterborne, Inc., LA, USA) and air-dried for 24 h followed by DFA staining using fluorescein isothiocyanate (FITC)-conjugated antibody and DAPI (4′6′-Diamidino-2-Phenylindole, Dihydrochloride) (EasyStain™, BTF Precise Microbiology, Inc., PA, USA) per manufacturer's instructions. (Oo)cysts were identified with a Zeiss Axioskop epifluorescence microscope equipped with a FITC filter set using the following criteria: *C. parvum* oocysts were enumerated when spherical to elliptical organisms of 4–6 μm diameter outlined in apple green were observed with up to 4 blue-staining nuclei, and as *Giardia* when oval cysts with a diameter of 9–14 μm were outlined with apple green and contained 2-4 blue-staining nuclei.

Toxoplasma gondii oocysts were recovered from the supernatant during the IMS procedure after the initial incubation of *C. parvum* and *G. enterica* (oo)cyst with magnetic beads. Membrane filtration was conducted to concentrate the oocysts on a 25-mm mixed cellulose hydrophilic membrane filter with a 5-μm pore size (Millipore, MA, USA). The entire filter was scanned using a Zeiss Axioskop epifluorescent microscope equipped with a UV emission filter set. The numbers of sporulated *T. gondii* oocysts were enumerated as previously described (Shapiro et al., 2010). Quantification of *Cyclospora* using microscopy is not reported here due to the lack of previously published systematic validation for using membrane filtration followed by UV excitation for quantifying oocysts from this protozoan parasite.

2.4. Application of mPCR for simultaneous detection of protozoan parasites on leafy greens

Laboratory spiking experiments were conducted using spinach as a model leafy green vegetable, and two treatments were compared for parasite recoveries including i) a wash procedure (Experiment 1a), and ii) a stomacher instrument (Experiment 2) (Fig. 1). After initial results demonstrated higher parasite recoveries using the washing technique, a second wash experiment (Experiment 1b) was conducted with added replicates to increase statistical power for evaluating the sensitivity of the mPCR assay when applied on leafy greens.

In all experiments, spinach samples were prepared by placing 10 g of spinach purchased from a local grocery store in individual weighing dishes (Fisher Scientific, CA, USA). Ten-fold serial dilutions of parasite mixture (10-10,000 (oo)cysts) were spiked on spinach by pipetting numerous small droplets (5 μL) onto the surface of leaves. A parasite-free PBS solution was used as a negative control. Three replicates (Experiment 1a and Experiment 2) or five replicates (Experiment 1b) were included at each parasite mixture concentration. Various drying times were initially tested, and a 2-h incubation time at room temperature was determined optimal for complete drying without visual wilting of the leaves. After the 2-h incubation, samples were placed in Whirl-pak® bags (Nasco, WI, USA). In Experiments 1a and 1b (washing technique), spinach leaves were placed in an 18-oz Whirl-pak® bag with 100 mL of 0.1% Tween 80 and subjected to manual wash for 2 min by externally rubbing leaves back and forth within the wash solution of the bag. In Experiment 2 (stomacher), spinach leaves were placed in a 24-oz filtered Whirl-pak® bag with 40 mL of 0.1% Tween 80 and mechanically agitated using Smasher™ Blender/Homogenizer (bioMérieux, NC, USA) at FAST mode for 2 min. The eluent solution from each spinach sample was transferred to Falcon tubes and centrifuged at 3,000 × g for 15 min at 4 °C. In Experiment 2, an additional 40 mL of 0.1% Tween80 was applied to rinse the filter. After centrifugation, supernatant was aspirated to reserve approximately 3 mL of pellets for each sample, which was subsequently divided into 3 microcentrifuge tubes for individual detection assays: 1) the newly developed multiplex nested PCR (mPCR), 2) qPCR assays, and 3) microscopy-based methods including IMS-DFA and membrane filtration. For nucleic acid extraction, the 1-ml (oo)cyst suspensions were further centrifuged at 14,000 × g for 5 min to retain a 100-μL pellet. The remainder of the parasite detection procedures were conducted for each method as described above.

2.5. Quality control

To minimize and monitor cross-contamination during sampling handling and analysis, multiple quality control measures were applied. Blank spikes using PBS without target parasites were used in all spiking experiments to assess potential cross-contamination during parasite spiking and processing. Extraction and PCR reagent negative controls were processed through the entire analytical procedure in molecular analysis and included in every experiment. PCR positive controls consisted of target parasite DNA from 1,000 (oo)cyst stock solutions.

2.6. Data analysis

The detection limits of mPCR assays on spinach were determined by the lowest positive amplification demonstrated for every experiment. Recoveries of target protozoa in the spinach spiking experiments were also estimated using qPCR and microscopy methods by dividing the detected parasite numbers by the number of spiked parasites. Recoveries of protozoan pathogens derived from parasite enumeration via microscopy-based methods were compared between Experiments 1a and 1b (wash) and Experiment 2 (stomacher) using a Mann Whitney test statistic with significance level set at $P \leq 0.05$. The probability of detecting spiked protozoan parasites on spinach was derived by fitting binomial detection data from the mPCR results to a logistic regression model. Each regression model was evaluated using the Hosmer-Lemeshow goodness-of-fit test. Regression coefficients were then used to estimate the probability of parasite detection across a range of potential (oo)cyst contamination levels per 10 g of spinach. All statistical analyses were performed using STATA software (StataCorp LLC, TX, USA).

3. Results

3.1. Development of multiplex-PCR

The mPCR assay limits of detection (ALODs) when applied on parasite mixtures were 1 (oo)cyst per reaction for all targeted protozoan

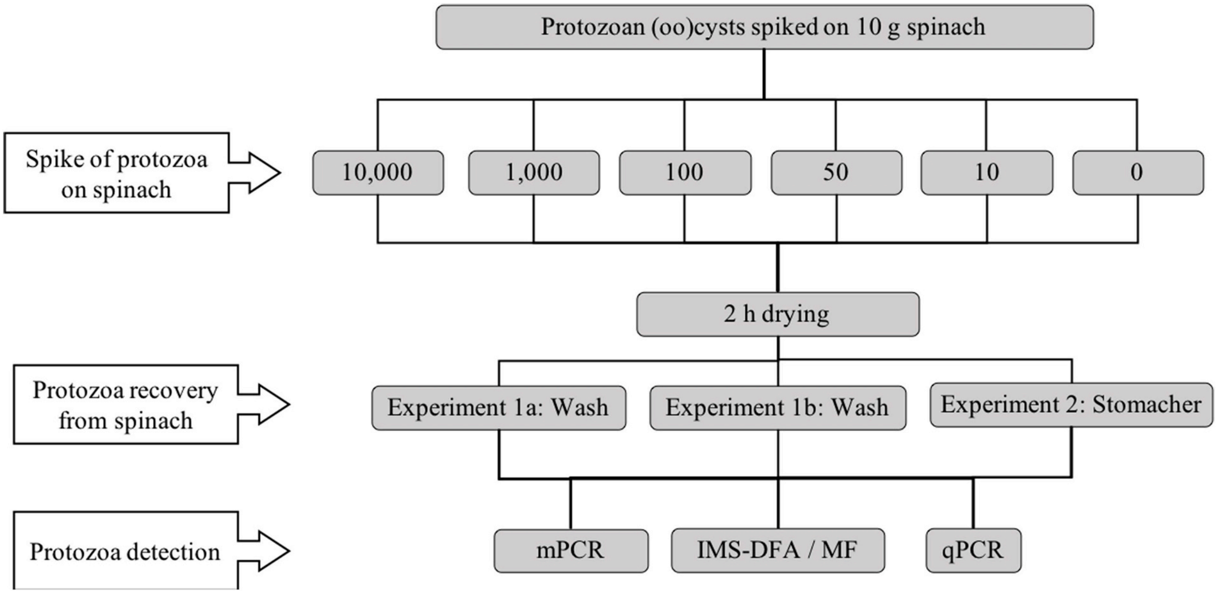

Fig. 1. Experimental study design for evaluating the performance of a newly designed multiplex PCR (mPCR) assay for simultaneous detection of protozoan parasites (*Cryptosporidium, Giardia, Toxoplasma gondii, and Cyclospora cayetanensis*) on spinach. Parasite recovery was initially assessed by comparing a wash technique (Experiment 1a, n = 3 for each dilution) vs. stomacher processing (Experiment 2, n = 3 for each dilution) of spiked spinach. A second wash experiment (1b) was then performed with more replicates (n = 5) to increase statistical power for estimating assay sensitivity. Performance of the nested mPCR assay was compared to parasite detection using real-time PCR (qPCR) and microscopy methods, including immunomagnetic separation (IMS) and direct fluorescence antibody (DFA) staining for *Cryptosporidium* and *Giardia*; and membrane filtration (MF) for *T. gondii* enumeration.

parasites (Table 2 and Fig. S1). ALODs were similar or lower (more sensitive) when each parasite was tested separately using the same primers but using a simplex (single primer set) approach. All amplification products from multiplex PCR assays were validated via sequence analysis. When evaluated for assay specificity, internal PCR products (359-543 bp; Table 1) were only present for the specific parasite targeted with each set of internal pathogen-specific primer set (Fig. S2). Virtual alignment of external and internal m18S primer sets with related protozoan parasites, spinach, and yeast demonstrated expected amplification of these organisms with the external, non-specific m18S primers. The internal primers did not align with sequences of these organisms (even when allowing for up to 5 mismatched base pairs), with the exception of *S. neurona*, *N. caninum* and *H. heydorni* which aligned with the *T. gondii* internal m18S primer set. When the sequences of these three apicomplexan parasites were aligned, parasites could be distinguished from *T. gondii* via the presence of single nucleotide polymorphisms (SNPs) – (96–99% identity).

Using the RFLP assay, the digestion of amplicons obtained from the external reaction of the mPCR assay yielded distinct banding patterns for each protozoan parasite, enabling differentiation of these protozoa (Fig. S3). While adequate parasite discrimination was obtained, further experimentation was not performed with RFLP assays due to a one- to three-log reduction in parasite detection sensitivity (Table S4).

3.2. Application of multiplex-PCR for protozoan pathogen detection on leafy greens

When the newly developed nested mPCR test was applied on spiked spinach leaves, *C. parvum, T. gondii* and *C. cayetanensis* could be detected at a concentration of 10 oocysts per 10 g of spinach, while *G. enterica* amplified only as low as 50 and 10,000 cysts per 10 g of spinach in Experiments 1 and 2, respectively (Table 3). Compared with stomacher processing (Experiment 2), manual washing (Experiments 1a and 1b) of spinach yielded equivalent or higher detection and recoveries of (oo)cysts, as measured by mPCR and microscopy methods, respectively (Figs. 2 and 3). Using probability curves for estimating the likelihood of parasite detection under a range of theoretical contamination levels, results further supported the washing technique as a more sensitive approach for detection of parasites as compared with processing spinach through a stomacher. Using *T. gondii* as an example, oocysts could be detected with 90% certainty at contamination levels of 10 or 100 oocysts per 10 g spinach, using either washing or a stomacher to recover parasites from leaves, respectively (Fig. 2). Applying probability curves to all four protozoan parasites demonstrated that when using manual washing, successful detection of parasites is expected to occur with 90% certainty when contamination levels on 10 g of spinach are 10, 35, 45 and 55 (oo)cysts for *T. gondii, C. parvum, G. enterica,* and *C. cayetanensis*, respectively (Fig. 4).

Parasite recoveries measured by microscopy (IMS-DFA/membrane filtration) and qPCR assays demonstrated especially poor detection of *G. enterica* in Experiment 2, where spinach was processed using a stomacher (10% median recovery via IMS-DFA (Fig. 3, Table S5)); and parasites were not detectable at all via qPCR (Table S6). The microscopy-based methods yielded detection of protozoa in nearly all replicates across all three experiments (Fig. 3). The exception was in Experiment 2 for *G. enterica*, where IMS-DFA yielded visualization of cysts in 1 of 3 replicates at the 10 and 50 dilutions levels, and in 2 of 3 replicates at the 1000 dilution level. For the wash experiments (1a and 1b), median recoveries measured by IMS-DFA were 31–38% for *G. enterica*, and 46–47% for *C. parvum*; and via membrane filtration recoveries were 22–43% for *T. gondii* (Table S5).

4. Discussion

Systematic validation studies of detection methods for foodborne pathogens are imperative for providing adequate tools to agencies and laboratories invested in screening food commodities for harmful microorganisms. The present study describes a nested multiplexed PCR (mPCR) assay for simultaneous detection and discrimination of *C. parvum, G. enterica, C. cayetanensis, and T. gondii*, validated using spinach as a model leafy green vegetable. Systematic spiking studies further demonstrated that manual washing of leaves yielded higher recoveries and more consistent detection of parasites as compared with stomacher processing. Compared with a nested mPCR approach, the

Table 3

Detection of protozoan pathogens using nested multiplex PCR (mPCR) in three spinach spiking experiments: Experiments 1a and 1b were performed by washing spinach (10 g), while Experiment 2 used a stomacher instrument to process leaves (10 g). Bolded dilution levels indicate the lowest concentration in which parasites were detected in one or more replicates. Negative control treatments did not produce amplification using mPCR.

(Oo)cysts/dilution	(Oo)cysts/extract[a]	No. detected/no. replicates											
		Cryptosporidium parvum			*Toxoplasma gondii*			*Cyclospora cayetanensis*			*Giardia enterica*		
		Experiment			Experiment			Experiment			Experiment		
		1a	1b	2	1a	1b	2	1a	1b	2	1a	1b	2
10,000	3,333	3/3	NA[b]	3/3	3/3	NA	3/3	NA	NA	NA	3/3	NA	**3/3**
1,000	333	3/3	5/5	3/3	3/3	5/5	3/3	3/3	5/5	3/3	3/3	4/5	0/3
100	33	3/3	4/5	3/3	2/3	5/5	2/3	2/3	2/5	2/3	2/3	1/5	0/3
50	17	**2/3**	2/5	3/3	**3/3**	4/5	1/3	**1/3**	1/5	0/3	**2/3**	**1/5**	0/3
10	3	NA	**1/5**	**1/3**	NA	**3/5**	**3/3**	NA	**1/5**	**1/3**	NA	0/5	0/3

[a] Samples were divided in three for detection via i) mPCR; ii) quantitative PCR; and iii) microscopy-based methods including immunomagnetic separation – direct fluorescence antibody (IMS-DFA) staining for *C. parvum* and *G. enterica*; and membrane filtration (MF) for *T. gondii*.

[b] NA = Not assessed. Spike dilutions ranged from 50 to 10,000 per 10 g spinach (Experiment 1a) and 10 to 1,000 per 10 g spinach (Experiment 1b). The highest oocyst concentration of *Cyclospora* was 1,000 oocysts due to limited oocyst availability.

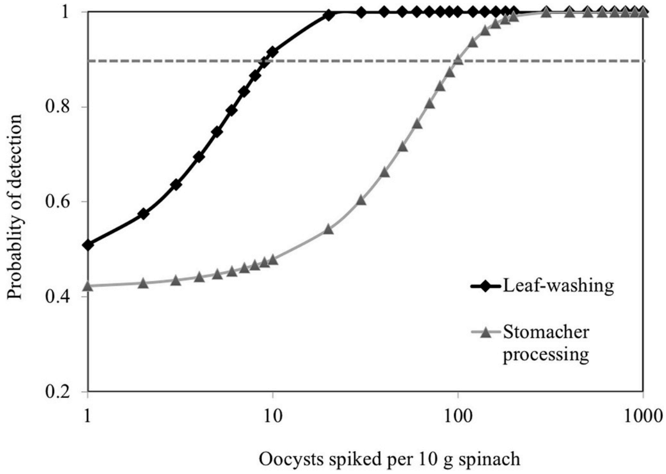

Fig. 2. Probabilities of detecting *Toxoplasma gondii* oocysts spiked on 10 g of spinach in Experiment 1b (leaf washing) and Experiment 2 (stomacher-processing). Probability curves were fitted using logistic regression coefficients obtained from the multiplex PCR parasite detection data. The dashed line indicates the 90% probability of parasite detection. A 10-fold increase in sensitivity was obtained at the 90% likelihood of detection (dashed line) threshold - with 10 oocysts per 10 g spinach detected when spinach was washed vs. 100 oocysts per 10 g spinach when leaves were processed using a stomacher instrument.

mPCR-RFLP method was approximately one to three orders of magnitude less sensitive for protozoan pathogen detection. The relative ease of execution, affordability, and speed of the mPCR assay for protozoan pathogen detection offers an important advance to the field of food safety and security.

In initial experiments (Experiment 1a and Experiment 2) two different methods were compared to evaluate parasite recovery from spiked spinach leaves: a manual leaf wash vs. stomacher instrument that beats material to loosen any adhered particles, such as pathogens. All detection methods (microscopy-based, mPCR and qPCR) yielded more consistent detection or higher recoveries of parasites when manual washing was applied, and this was particularly notable for *G. enterica*. Processing spinach with a stomacher resulted in more spinach tissue debris in the elution buffer during treatment, which may have resulted in increased inhibition of DNA amplification during PCR. The fine debris may have also adversely affected the recovery of the relatively larger *Giardia* cysts (12–15 μm) from solid mashed leaves to eluted solution through the filter mesh in the Whirl-pak® filter bags (pore diameter 330 μm). Additionally, *G. enterica* cysts are less environmentally resistant than *T. gondii* and *C. parvum* oocysts; thus, the physical action of the stomacher on the spinach leaves may have crushed the cyst walls – damaging their physical morphology for IMS-DFA recovery and/or DNA integrity for PCR amplification.

The current study design allowed comparison of protozoa detection in spinach among three different detection methods: the newly developed mPCR, previously published qPCR assays, and microscopy methods based either on IMS-DFA (*C. parvum* and *G. enterica*) or membrane filtration (*T. gondii*). Compared with qPCR, more sensitive detection of protozoa was achieved via mPCR for simultaneous detection of *C. parvum, T. gondii, C. cayetanensis*, and *G. enterica* (Tables 3 and S7). While qPCR performed similarly to mPCR for detection of *C. parvum* and *T. gondii,* it failed to detect *C. cayetanensis*, and *G. enterica* at lower spiking concentrations. Poor results for *G. enterica* were not entirely unexpected, given the relatively high qPCR assay limit of detection (ALOD) for this protozoan (16 cysts), as compared with qPCR ALOQs for the other protozoa (1-4 oocyst). The high limit of detection of the RFLP approach for discriminating parasites (100-1,000 (oo)cysts per reaction) was deemed too insensitive for application in food safety efforts. Therefore, this method was not included in the spinach spiking experiments.

The finding that microscopy methods were able to identify parasites in nearly all samples from all three experiments should not be understated. While the IMS-DFA method is expensive (~$US 88 per sample), in cases where sensitive detection of parasites outweighs cost considerations (in resource-abled regions or in an academic setting), this approach should still be considered for sensitive detection of protozoa. For *T. gondii*, the microscopy approach is also very affordable (~$2 per sample as no proprietary reagents are needed). However, for conclusive molecular identification, any samples with observed parasites should ideally be subjected to PCR and sequence confirmation. Coupling IMS-PCR or IMS-qPCR methods is also an option when additional cost and time can be accommodated (Hohweyer et al., 2016). For *C. cayetanensis*, validated immunoassays are not currently available.

In addition to confirming the specificity of the newly designed m18S primers among the four protozoans targeted in this study, other closely related coccidia were evaluated for potential amplification using primer alignment tools. While most coccidia, yeast and spinach DNA did not align with most primer sets, the *T. gondii* internal m18S primers were predicted to anneal with *S. neurona, N. caninum* and *H. heydorni* – although sequence analysis would enable parasite discrimination from *T. gondii*. Environmental contamination with *N. caninum* and *H. heydorni* has not been previously reported, though both would indicate contamination with canid (dog or coyote) feces (Dubey et al., 2002).

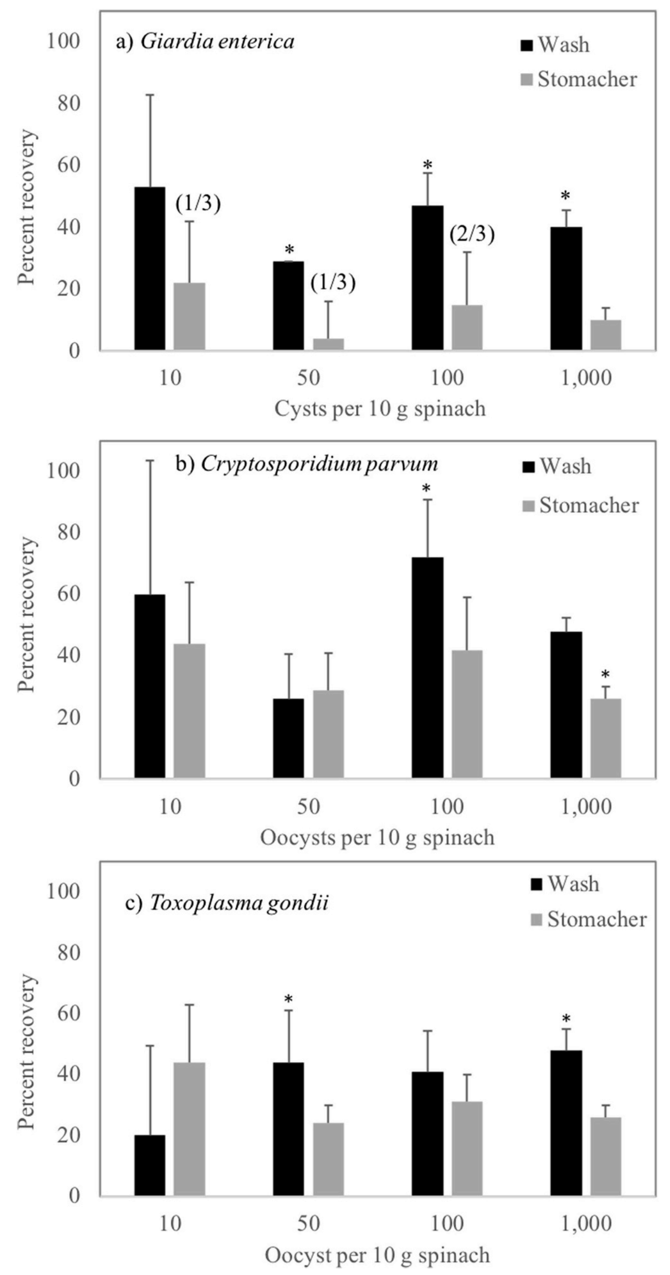

Fig. 3. Percent recoveries of *Giardia enterica* (panel "a"), *Cryptosporidium parvum* (panel "b"), and *Toxoplasma gondii* (panel "c"), as determined via microscopy methods in spinach spiking experiments with leaves processed either by washing (Experiment 1b) or via a stomacher instrument (Experiment 2). Immunomagnetic separation (IMS) followed by direct fluorescence antibody (IFA) staining was used to enumerate *G. enterica* and, *C. parvum*, while membrane filtration (MF) was used to enumerate *T. gondii* oocysts. Significance (*) is indicated where significantly greater recoveries were obtained using the wash method as compared with a stomacher (Mann Whitney Test $P \leq 0.05$). Parenthesis indicate number of detects per number of replicates in cases where not all replicates had parasites visualized.

Because unknown environmental organisms can also yield amplicons consistent with positive controls – even for assays targeting pathogen-specific genes such as the *T. gondii* RE element or B1 (Shapiro et al., 2015) – it is recommended that sequence confirmation of suspect positives be always performed for conclusive molecular identification.

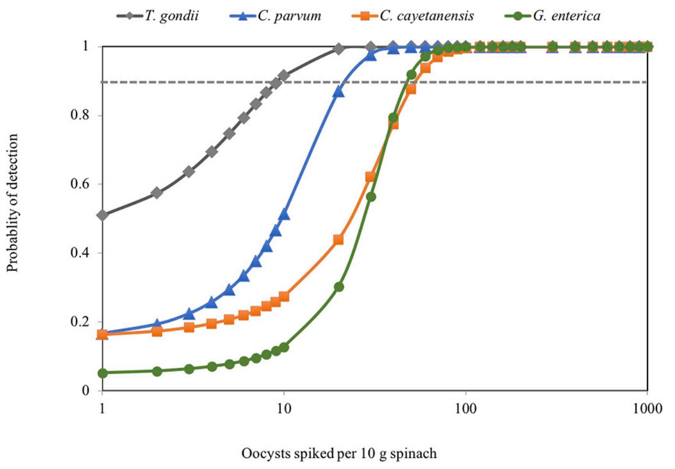

Fig. 4. Probabilities of detecting four target protozoa when recovery of spiked parasites was performed via washing (Experiment 1b). Probability curves were fitted using logistic regression coefficients obtained from the multiplex PCR parasite detection data. The dashed line indicates the 90% probability of parasite detection. Per 10 g of spinach, parasites could be detected with 90% likelihood at the following contamination levels: *Cyclospora cayetanensis,* 55 oocysts; *Giardia enterica,* 45 cysts; *Cryptosporidium parvum,* 35 oocysts; and *Toxoplasma gondii,* 10 oocysts.

Despite increasing numbers of publications on detection methods for protozoa on food matrices, there is a scarcity in systematic spiking studies that simultaneously tested for multiple parasites, compared different detection assays, and reported method limits of detection when applied on fresh vegetables. Detection of *C. parvum* and *G. enterica* was first reported by Robertson and Gjerde (2000), where IMS-DFA was applied for detection of parasites on leafy greens, strawberries and bean sprouts (Table 4). Cook et al. (2006, 2007) also reported the application of IMS-DFA for *C. parvum* and *G. enterica* detection on a variety of vegetables. However, limits of detection in these prior studies were not reported. While detection of *C. parvum* and *G. enterica* is fairly reliable using IMS-DFA (in prior reports as well as in the present study), limitations to this method include the cost and lack of molecular confirmation that is essential for accurately determining risk of infection to people.

Simultaneous detection of *C. parvum* and *C. cayetanensis* was described on herbs and berries using either microscopy (Shields et al., 2012) or PCR (Shields et al., 2013). Detection limits were not reported in either study. A more recent investigation by (Sim et al., 2017) applied a multiplex qPCR assay for detection of *C. parvum* and *C. cayetanensis*; however, the assay was not validated in spiking experiments and no information is available regarding sensitivity (e.g. limit of detection) or specificity.

Only two prior reports describe detection of three of the four protozoan pathogens included in the present investigation. Molecular methods (three separate conventional PCR assays) were evaluated for detection of *C. parvum, C. cayetanensis, and T. gondii* in basil, but detection limits for these assays were not reported (Chandra et al., 2014). An approach for simultaneous detection of *C. parvum, G. enterica, and T. gondii* on basil and raspberries was demonstrated using IMS-DFA followed by qPCR (Hohweyer et al 2016). This investigation utilized separate qPCR assays for each protozoan and reported sensitive parasite detection at 1-3 (oo)cysts/g. These low detection levels were similar or lower than the ones reported in the current study (1–5.5 (oo)cyst/g). An important difference between the two approaches is that in the spiking experiments described by Hohweyer et al. (2016), IMS was initially used to concentrate *C. parvum* and *G. enterica* from produce, whereas IMS-DFA and nested mPCR were performed in parallel in the current investigation (to compare their performance). Thus, the observed limits of detection (5.5 *C. parvum* and 4.5 *G. enterica* (oo)cyst/g) were obtained without the use of costly proprietary reagents and additional time required for parasite concentration (3–4 h for IMS).

The need for efficient and affordable methods for simultaneous

Table 4

Published methods that have been evaluated using parasite spiking studies for detection of *Cryptosporidium, Giardia, Toxoplasma gondii,* and *Cyclospora cayetanensis* in fresh produce. Most prior investigations have targeted detection of one or two of these protozoan pathogens, while two publications describe methods for detection of three of the four parasites.

Matrix (grams)	Parasite(s)	Recovery (%)[a]	LOD (oo)cysts	Processing Method	Detection Method	Reference
Salad mix (50) Bean sprouts (50) Lettuce (100) Chinese leaves (100) Strawberries (100)	*Cryptosporidium Giardia* (and *Ascaris*)	*Cryptosporidium* 42% *Giardia* 67% (Salad, lettuce, leaves and strawberries) *Cryptosporidium* 22–35% *Giardia* 4–37% (Bean sprouts)	NR[b]	Wash in rotating drum and sonication	IMS-DFA[c]	Robertson and Gjerde, (2000)
Mushrooms (100) Lettuce (100) Raspberries (100) Bean sprouts (50)	*C. cayetanensis*	12% (Mushrooms, lettuce and raspberries) 4% (Bean sprouts)	NR	Wash in rotating drum and sonication	Lectin-coated beads and Microscopy	Robertson et al., (2000)
Lettuce (30) Raspberries (60)	*Cryptosporidium*	59% Lettuce 41% Raspberries	NR	Stomacher, pulsification, rolling and shaking	IMS-DFA	Cook et al., (2006)
Various[d]	*Cryptosporidium Giardia*	*Cryptosporidium* 3–70% *Giardia* 5–65%	NR	Wash	IMS-DFA	Cook et al., (2007)
Herbs (0.2–25) Raspberries (10)	*Cryptosporidium C. cayetanensis*	*Cryptosporidium* 73% (Basil) *C. cayetanensis* 70–80% (Herbs); 35% (Raspberries)	NR	Wash (Alconox)	Microscopy	Shields et al., (2012)
Washes (80–230 mg) from Raspberry (25 g) and Basil (50 g)[e]	*Cryptosporidium C. cayetanensis*	NR	50/wash		PCR and qPCR	Shields et al., (2013)
Basil (25)	*Cryptosporidium C. cayetanensis T. gondii*	NR	4/g	Wash	PCR[F]	Chandra et al., (2014)
Lettuce (50)	*Giardia* (and *E. coli*)	20%	20/g	Wash	Microscopy and PCR	Ramirez-Martinez et al., (2015)
Lettuce (30)	*Cryptosporidium Giardia*	*Cryptosporidium* 53% *Giardia* 33%	NR	Wash and stomacher	IMS-DFA	Utaaker et al., (2015)
Lettuce (25)	*Giardia*	68%	1.4/g		Inertial Microfluidic Separation and DFA	Ganz et al., (2015)
Basil (30) Raspberries (30)	*Cryptosporidium Giardia T. gondii*	Basil: *Cryptosporidium* 11% *Giardia* 2% *T. gondii* 35% (no IMS) Raspberries: *Cryptosporidium* 14% *Giardia* 21% *T. gondii* 2.5% (no IMS)	Basil: *Cryptosporidium* and *Giardia* 3/g *T. gondii* < 1/g Raspberries (all): < 1/g	Wash, mechanical agitation	IMS- qPCR[f]	Hohweyer et al., (2016)
Basil (30)	*Cryptosporidium Giardia T. gondii*	NR	All 3/g	Wash, mechanical agitation	Reverse transcriptase (RT) qPCR Bioassay	Travaille et al., (2016)
Baby lettuce (50)	*T. gondii*	NR	0.5/g		LAMP- Chromatographic Lateral-Flow Dipstick	Lalle et al., (2018)
Spinach (10)	*Cryptosporidium C. cayetanensis Giardia T. gondii*	*Cryptosporidium* 46% *Giardia* 38% *T. gondii* 43%	*Cryptosporidium* 3.5/g *Giardia* 4.5/g *C. cayetanensis* 5.5/g *T. gondii* 1.0/g	Wash	IMS/DFA or MF; multiplex PCR	Current study

[a] Note that different initial spiking levels, drying conditions, and reagents used during processing hinders direct comparison of recoveries among studies; only studies that evaluated a quantitative method for parasite recovery are included in this column (i.e. percent of samples that amplified via qualitative conventional PCR is not listed).

[b] NR = Not reported.

[c] Immunomagnetic separation – Direct fluorescence antibody staining.

[d] Twenty different vegetable products (e.g. lettuce, corn, herbs, etc.) were tested, varying in amounts from 1 piece to 150 grams

[e] Parasites spiked into wash obtained from produce (not applied directly on produce).

[f] Different PCR assays were used for each target parasite.

detection of protozoan on produce is exemplified by field studies that have demonstrated the presence of these pathogens on ready to eat (RTE) salads. To date, these studies have relied on separate molecular assays for each targeted pathogen, or immunoassay-dependent approaches that may be cost prohibitive in some regions. In Italy, Caradonna et al. (2017) applied two separate conventional PCR assays for detection of *G. enterica* and *Cryptosporidium* spp., and two separate qPCR assays followed by melting curve analysis for detection of *T. gondii* and *C. cayetanensis*. Notably, all four pathogens were detected on RTE salads in this study. Separate PCR methods, as well as immunofluorescence microscopy, were also used to identify *C. cayetanensis*, *C. parvum,* and *G. enterica* on RTE salads in Canada (Dixon et al., 2013).

One limitation of the current investigation is the relatively small volume of inoculated produce (10 g). Most investigations that applied protozoa on vegetables describe volumes ranging from 25 to 250 g (Table 4). Produce contamination studies with bacteria have also applied small volumes of leafy greens (one leaf – 10 g) to detect bacteria using molecular methods (without culture) (Dinu and Bach, 2013). Validation of pathogen detection methods should be performed on larger quantities of produce that represent (at least) a consumed meal or purchased product. In this investigation, a spiking approach was developed for application of both nonviable and viable organisms. Priority was given to performing the spiking study using multiple concentration levels (4 dilutions) and replicates (n = 5) in a limited space available for conducting studies on organisms considered as biosafety level 2 pathogens. Further studies are thus needed to evaluate the performance of the mPCR assay on larger volumes of produce, as well as in field investigations. In addition, there is currently a critical need for establishing efficient assays for discriminating the viability or infectivity of parasites that are detected on food. Recent studies have demonstrated the application of such assays (Hohweyer et al 2016), with the hope that more accurate determination of health risk to consumers can be obtained through insight on pathogen viability when detected on produce.

5. Conclusions

Efficient and accurate detection of pathogens on food is critical for reducing foodborne illness, increasing consumer confidence, protecting economic livelihoods of producers, and ensuring food security. Improving methods for detection of protozoan pathogens on produce is especially relevant because of the increasing pressures a changing climate is exerting on agricultural areas. As temperatures increase, it is expected that many disease agents that have been mostly found in semitropical and tropical regions will gradually move north and become more common in the northern hemisphere (Myers et al., 2013). In addition, resource scarcity affecting availability of freshwater irrigation implies application of alternative approaches to grow food, including reuse of wastewater effluents following treatment. While effective means for safe application of treated effluents for produce irrigation are emerging (Orlofsky et al., 2013), breaks in treatment systems or ineffective pathogen removal in resource-limited regions implies that surveillance for presence of harmful pathogens on vegetables and fruit will become even more critical. The mPCR approach developed in this investigation offers a new tool for the produce industry, regulatory agencies charged with food safety, and academics to perform routine screening of fresh produce for the presence of protozoan pathogen contamination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103252.

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