Optimization and validation of a loop-mediated isothermal amplification (LAMP) assay for detection of *Giardia duodenalis* in leafy greens

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ABSTRACT

Giardia duodenalis is one of the most common food and water-borne intestinal parasites of humans and animals worldwide. Fresh, ready-to-eat produce such as leafy greens and salad mixes are considered potential transmission vehicles for *Giardia* infection in humans. Therefore, a specific, sensitive, and reliable method for *Giardia* detection in leafy greens is needed. We optimized washing procedures for the recovery of *Giardia* cysts from leafy greens and adapted and validated an existing EF1α LAMP assay for the detection of *Giardia* DNA to support routine diagnostic surveillance and disease outbreak investigations. Four leafy green types (35 ± 1 g) were spiked with 100 *Giardia* cysts and we compared washing by shaking with 1 M glycine ($n = 20$) or 0.1% Alconox ($n = 20$). DNA was extracted from washes, tested by LAMP and melt curve analysis, and time to positive (TTP) values compared. The detection limit was determined by spiking 10 ($n = 40$) *Giardia* cysts onto these same types of leafy greens and processing as above with 0.1% Alconox. Method robustness was assessed by subjecting spring mix ($n = 45$ total) to aging (1, 3 or 7 days) and washes to aging and freezing conditions prior to testing. Assay repeatability and specificity were evaluated, and an artificial positive control (APC) distinguishable by melt temperature (Tm) from DNA of *Giardia* spiked on leafy greens was designed to rule out cross-contamination from the control. *Giardia* detection rates were higher and TTP was lower ($P < 0.05$) for 0.1% Alconox (19/20, 8.85 ± 0.3 min) compared with 1 M glycine (15/20, 14.53 ± 7.2 min). The LAMP assay detected 10 *Giardia* cysts spiked on leafy greens in 13–34 min in 14/40 samples tested. Robustness assessment showed that TTP was higher ($P < 0.0001$) when spiked produce was stored for 7 days (13.09 ± 1.14 min) compared to fresh (9.72 ± 0.43 min). No unspiked samples were positive by LAMP, and the Tm for DNA of *Giardia* spiked on leafy greens was higher ($P < 0.0001$, 87.43 ± 0.05 °C) than the APC (86.43 ± 0.12 °C). Within-assay repeatability co-efficient of variation (CV) for TTP was 5.4% and no cross-contamination occurred when spiked and un-spiked samples were processed in alternate order. The optimized sample processing procedure combined with the EF1α LAMP assay is a sensitive, specific, labour-saving, and rapid method for the detection of *Giardia* cysts in leafy greens.

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1. Introduction

The flagellated protozoan *Giardia duodenalis* (also known as *Giardia intestinalis* and *Giardia lamblia*) is one of the most common intestinal parasites of humans identified worldwide and causes more than 280 million human infections each year. Currently, eight genetic assemblages (assemblage A–H) are recognized, and although assemblages A and B are reported to infect humans and considered to be zoonotic (Feng and Xiao, 2011; Ryan and Cacciò, 2013), assemblages C, D, E, and F are also reported in humans (Cacciò et al., 2018). *Giardia* has been detected in leafy green vegetables, which are eaten raw with minimal processing or washing, in both developing and developed countries (Dixon et al., 2013; Caradonna et al., 2017; Utaaker et al., 2017a) and has been recognized by the FAO/WHO as a parasite of concern in fresh produce (FAO/WHO, 2014). People may become infected by consuming contaminated produce that was grown or processed in an environment contaminated with animal or human faeces, or even from infected food handlers (Feng and Xiao, 2011; Utaaker et al., 2017b; Ryan et al., 2019). Overall, an estimated 15% of the total global *Giardia* infections are food-borne (Torgerson et al., 2015); however, many outbreaks are not documented due to the limitations of current detection and surveillance methods (Ryan et al., 2019; Chalmers et al., 2020).

In order to adequately mitigate the food safety risks for contaminated, perishable, fresh produce items, tests need to be rapid, sensitive, and specific (Chalmers et al., 2020). The international standard method 18,744 for detection of *Giardia* in leafy greens and berry fruits has been recently published (International Standards Association, 2016); however, the reagents are expensive and the method is time-consuming and microscopy-based, thus leaving limited potential for further molecular analysis (Utaaker et al., 2017a; Utaaker et al., 2015). Our laboratory has used a nested PCR targeting the SSU-rRNA gene (Appelbee et al., 2003) for surveillance testing of leafy greens for *Giardia*. Although sensitive, the nPCR test requires several days to complete and additional time to confirm presumptive positive results by sequencing. Nested PCR also has a higher risk of cross-contamination, especially when opening tubes with amplified PCR product and transferring PCR products from the primary to the secondary round of amplification. The potential for cross-contamination complicates the interpretation of results. Timely testing is required to ensure that appropriate regulatory action can be taken while produce is still in suitable condition for testing on the shelves or in the fridges of consumers.

Loop-mediated isothermal amplification (LAMP) of DNA was first introduced by Notomi et al. (2000) as a highly specific, efficient and rapid method that can accumulate 10^9 copies of target DNA in under an hour and be performed at a single temperature (~65 °C) using simple equipment (water bath, block heater, or incubator). LAMP assays use a strand displacing *Bst* polymerase and employ 4–6 primers that bind to 6–8 regions of the target DNA, thus reducing the non-specific or background amplification that plagues traditional PCR. LAMP assays are more specific than PCR due to the additional primers, simple to perform, inexpensive, and can result in faster turn-around times. LAMP assays have also been designed and tested for use in traditionally difficult matrices such as water, faeces, and food (Karanis et al., 2007; Lalle et al., 2018); the method performs well using relatively impure sample template preparations, unlike PCR. In addition to the direct measurement of turbidity of the positive LAMP reactions by the naked eye, usage of fluorescent dyes such as SYTO, SYBR Green, and Eva Green also allow real-time monitoring and detection of LAMP products (Patel et al., 2013; Fischbach et al., 2015; Maeda et al., 2005; Oscorbin et al., 2016). LAMP assays may not require post-amplification processing procedures such as electrophoresis and gel staining, which allows for a lower risk of cross-contamination, and are more suitable for high-throughput analysis and amenable to field testing (Law et al., 2014). The objectives of the current study were to optimize the produce washing procedures for the isolation of *Giardia* cysts from leafy greens, adapt and validate an existing elongation factor 1 *alpha* (EF1α) gene LAMP assay (Plutzer and Karanis, 2009) for detection of *Giardia* gDNA in concentrated produce washes, and implement the method for routine testing of leafy greens at the Canadian Food Inspection Agency, Centre for Food-borne and Animal Parasitology (CFIA-CFAP).

2. Materials and methods

2.1. Giardia cysts and produce samples

Giardia duodenalis cysts used for spiking experiments were purchased from Waterborne, Inc. (H3 isolate; New Orleans, USA) and stored in PBS with penicillin, streptomycin, gentamicin, amphotericin B and 0.01% Tween 20 at 4 °C. Cysts were counted by haemocytometer and stocks containing 5×10^3/mL of viable *Giardia* cysts were prepared for spiking experiments. For confirmation of the assay's inclusivity, an additional 14 isolates of *Giardia* were obtained as trophozoites in culture from the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, and several isolates were obtained from human faeces collected in Nepal and the Dominican Republic (CFAP collection, Supplementary Table 1).

Produce samples used for spiking experiments were purchased from local grocery stores. Matrices included pre-packaged, ready-to-eat kale salad mix (containing broccoli, Brussel sprouts, kale, cabbage, and chicory), romaine lettuce, spring mix (containing a mix of baby lettuces, arugula, radicchio and spinach), and 'American'-type salad mix (containing carrots, radishes, iceberg and romaine lettuces). For spiking experiments, samples (35 g ± 1 g, n = 5–10 for each experiment) were weighed and placed into BagPage® + filter bag (400 mL size with micro-perforated filter; Cole-Parmer, Montreal, Canada) and then spiked with 500, 100 or 10 *Giardia* cysts. Cysts were applied directly onto the samples in 3–4 droplets using an air displacement variable volume pipette fitted with a plastic tip, and allowed to dry for 2–3 h at room temperature. Un-spiked samples of each matrix (negative control) were tested alongside spiked samples for each spiking experiment. All spiked and un-spiked samples were stored at 4 °C overnight (16–18 h) prior to processing unless otherwise specified.

2.2. Wash solutions

Two wash solutions were evaluated in this study: 1 M glycine buffer (pH 5.5; Millipore Sigma, Oakville, Canada) and 0.1% Alconox (Millipore Sigma). To isolate the *Giardia* cysts, 200 mL of the selected wash solution was added to the filter bag containing the sample. Bags containing samples and wash solution were gently massaged to remove most of the air and sealed with bag clips. The sealed bags were laid flat on a platform shaker and shaken for 30 min at 130 rpm, and flipped over for another 30 min of shaking to ensure complete submersion of contents. The wash solution was transferred into a 250 mL centrifuge bottle from the back (non-produce) side of the filter bag using a serological pipette. An additional 50 mL of wash solution was used to rinse the sample at the end of the shaking and added to the 250 mL centrifuge bottle. All wash was concentrated by centrifugation at 2050 ×*g* for 15 min. The supernatant was removed by vacuum aspiration down to a volume of approximately 2 mL without disturbing the pellet. All remaining wash solutions and pellets were transferred to 2 mL microcentrifuge tubes and concentrated by centrifugation at 20,000 ± 1000 ×*g* for 15 min. The supernatant was removed by vacuum aspiration down to a volume of approximately 100 μL, and the resulting samples stored at 4 °C prior to DNA extraction unless otherwise specified.

2.3. DNA extraction

Two DNA extraction procedures were evaluated for use together with the LAMP assay for detection of *Giardia* DNA in the concentrated produce wash: a freeze/thaw method and a bead-beating method. For the free-thaw method, DNA was extracted from produce wash pellets using the QiaAmp DNA Micro Kit (Qiagen, Toronto, Canada) with modifications to the manufacturer's protocol. Briefly, 200 μL of Buffer ATL was added to each sample, followed by eight cycles of freezing in liquid nitrogen and thawing in a 95 °C water bath. Samples were incubated at 56 °C overnight with 20 μL of proteinase K, and then 300 μL of Buffer AL was added into each tube and the samples were incubated at 70 °C for 10 min in a thermomixer with shaking at 900 rpm. After adding 150 μL of 100% ethanol and vortexing, the samples were loaded into MinElute spin columns. The columns were washed with 500 μL of Buffer AW1, then 700 μL of Buffer AW2, and finally 700 μL of 100% ethanol. After two consecutive elutions with 20 μL of Buffer AE, the DNA samples were stored at −20 °C until use. For the bead-beating method, DNA was extracted from the produce wash pellets using the FastDNA SPIN Kit for Soil in conjunction with a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA) following a slightly modified protocol (Murphy et al., 2017). To compare these two extraction methods, we extracted DNA from samples of 200 ($n = 14$), 100 ($n = 14$) and 50 ($n = 14$) *Giardia* cysts in 100 uL produce wash carrier; half the samples were processed by the freeze-thaw method and half by the bead-beating method.

2.4. LAMP assay

All primers and oligonucleotides were purchased from Integrated DNA Technologies (Coralville, USA). The oligonucleotide sequences and amplification protocols are shown in Table 1. For this study, we selected a previously developed EF1α LAMP assay to detect *Giardia* in faecal and environmental samples (Plutzer and Karanis, 2009) and validated it for detecting *Giardia* in leafy greens. The LAMP reaction was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Mississauga, Canada) and the *Bst* 2.0 WarmStart® DNA Polymerase Kit (New England Biolabs, Whitby, Canada). The 25 μL reaction mix contained *Bst* 2.0 DNA Polymerase (0.32 units/μL), 1.6 μM of each FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.2 mM dNTPs (New England Biolabs), 6 mM MgSO$_4$, 3.34 μM STYO-9 (Invitrogen, Waltham, USA), 0.2 M Betaine (Millipore Sigma), and 2 μL of DNA template. In early development and optimization experiments, gDNA from *Giardia* cysts was included as a positive control, and the assay was performed for 60 min and the products were examined by electrophoresis on a 1.5% agarose gel. The samples were considered positive if the characteristic LAMP "ladder" pattern was observed on the gel. For validation experiments, the LAMP assay was conducted using a real-time PCR instrument, as indicated above, according to the conditions in Table 1 to avoid post-amplification handling steps. Samples with time to positive value (TTP) ≤ 40 min were considered positive, and data are presented as means ± standard error of the mean (SEM).

To directly sequence the LAMP products for confirmation, we designed sequencing primers (Seq1 F/ Seq1 R) that anneal within the predicted LAMP amplicon using the EF1α gene sequence of *Giardia duodenalis* Assemblage B (GenBank: AF069570.1, Table 1). An artificial *Giardia* EF1a (AGE) consisting of the LAMP assay primer binding sites for the *Giardia* EF1α gene and random DNA sequence was designed using the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/random_dna.html, accessed 2019-03-07) and included in the LAMP assay as a positive control to assist with data interpretation and ruling out cross-contamination (Supplementary Fig. 1). The LAMP assay was further improved by adding a melt curve analysis step (LAMP-MCA) following the amplification step, and the melting temperatures of LAMP products generated from DNA of *Giardia* spiked on leafy greens and the AGE were compared.

2.5. Nested and conventional PCR assays

A nested PCR assay targeting the small subunit ribosomal RNA (SSU-rRNA) (Appelbee et al., 2003; Hopkins et al., 1997) was used for comparison to the EF1α LAMP assay on spiked produce samples. For this assay, the first PCR amplification mixture (final volume 25 μL) contained 0.625 U GoTaq DNA Polymerase, 1× Colorless GoTaq Buffer (Promega, Madison, USA), 0.2 mM dNTPs (Qiagen), 12.5 pmol each of Gia F/Gia R primers, 1.5 mM MgCl$_2$, 5% DMSO, 1× PT enhancer (Horakova et al., 2011) [5× PT enhancer, 0.76 g trehalose dehydrate (Millipore Sigma) dissolved in 735 μL of 1, 2-Propanediol (Millipore Sigma) and QS to 2 mL

Table 1
Molecular assays, primer sequences and amplification conditions used in this study.

Assay name	Product size (bp)	Primer name	Primer sequence (5′-3′)	Amplification conditions	Reference
EF1α LAMP	178	F3	ATGGACGACGGCCAGG	LAMP-1 × [67 °C-40 min]; 1 × [80 °C 4 min]; melt curve 60 °C to 98 °C, increasing by 0.2 °C increments with 5 s hold at each step	Plutzer and Karanis, 2009
		B3	CCCTCGTACCAGGGCATC		
		FIP	AGCCGATGTTCTTGAGCTG CTT-GTACTCGAAGGAGCGC TACG		
		BIP	GAAGAAGGCCGAGGAG TTCG-TTGTCGGACCTCTCCATGA		
		LF	ATCATCTCGCCCTTGATCTCG		
		LB	CTGGACCGGGGACAACA		
SSU-rRNA nPCR	497	Gia F	AAGTGTGGTGCAGACGGACTC	1 × [95 °C-2 min]; 35 × [95 °C-45 s, 58 °C-30 s, 72 °C-45 s]; 1 × [72 °C-4 min]	Appelbee et al., 2003
		Gia R	CTGCTGCCGTCCTTGGATGT		
	292	RH 11	CATCCGGTCGATCCTGCC	1 × [95 °C-2 min]; 25 × [95 °C-45 s, 55 °C-30 s, 72 °C-30 s]	Hopkins et al., 1997
		RH 4	AGTCGAACCCTGATTCTCCGC CAGG		
β-Giardin nPCR	729	BGN1-F	TACGCTCACCCAGACGATG	1 × [95 °C-5 min]; 35 × [95 °C-30 s, 52 °C-30 s, 72 °C-1 min]; 1 × [72 °C-5 min]	This study
		BGN1-R	ATCTTGTCCTCYGCCTCCTTG		
	542	BGN2-F	ATGGAGAACGAGATCGAGGTC	1 × [95 °C-5 min]; 25 × [95 °C-30 s, 60 °C-30 s, 72 °C-40 s]	This study
		BGN2-R	TTCTCGAGCTGCTCGTTGAC		
Sequencing	102	Seq1F	AGGGCGAGATGATGAAGCAG	Sanger sequencing	This study
		Seq1R	TCTCCATGATGTTGTCCCCG		
EF1α PCR	1305	F	ATGGGCAAGGAGAAGAAGC	1 × [98 °C-30 s]; 35 × [98 °C-10 s, 61 °C-30 s, 72 °C-1 min]; 1 × [72 °C-2 min]	This study
		R	GAACTCCTCCTTGTCGATCTC		

with PCR water] and 2 μL of DNA template. The second PCR amplification mixture (final volume 25 μL) contained 0.625 U GoTaq DNA Polymerase, 1× Green GoTaq Buffer (Promega), 0.2 mM dNTPs (Qiagen), 12.5 pmol each of RH 11/RH 4 primers, 1.5 mM MgCl$_2$, 5% DMSO, 1× PT enhancer and 1 μL of DNA template. An artificial positive control gene fragment, consisting of the SSU-rRNA nPCR primer landing sites and otherwise random DNA (Integrated DNA Technologies) and a no-template control (NTC) consisting of reagents only were included in each run.

A β-giardin nPCR assay (in-house-developed) was used to genotype the isolates used in this study via amplicon sequencing. For this assay, the first PCR amplification mixture (total volume 25 μL) contained 1× AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Waltham, USA), 10 pmol each of BGN1-F/R primers, 0.16 μg/ μL bovine serum albumin (Millipore Sigma) and 2.5 μL of DNA template. The second PCR amplification mixture (final volume 25 μL) contained 1 × AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific), 10 pmol of each BGN2-F/R primers and 1 μL of DNA template. The nested PCR products were analyzed using the QIAxcel Advanced System (Qiagen) with the QIAxcel DNA High Resolution Kit (Qiagen) using method OM500 with the combination of 100 bp-2.5 kb QX DNA Size Marker (Qiagen) and 15 bp-3 kb QX Alignment Marker (Qiagen), according to the manufacturer's manual. A positive control consisting of gDNA extracted from *G. duodenalis* cysts, and a NTC consisting of reagents only were included in each run. The β-giardin PCR products were purified using the QIAquick PCR purification Kit (Qiagen) according to the manufacturer's protocol and sequenced in both directions using BGN2-F/R primers. Sequences of the forward and reverse reads were assembled and trimmed using Clone Manager 9 Professional (Sci-Ed Software) and compared to the β-giardin sequences available in GenBank using NCBI-BLAST.

2.6. Evaluation of method performance characteristics

To evaluate the specificity of the *Giardia* LAMP assay, we tested *Giardia muris*, *Cryptosporidium parvum*, *Tritrichomonas foetus*, *Entamoeba* sp., *Toxoplasma gondii*, *Hammondia hammondii*, *Eimeria papillata*, un-spiked produce wash DNA, and 14 different human *G. duodenalis* isolates (confirmed as Assemblage A or B by β-giardin nPCR, Supplementary Table 1). To confirm the identity of LAMP products generated from *G. duodenalis* DNA, they were purified as described above and sequenced in both directions using the in-house Seq1 F/ Seq1 R primer set.

To evaluate the degree of sequence heterogeneity at the LAMP primer binding sites among different geographical isolates and its effect on TTP, we amplified a 1305 bp region of the EF1α gene using in-house primers (Table 1) from six *Giardia* isolates (denoted by an asterisk in Supplementary Table 1). For amplification of the EF1α gene, the PCR amplification mixture (total volume 50 μL) contained 1 × Q5 Reaction Buffer (New England Biolabs), 20 pmol each of EF1α F/R primer, 0.2 mM dNTPs (Qiagen), 1 U Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs), 1 × Q5 High GC Enhancer (New England Biolabs) and 2 μL DNA template. The amplified fragments were cloned using the pGEM®-T Easy Vector system (Promega, Madison, WI, USA) according to the manufacturer's instructions. For each isolate, 6–7 clones were sequenced and aligned with a reference *Giardia duodenalis* EF1α sequence (XM_001704495). By examining the sequence heterogeneity in the LAMP primers' annealing sites, we identified clones with either 100% homology with the reference sequence, one single nucleotide polymorphism (SNP) (A to G at position 614), or three SNPs (A to G at position 614, G to A at position 615, and T to C at position 524). Then we compared the TTP values of LAMP reactions with plasmid DNA of one or two representative isolates from each of these groups (100% homology, 1 SNP, or 3 SNPs) diluted to 0.5 ng/μL and 0.05 ng/μL.

To determine the detection limit and to verify the method's suitability for different matrices, we spiked four types of leafy greens (American salad mix, kale salad mix, romaine lettuce, and spring mix) with 10 ($n = 10$ for each type) or 0 ($n = 1$ for each type) *G. duodenalis* (H3 isolate) cysts, washed these with 0.1% Alconox, and tested by *Giardia* LAMP as described above. The method's robustness was evaluated by testing spring mix samples spiked with 500 *Giardia* cysts in a 3×3 factorial design. Spiked spring mix samples were divided into three groups ($n = 15$/group) and stored at 4 °C overnight, for 3 days, or 7 days and processed as described. For each group, the final produce wash either proceeded to DNA extraction immediately ($n = 5$), was stored at 4 °C ($n = 5$), or frozen ($n = 5$) for 2 days and then extracted. The impact of these changes on TTP and the detection rate was then calculated. To evaluate the method repeatability, two different analysts tested two different panels consisting of five spring mix samples spiked with 500 *Giardia* cysts and one un-spiked sample on different days, and the coefficient of variation between TTP values of each analyst's results was calculated. In addition, five samples of spring mix spiked with 500 *Giardia* cysts were processed in alternating order with five un-spiked negative control samples to examine the potential for cross-contamination during testing.

2.7. Method implementation and proficiency testing

Our laboratory is accredited by the Standards Council of Canada (SCC) for testing under the International Organization for Standardization (ISO) 17025: 2017 standard and research according to the SCC Requirements and Guidance for Accreditation of Laboratories Engaged in Test Method Development and Non-Routine Testing. As such, all method development and validation work was carried out in accordance with these requirements. To facilitate the implementation of the *Giardia* LAMP method for routine surveillance testing of leafy greens, we trained four analysts to perform the method and administered proficiency tests to each analyst. The proficiency tests consisted of a panel of six leafy green samples, five of which were spiked with 200 *Giardia* cysts, and one randomly selected un-spiked sample. Analysts were required to correctly identify all six samples in their panel to be authorized to perform the method routinely for diagnostic testing. The panels were prepared as above for method validation experiments and analyzed by four different analysts on different days. Each panel also included the positive AGE control, a no-template control, a DNA extraction positive control (200 *Giardia* cysts), and a DNA extraction negative control (extraction reagents only), in addition to the six panel samples.

2.8. Statistical analysis

All TTP data are presented as means $\pm$ SEM. For experiments of factorial design, a two-way ANOVA was performed using GraphPad Prism (version 4.03) with an *alpha* level of 0.05, and significant difference was considered if *P value* < 0.05. For comparing means, an unpaired *t*-test or one-way ANOVA was performed using GraphPad Prism and significant difference was considered if *P value* < 0.05.

3. Results

3.1. Evaluation of Giardia EF1α LAMP assay specificity

The LAMP assay did not amplify DNA from *Giardia muris*, *Cryptosporidium parvum*, *Tritrichomonas foetus*, *Entamoeba* sp., *Toxoplasma gondii*, *Hammondia hammondii*, *Eimeria papillata* or un-spiked produce wash DNA. Ladder-like LAMP products were detected (TTP 54 min) and observed on the gel when the assay was used on DNA from a bovine gut flagellate. However, conventional PCR on this sample to generate product for sequencing was negative. The standard run time for the LAMP assay was shortened to 40 min, as the likelihood of generating non-specific products is high when the assay runs for an extended period.

When examining the degree of sequence heterogeneity among different geographic isolates of *Giardia* at the EF1α gene LAMP primer landing sites, only a very low level of heterogeneity was found. No amplification delay or increase in TTP was observed ($P > 0.05$) when different concentrations (0.5 μg/μL or 0.05 μg/μL) of plasmids with either 100% homology to the reference isolate, a single SNP (A to G at site 614, GenBank accession number MW8035588), or three SNPs (T to C at 524, A to G at 614, and G to A at 615, GenBank accession number MW803559) in the cloned EF1α sequence were used as templates for LAMP (Supplementary Fig. 2). The mean TTP for the 100% homology group ($n = 6$) was 5.4 min, while the TTP for the one SNP group was 5.8 ($n = 6$), and 5.9 min for the three SNPs group ($n = 3$).

To confirm and evaluate the *Giardia* EF1α assay's specificity, we performed direct sequencing of the LAMP products using primers Seq1 F/R. Selected LAMP products amplified from spiked produce samples were confirmed by sequencing for 5/15 samples. The sequences showed high homologies (96–99%) with the EF1α gene from *Giardia duodenalis* strain ATCC 50803 (Accession: KX131163.1). We also applied the LAMP assay to detecting a variety of animal and human *Giardia* isolates. DNA was extracted from 15 *Giardia* isolates (Supplementary Table 1) originating from human or beaver faeces and identified as assemblage A or B by β-giardin nPCR. All isolates tested positive by LAMP. Sequencing with Seq1 F/R primers was successful for all (15/15) LAMP products, and the obtained sequences demonstrated high homology (95–100%) with the EF1α gene from *Giardia duodenalis* strain ATCC 50803.

3.2. Melt curve analysis

The LAMP amplicon generated from DNA of *Giardia* spiked on leafy greens had a melting temperature (Tm) of 87.43 ± 0.05 °C ($n = 50$), and the AGE had a Tm of 86.43 ± 0.12 °C ($n = 17$). The melt temperature ranges do not overlap and are significantly different (*t*-test: $P < 0.001$). LAMP products generated from the DNA of *Giardia* spiked on leafy greens can be distinguished from LAMP products amplified from the AGE, which enables differentiation of true and false positives (Fig. 1).

3.3. Comparison of DNA extraction methods

For *Giardia* cysts extracted by the freeze/thaw method, 21/21 samples were positive by LAMP and the overall average TTP was 6.7 min (Table 2). For *Giardia* cysts extracted by the bead-beating method, the overall average TTP was 24.2 min for and the proportion of samples positive by LAMP was 15/21. Since the mean TTP was lower (*t*-test: $P < 0.0001$) for samples extracted by the freeze-thaw method, all further validation experiments were performed using that procedure.
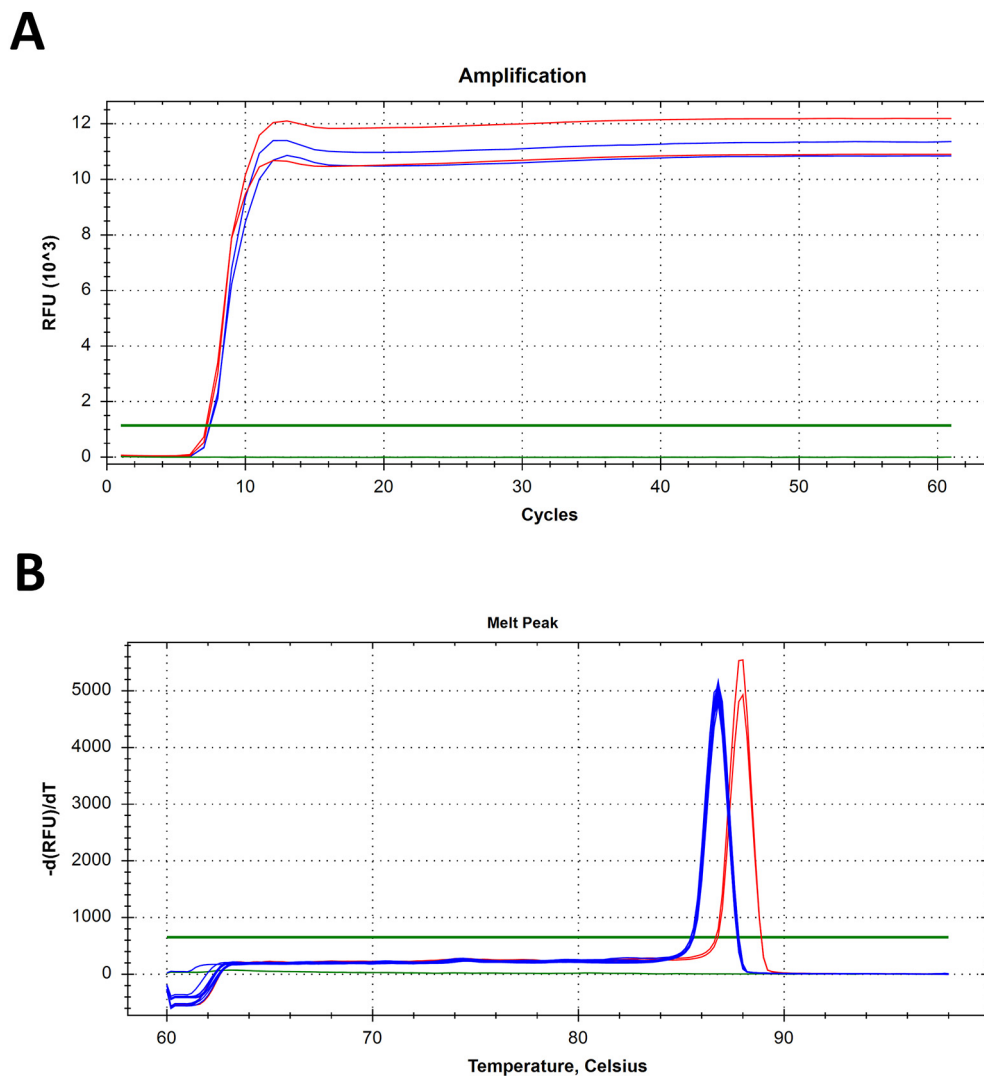

Fig. 1. A. Amplification plot showing the LAMP products generated from DNA of *Giardia* spiked on leafy greens (red) and artificial *Giardia* EF1α gene (blue), each cycle = 1 min. B. Melt curve graphs showing the melting peaks and melt temperature comparison of DNA of *Giardia* spiked on leafy greens (red, 87.43 ± 0.05 °C) and artificial *Giardia* EF1α gene (blue, 86.43 ± 0.12 °C) amplified by LAMP-MCA.

Table 2
Time to positive (TTP) and proportion positive for *Giardia* cysts extracted by freeze-thaw or bead-beating methods and tested by LAMP (samples were considered positive if TTP ≤ 40 min).

DNA extraction method	Number of *Giardia* cysts	Mean TTP min (SEM)	Proportion positive
Freeze-thaw	200	6.2 (0.04)	7/7
	100	6.7 (0.08)	7/7
	50	7.1 (0.22)	7/7
Bead-beating	200	15.1 (2.24)	6/7
	100	25.1 (2.49)	4/7
	50	32.3 (2.65)	5/7

3.4. Comparison of wash solutions

The proportion of samples positive and TTP of the *Giardia* LAMP for different produce matrices spiked with *Giardia* and processed with 0.1% Alconox or 1 M Glycine pH 5.5 is presented in Table 3. For American salad, romaine lettuce and spring mix, lower TTP values were observed ($P < 0.05$) when samples were washed with 0.1% Alconox (8.48 ± 0.48 min, 8.52 ± 0.64 min, and 9.27 ± 0.75 min) as compared with those washed with 1 M Glycine pH 5.5 (10.94 ± 2.61 min, 24.62 ± 0.44 min and 14.05 ± 1.18 min), whereas similar TTPs were found in kale mix salad (8.75 ± 0.88 vs 8.07 ± 0.27) tested with either of the wash solutions. In addition, samples processed with 0.1% Alconox also had higher proportion positive when tested by LAMP: 100% of samples were positive for American salad mix, kale mix salad, and romaine lettuce, and 80% for spring mix. Although 100% of American salad mix and 80% of spring mix samples processed with 1 M Glycine were also positive, only 60% of samples were positive for both the kale mix salad and romaine lettuce. Therefore, 0.1% Alconox was selected for further spiking experiments.

3.5. Detection limit

The LAMP positive rate and TTP for leafy greens spiked with 10 *Giardia* cysts are shown in Table 4. The TTP ranged from 13.86 ± 4.92 min for American salad to 34.16 ± 1.91 min for spring mix. American and kale mix salads were 40% positive by LAMP when spiked with 10 *Giardia* cysts, while romaine lettuce and spring mix were 30% positive. The positive rate for nPCR is also shown in Table 4. The nPCR positive rate for American and kale mix salads was 100%, but none of the romaine lettuce and spring mix samples were positive when tested by nPCR. All un-spiked samples were negative by both methods.

3.6. Robustness

The effect of produce condition (fresh, 3 days, ≥ 7 days old), and time between processing and DNA extraction (0 day, 2 days at 4 °C, and frozen), using a factorial design which compared all possible combinations of changes is shown in Table 5, and Fig. 2. All samples ($n = 5$ per group) tested positive by LAMP. For spiked samples aged overnight, LAMP gave TTP of 9.72 ± 0.43, 9.12 ± 0.15 and 9.23 ± 0.19, respectively, when produce washes proceeded to DNA extraction immediately, were stored at 4 °C, or frozen for 2 days followed by DNA extraction. For spiked samples aged for 3 days, similar TTPs of 9.50 ± 0.24, 9.43 ± 0.12 and 9.60 ± 0.35 min were observed. When spiked samples were aged for 7 days, slightly higher TTPs of 13.09 ± 1.14, 12.49 ± 0.82 and 10.87 ± 0.62 min were obtained. Two-way ANOVA with replication demonstrated that only the age of spiked produce had a significant impact on mean TTP ($P < 0.0001$). There was no significant effect of the storage condition of the produce washes or time between processing and DNA extraction (0 day, 2 days at 4 °C, and frozen, Supplementary Tables 2 and 3).

3.7. Repeatability

When performed by two different analysts, the LAMP assay was repeatable with a coefficient of variation (CV) of TTP <10%. Cross-contamination risk assessed by alternatively processing five positive and five negative produce samples yielded no false positive or negative results, indicating no occurrence of cross-contamination between spiked and un-spiked samples (data not shown).

3.8. Proficiency testing

The results of proficiency testing for four analysts trained in the *Giardia* LAMP method are summarized in Table 6. All analysts successfully achieved the correct result for each sample. The TTP for all spiked matrices ranged from 9.40 (±0.86) to 11.80 (±1.99) minutes, and all un-spiked samples were negative. The mean TTP for the AGE control, tested by each of the four analysts as part of their panel, was 9.04 (± 0.49) min, and the coefficient of variation between analyst runs was 5.4%.

Table 3

Time to positive (TTP) and proportion positive for leafy green matrices spiked with *Giardia* cysts and processed with 0.1% Alconox or 1 M glycine and tested by LAMP ($n = 5$; samples were considered positive if TTP ≤ 40 min).

Produce type	Number of *Giardia* cysts spiked	Wash solution	Mean TTP min (SEM)	Proportion positive
American salad	100	0.1% Alconox	8.84 (0.48)	5/5
	100	1 M Glycine	10.94 (2.61)	5/5
	0	1 M Glycine	NEG	0/1
Kale mix salad	100	0.1% Alconox	8.75 (0.88)	5/5
	100	1 M Glycine	8.07 (0.27)	3/5
	0	1 M Glycine	NEG	0/1
Romaine lettuce	100	0.1% Alconox	8.52 (0.64)	5/5
	100	1 M Glycine	24.62 (0.86)	3/5
	0	1 M Glycine	NEG	0/1
Spring mix	100	0.1% Alconox	9.27 (0.75)	4/5
	100	1 M Glycine	14.50 (1.18)	4/5
	0	1 M Glycine	NEG	0/1

Table 4

Time to positive (TTP) and proportion positive for leafy green matrices spiked with *Giardia* cysts, processed with 0.1% Alconox, and tested by LAMP (samples were considered positive if TTP ≤ 40) and nPCR (samples were positive if a 292 bp band was amplified).

Matrix	Number of *Giardia* cysts spiked	Mean TTP min (SEM)	LAMP proportion positive	nPCR proportion positive
American salad	10	13.86 (4.92)	4/10	10/10
	0	NEG	0/1	0/1
Kale mix salad	10	30.26 (3.45)	4/10	10/10
	0	NEG	0/1	0/1
Romaine lettuce	10	17.02 (8.89)	3/10	0/10
	0	NEG	0/1	0/1
Spring mix	10	34.16 (1.91)	3/10	0/10
	0	NEG	0/1	0/1

Table 5

Design of 3 × 3 factorial experiment to determine the impact of produce age and produce wash storage condition (post-processing) on time to positive (TTP) and proportion positive for *Giardia* LAMP assay.

Produce wash storage condition (Post-processing)	Produce storage time after cyst spiking		
	1 day (overnight)	3 days	7 days
	Mean TTP (SEM) and proportion positive		
0 day (no storage)	9.72 (0.43)	9.50 (0.24)	13.09 (1.14)
	5/5	5/5	5/5
2 days (4 °C)	9.12 (0.15)	9.43 (0.12)	12.49 (0.82)
	5/5	5/5	5/5
2 days (frozen)	9.23 (0.19)	9.60 (0.35)	10.87 (0.62)
	5/5	5/5	5/5

4. Discussion

Since the introduction of LAMP technology over 20 years ago, it has been applied in many settings, from point-of-care rapid clinical testing for coronaviruses (Augustine et al., 2020) to multiplex lateral-flow dipstick tests for simultaneous rapid detection of multiple pathogens in foods (Lalle et al., 2018; Jiang et al., 2020). Here, we applied a previously developed LAMP test to the detection of *Giardia* in leafy greens, using a comparatively simple approach that could be readily employed in most food testing laboratories for routine surveillance, using existing equipment and expertise and commercially available reagents. Application of the LAMP method to detection of *Giardia* in leafy greens is appropriate for several reasons. First, the cysts are generally present in low numbers on produce samples, and enrichment methods, such as those used for bacterial detection, are not applicable for routine diagnostic use with parasites, so a highly sensitive molecular test is required. Second, produce washes contain high amounts of debris, and "background" contaminants such as soil, fungus, bacteria, and PCR-inhibiting substances, so an effective molecular test must be highly specific and robust. Third, a test method employed for routine surveillance and disease outbreak investigations of *Giardia* in leafy greens should have a rapid turn-around time to allow for regulatory action or recall to occur while the perishable product is still available on the shelf or in the consumer's refrigerator. We have demonstrated the *Giardia* LAMP assay to be
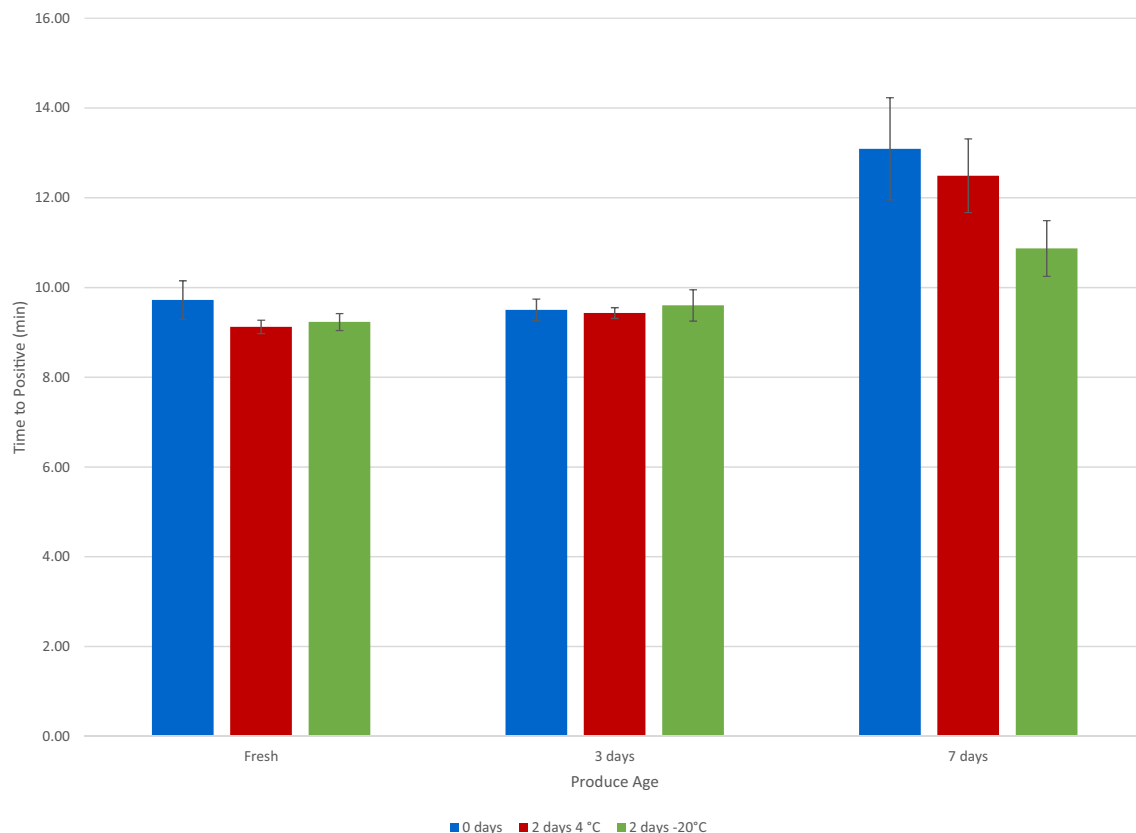
Fig. 2. Evaluation of LAMP robustness by testing different ages of spring mix and times between processing and DNA extraction in factorial design experiment ($n = 5$ per group).

Table 6
Proficiency testing results (TTP, min) for four analysts trained in the *Giardia* LAMP assay.

	Analyst 1		Analyst 2		Analyst 3		Analyst 4		Mean TTP min (SEM)
	Panel	Result	Panel	Result	Panel	Result	Panel	Result	
AGE Control	Positive	9.65	Positive	8.46	Positive	9.07	Positive	8.97	9.04 (0.49)
Iceberg lettuce	Negative	Negative	Spiked	10.24	Negative	Negative	Spiked	9.33	9.79 (0.46)
Spinach	Spiked[a]	9.05	Spiked	14.22	Spiked	9.03	Spiked	11.89	11.04 (1.25)
Spring Mix	Spiked	10.14	Spiked	12.01	Spiked	10.36	Spiked	8.76	10.31 (0.67)
Kale salad	Spiked	8.02	Negative	Negative	Spiked	14.75	Spiked	12.64	11.80 (1.99)
American salad	Spiked	15.65	Spiked	10.52	Spiked	11.36	Spiked	8.30	11.46 (1.54)
Romaine lettuce	Spiked	7.67	Spiked	10.31	Spiked	10.21	Negative	Negative	9.40 (0.86)

[a] Spiked with 200 *Giardia duodenalis* cysts.

sensitive, with a detection limit as low as 10 cysts/35 g produce, specific for the detection of *Giardia* to the exclusion of other parasites and matrix DNA, and faster to perform than our previously employed nPCR method.

The efficiency in isolating and concentrating the cysts from the leafy greens is a critical step in the *Giardia* LAMP procedure. We evaluated two different commonly employed wash solutions, 0.1% Alconox (Shields et al., 2012) and 1 M Glycine pH 5.5 (Cook et al., 2006), which have both been reported extensively for use in the recovery of parasites from leafy vegetables (Murphy et al., 2018; Lalonde and Gajadhar, 2016). In this study, the 0.1% Alconox solution was more effective. The high efficacy of 0.1% Alconox in isolating *Giardia* cysts from selected produce could be explained by the combination of its two components, sodium dodecylbenzenesulfonate ($C_{12}H_{25}C_6H_4SO_3Na$) and tetrasodium pyrophosphate ($Na_4P_2O_7$). These two agents were effective in isolating the cysts presumably by sufficiently wetting, swelling, and plumping the surface of the leafy greens, which increased the surface area, exposing more cysts to the wash (Shields et al., 2012). Alconox is a detergent, and therefore not suitable for processing leafy greens by stomacher (another common technique) possibly due to the excessive foam produced from the agitation of the detergent combined with the release of additional saponins contained in some vegetables, such as spinach. Our laboratory

also performs testing of produce for *Cyclospora* according to the US FDA's BAM Chapter 19b, which utilizes shaking with 0.1% Alconox to wash the produce, so shaking is the preferred approach for *Giardia* to streamline our lab work flow.

A flotation technique is commonly used in the concentration and recovery of protozoan oocysts or cysts by using a solution with a relatively higher specific gravity (such as sucrose or zinc sulfate) to facilitate separation of produce wash debris from the cysts or oocysts, which float to the top. Although fairly simple to perform, the recovery efficiency of flotation is poor, and degenerated cysts or oocysts might not be recovered if they do not float and are discarded with the sample debris (Kar et al., 2011). Our modified sample processing procedure eliminated the flotation step; once the produce samples were washed and the resulting wash buffer concentrated by centrifugation, DNA was extracted directly from the pellets, which allowed us to maximize the recovery rate of the *Giardia* cysts. This approach is not novel and has also been demonstrated as effective for recovery of *Cyclospora* (Murphy et al., 2018) and *Toxoplasma* (Lalle et al., 2018) from leafy greens. Eliminating the flotation step may also reduce the risk of cross-contamination, and it simplifies and speeds up the sample processing, especially when working with larger numbers of samples. The centrifugation times and speeds used here were selected to streamline our overall laboratory workflow, however the processing time could be further reduced by shortening the centrifugation steps from 15 min to 5 min at 2500 ×g or 10 min at 1000 ×g (Chaste et al., 1992).

The infectious dose for *Giardia* cysts is low; 10 or fewer cysts may cause illness in humans (Ortega and Adam, 1997). Therefore, our aim was to develop an assay that could detect 10 *Giardia* cysts in a single "serving" size (sample weight of 35 g) of leafy greens. We established that the detection limit for this method is 10 spiked *Giardia* cysts, as 30–40% of American salad, kale mix salad, romaine lettuce, and spring mix spiked with 10 cysts were positive by LAMP. Interestingly, the nPCR positive rate for American and kale mix salads was higher (100%) than that of the LAMP but lower (0%) for romaine lettuce and spring mix. This could be explained by the higher amounts of debris and PCR-inhibitors generated from certain types of more tender leafy greens, such as spring mix and romaine lettuce. The strand-displacing *Bst* polymerase used in the LAMP assay has a higher tolerance to PCR inhibitors than the *Taq* DNA polymerase used in nPCR (Kaneko et al., 2007). Additionally, the nPCR targets SSU rRNA of *Giardia,* which is a multi-copy gene (Boothroyd et al., 1987), while the LAMP targets EF1α, which is represented by a single copy (Thompson and Ash, 2016), so in samples containing fewer PCR inhibitors, the nPCR may outperform the LAMP assay.

LAMP assays are purported to be more specific than PCR assays due to the additional primers and more capable of discriminating SNPs (Plutzer and Karanis, 2009). If there are mismatches in the FIP or BIP sites, the dumbbell-like structure of the initial LAMP amplicons may fail to form, abrogating subsequent amplification (Lin et al., 2018). However, our examination of the impact of one or three SNPs in the LAMP primer landing sites on TTP showed the effect was negligible. Since we had access to a fairly limited number of unique *Giardia* isolates during the validation of this method, determining that the impact of SNPs in the primer landing sites did not increase TTP or reduce detection significantly provided confidence that the assay would be suitable for field samples. The accommodation of too many mismatches in the primer landing sites by the LAMP could lead to non-specific amplification and false-positive results, however, the EF1α gene is highly conserved (Wielinga et al., 2015).

Although the LAMP method described here is a sensitive and reliable screening tool for detecting *Giardia* in leafy greens, a positive result simply indicates the presence of *Giardia* DNA and does not provide information on the potential viability or infectivity of cysts present. Similarly, the ISO 18744:2016 method for the detection of *Giardia* cysts does not determine the viability or infectivity of any cysts detected. Some approaches to assessing viability and infectivity include bioassay, excystation, and vital dye exclusion. However, these approaches are not practical or economical for screening purposes and often require purified cysts (Rousseau et al., 2018). Propidium monoazide (PMA) is a DNA-intercalating dye that can selectively permeate dead cells and form stable DNA monoadducts upon photolysis, resulting in DNA that cannot be amplified by PCR or LAMP (Rawsthorne et al., 2009). Treatment of extracted DNA with PMA before LAMP has been employed in the detection of viable bacteria in foods (Fang et al., 2018), and viable *Cryptosporidium* oocysts and *Giardia* cysts in wastewater (Alonso et al., 2014) and could be explored together with the *Giardia* LAMP optimized in this study for the detection of viable *Giardia* cysts. The detection of *Giardia* DNA in a produce sample is indicative of faecal contamination, and therefore a cause for concern regardless of the potential viability or infectivity of the contaminating cysts.

A common concern with the ISO 18744:2016 method for detection of *Giardia* in leafy greens [which is based on concentration by centrifugation and immunomagnetic separation (IMS), and detection by immunofluorescent antibody testing (IFAT)] is the cost of the reagents required to perform the test (Utaaker et al., 2015). A rough estimate of the cost to perform the *Giardia* LAMP assay, including all reagents and consumables, is approximately $14 CAD per sample. This does not include the start-up costs for required laboratory equipment or positive controls; however, it is significantly less expensive than the advertised price for IMS and IFAT kits, estimated at approximately $50 CAD per sample. The hands-on time required to process a batch of 10 samples by *Giardia* LAMP is estimated to be 3–4 h to process the samples and 1 h to set up the LAMP assay compared to nPCR, which requires 2–3 h for set up due to the additional round of amplification and gel electrophoresis.

Sequencing suspect positive amplification products of molecular diagnostic tests is one strategy to confirm the results and rule out cross-contamination from controls. Due to the complex nature of LAMP amplification products, direct sequencing using the outer F3/B3 primers resulted in very few readable sequences in this study. Multiple overlapping traces were observed in some sequence data, making it impossible to assemble the sequences for analysis. Therefore, we designed specific primers for direct sequencing of the *Giardia* LAMP products to enable confirmation. The in-house designed primers were suitable for reliably sequencing LAMP products generated from pure gDNA samples. However, sequencing of LAMP products generated from spiked produce samples was successful only in less than half of the samples submitted. To assist with results interpretation in the event that sequencing is not successful, we included an artificial positive control (AGE) and melt curve analysis following amplification. The melt peaks generated from the AGE and DNA of *Giardia* spiked on leafy greens do not overlap; the AGE generates a

melt peak that can be distinguished from a true positive due to differences in sequence and G-C content (AGE: 50.6% vs DNA of *Giardia* spiked on leafy greens: 64.5%), thus ensuring that cross-contamination can be ruled out without relying on sequencing for confirmation. Using the AGE instead of *Giardia* gDNA as a LAMP positive control further reduces the chance of cross-contamination when the test is performed in a routine diagnostic setting. The nPCR assay can also be employed as an ancillary test if further confirmation of suspect positive samples is required.

Our robustness experiment examined the impact of several common changes that could be expected to occur in routine surveillance testing in a diagnostic laboratory (e.g., produce may need to be stored for several days prior to testing, or the testing process may need to be halted temporarily and resumed following a holiday or staff absence). Therefore we examined the effect of aging the produce for up to 7 days, and either extracting DNA the day after processing, waiting 3 days (i.e., duration of a long weekend), or freezing for more extended periods prior to extraction. The TTP was negatively affected when spiked produce was stored for 7 days. The higher TTP may be explained by the debris generated during processing steps of more aged and deteriorated produce, which negatively affect DNA extraction efficiency, although we did not quantify the degree of deterioration of the produce or the size of the resulting wash pellets. In addition, others have observed a reduction in cyst integrity of *Giardia* on lettuce leaves over a period of 9 d, even when stored at 4 °C (Utaaker et al., 2017b). However, our results showed that all spiked produce still tested positive even when the samples were aged for 7 days at 4 °C, suggesting that our processing method and LAMP assay are robust enough to detect *Giardia* cysts from such samples. Since the clinical symptoms of giardiasis usually begin 1–3 weeks after consuming food contaminated by *Giardia* cysts, reliability in testing aged produce would be particularly important in a giardiasis outbreak investigation, should there be the requirement to test leftover produce samples implicated in human cases.

In conclusion, we have adapted and validated a previously developed EF1α LAMP assay for use in routine surveillance of leafy greens for *Giardia*. Compared to the nPCR method previously employed in our laboratory, the *Giardia* EF1α LAMP assay is more sensitive in tender leafy greens such as romaine and spring mix, and performed robustly on aged and deteriorated samples. The nPCR may be more sensitive in matrices that generate few inhibitors, and direct sequencing of nPCR products for confirmation or further molecular analysis is more reliable than direct sequencing of LAMP products. However, the LAMP is straightforward to perform and has a faster turnaround time, saving hands-on time at the bench. An inter-laboratory trial would further validate the assay and demonstrate suitability for use by different labs using comparable equipment and reagents. The EF1α LAMP assay for the detection of *Giardia* in leafy greens has now been implemented in our laboratory for routine monitoring and surveillance testing and to provide more rapid diagnostic support to disease outbreak investigations at the CFIA-CFAP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2021.e00123.

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