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Detection of *Giardia spp.* with formalin/ether concentration in *Brassica oleracea* (cabbage) and *Lactuca sativa* (lettuce)

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ABSTRACT

Methods to detect protozoa are needed for food safety monitoring. We evaluated protocols to recover *Giardia spp.* cysts in *Brassica oleracea* (cabbage) and *Lactuca sativa* (lettuce) and then detection was performed by concentrating with formalin/ether solutions and microscopy or immunofluorescence or DNA amplification via PCR. To evaluate this methodology, *G. duodenalis* cysts were inoculated in triplicate (10 cysts) in 35-g samples of lettuce and cabbage. The method obtaining the highest percentage of recovery in cabbage was sulfamic acid solution plus stirring with stomacher (47.7% ± 7.5). For lettuce, the best method was glycine solution plus stirring with stomacher (46.6% ± 5.3). Inter-observer agreement was of 0.99. *Giardia* was detected by amplifying specific sequences for the DNA coding SSU rRNA. In 27 lettuce samples and 27 cabbage samples, obtained from supermarkets and street vendors, two lettuce samples (7.4%) and one cabbage sample (3.7%) were positive for *Giardia* via PCR assay and were sequenced, determining that they were two of assemblage B and one of lettuce to assemblage E. This method is proposed to detect *Giardia* in vegetables by PCR detection, enabling public health authorities to identify genotypes circulating in food, which will help to establish measures that reduce outbreaks of parasitic diseases associated with contaminated food.

1. Introduction

Giardia duodenalis (also known as *G. intestinalis*) is ranked as the 11th foodborne parasite of priority to address risk management international measures (FAO; WHO, 2014). *Giardia duodenalis* is the only species found in humans and many other mammals; it is now considered a multispecies complex with at least seven distinct assemblages or groups of strains (Adeyemo et al., 2018). Only assemblages A and B have been detected in humans and in a wide range of other mammalian hosts, whereas the remaining assemblages, C to G, have not yet been described infecting humans (Adeyemo et al., 2018; Smith et al., 2007). Vegetables for human consumption can be contaminated with *Giardia* and other protozoan cysts or oocysts during their production if irrigated with untreated wastewater and by using manure as fertilizer, which is a common practice in some regions of developing countries or by irrigating with contaminated water or by animals living near the production sites or during transport by food handlers (Robertson, 2016; Smith et al., 2007). In the United States, foodborneoutbreaks by *Giardia* were associated with raw vegetables (Adam et al., 2016). To detect protozoa in vegetables is challenging given that cyst and oocyst recoveries differed considerably for individual matrices (Cook et al., 2007). Current standardized methods to monitor protozoa in vegetables use immunomagnetic separation as a step to concentrate the protozoa; however, this includes using expensive reagents, such as immunomagnetic separation kits that hindered the method for many countries (Utaaker et al., 2015). Indeed, an ISO international standard to detect and enumerate *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits, which includes an immuno-separation step, is now available and may have implications for the international food trade ("Microbiology of the food chain -Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits (ISO, 18744:2016)," n.d.). Recent efforts were undertaken to diminish costs by reducing the amount of immunomagnetic reagents with protozoa recovery rates ranging from 4% to 88% with a mean of 53% for *Cryptosporidium* and 33% for *Giardia* (Utaaker et al., 2015). This method is still of high analytical cost by using immunomagnetic reagents and this method does not distinguish between the *iardia. duodenalis* assemblages or establishes its infectivity. An alternative to identify the presence of the A and B assemblages, responsible for the

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vast majority of human infections, is to conduct molecular detection and sequencing of the PCR products of amplification (Adeyemo et al., 2018).

We previously developed the formalin-ether concentration method to monitor protozoa in water as an affordable alternative for low-income countries (Lora-Suarez et al., 2016). For present work on salads, sulfamic acid or glycine solutions combined with stirring are used first to separate the spiked cysts from salads as described previously (Cook et al., 2007). Then, the elution is submitted to the formalin ether to concentrate the protozoa. The formalin-ether uses solutions of lower specific gravity than the parasitic organisms, thus, concentrating the latter in the sediment and consequently improving the sensitivity of the parasite detection methods (Methanitikorn et al., 2003). The aim of this work was to evaluate different protocols to recover *Giardia* cysts in different vegetable matrices and then the eluates were submitted to the formalin-ether method that concentrates *Giardia* cysts. We first used microscopy to establish the best protocol to recover *Giardia spp* and then we established the recovery percentage by using immunofluorescence and PCR. We evaluated the use of these detection methods in two widely consumed vegetables (cabbage and lettuce). While PCR detection cannot differentiate between viable or non-viable parasites, it can be a useful tool for epidemiological purposes, food monitoring and to identify where potential contamination is occurring (Rousseau et al., 2018). The available methodology for green vegetables is based on immunomagnetic separation techniques (Utaaker et al., 2015). Here we describe an alternative, cheap, rapid, simple methods for analyzing fresh produce (cabbage, lettuce) for contamination with *Giardia* cysts (elution followed by formol-ether concentration followed by detection by PCR).

2. Materials and Methods

2.1. Preparation of Giardia cysts to evaluate the concentration methods

Purified *Giardia duodenalis* cysts at 10^5 cyst/ml concentration, originally isolated from human stools, were kindly donated by Dr. Fidel Angel Nuñez from Instituto Pedro Kouri at Havana (Cuba) and kept at 4 °C until use. The parasite suspension concentration for dilution experiments was obtained by counting via hemocytometer.

2.2. Vegetables and artificial inoculation with Giardia cysts

Brassica oleracea (cabbage) and *Lactuca sativa* (lettuce) were purchased from local wholesalers. Outer leaves were discarded and the remaining leaves removed individually and mixed prior to artificial contamination. Thereafter, the vegetables were washed with 1 l of sodium chloride solution at 0.9% and then the vegetables were divided into pieces of leaves 200 g each and stored on polypropylene sterile bags. Some of the pieces obtained from vegetables (lettuce and cabbage) were examined by microscopy for previous presence of *Giardia* before inoculation. The rest of the pieces of leaves were inoculated with 50 ml of saline solution at 0.9% containing 10 cysts *of Giardia* volumes confirmed by hemocytometry and obtained by serial dilutions and deposited into the polypropylene bags. Afterwards, the bags were incubated in a mechanical shaking mixer during 18 h at room temperature; thereafter, the isolation procedure was undertaken. From each vegetable, a separate leaf sample was inoculated with sodium chloride solution at 0.9% without *Giardia* cysts as blank control.

2.3. Isolation and concentration of Giardia cysts from salad produce

As previously described (Cook et al., 2007), the noncovalent interactions responsible for *Giardia* attachment can differ between salad, doing necessary to test the best conditions (washing solution, pH and time of shaking) for each vegetable. To do this, we used 40 spiked samples of leaves from each vegetable inoculated with 10 *Giardia* cysts as determined by hemocytometer count plus one blank to test three washing solutions:

a) Glycine 1 M in distilled water pH 5.5 (**M1**)
b) Sulfamic acid 10 g/l, PBS 1X, Tween 80 0.1% pH 3.5 (**M2**)
c) Sodium chloride solution (NaCl 9 g/l) pH 3.8–4.0 **(M3)**

Each sample of leaves was kept in the bag containing 200 ml of the washing solution during 24 h and, thereafter, the bag was subjected to mechanical agitation (stirring) by one of two methods:

a) Mechanical shaking in Labline 4625 Titer Shaker (Marshall Scientific Ltd, USA) at 125 rpm for 30 min **(S1)**
b) Agitation in a Stomacher 400 circulator (Seward Ltd., United Kingdom) at 260 rpm, five times for 30 s **(S2).**

After stirring, the eluate obtained from the bag was filtered through a Whatman filter paper into a 50-ml tube and centrifuging this tube for 5 min at 1,600 g at 4 °C in a Hermle model Z 446 K centrifuge (Hermle Labor Technik GmbH, Wehingen, Germany). We discarded the supernatant from each tube obtaining approximately a total volume of 15 ml. We centrifuged this 1,600-g tube for 3 min and then discarded the supernatant. Then we added 5 ml of formalin solution at 10% and 3 ml of diethyl ether at 99% (Sigma, USA). We sealed and rigorously shook the tube to bring the diethyl ether in contact with all parts of the sediment and performed a new centrifugation at 1,000 g for 2 min. After that, four different layers were formed, as follows: (i) diethyl ether on the top, (ii) debris, (iii) saline, and (iv) a sediment at the bottom. We discarded the upper three layers so that only the sediment remained in the tube; the sediment was re-suspended in 500 μl of 0.85% sodium chloride solution and, subsequently, placed on a slide for direct microscopy identification and enumeration at 40X. The whole volume was examined and identification was done by size and morphological characteristics as accepted by the EPA (U.S. Environmental Protection Agency (U.S. EPA) Office of Water, 2012).

2.4. Microscopic identification and statistical performance of formalin/ether method for recovery of Giardia in cabbage and lettuce leaves

Microscopic determination on spiked samples of *Giardia* cysts was used initially to establish the best isolation and concentration conditions. Three different statistical evaluations were performed:

i) percent recovery of three washing solutions combined with two different stirring methods,
ii) performance of the method in terms of detection and quantification limit by using different concentrations of *Giardia* cysts,
iii) intra-observer variation of the method

To select the best protocol for washing and stirring, 10 *Giardia* cysts were spiked on cabbage and lettuce leaves. The percent recovery (**R**) was calculated with the following equation: R = N/T x 100; where: R = the percent recovery, N = the number of cysts counted, and T = the number of cysts spiked. The ANOVA analysis for both samples (cabbage and lettuce) was performed with 95% confidence interval. The percent recovery was estimated as the percentage of the initial spiked dose recovered at the end of the method trial, from initial concentration to final sample enumeration.

Once the protocol was established for washing and stirring, one calibration curve was constructed by testing 0, 10, 50 and 100 cysts of *Giardia* spiked on leaves of cabbage samples. The suspension of parasites was enumerated by microscopy at 40X magnification.

2.5. Calculation of the limit of detection and intra-observer variation of the method

The relative standard deviation (RSD) was obtained with the standard deviation divided by the mean, times 100. The uncertainty was determined with the confidence interval as follows: IC = X-(+1- α/2 x SE),

where: X = average, α = 0.09 degrees of freedom of the t value, and standard error (SE) = (standard deviation)/$\sqrt{}$ n. The limit of detection (**LOD**) was estimated as: DL = 3 x σ/S; where σ = the standard deviation of the response and S = the slope of the calibration curve. The limit of quantification (**LOC**) was estimated as: DL = 10 x σ/S; where σ = the standard deviation of the response and S = the slope of the calibration curve. The slope, S, was estimated from the calibration curve with 0, 10, 50, and 100 *Giardia* cysts. Statistical analyzes were performed by using Statgraphics Centurion XVI.II software.

Intra-observer variation of the method was evaluated on 10 repetitions of the entire method in cabbage leaves spiked with 10 parasites and one blank sample prepared in reagent-grade water and read by three different observers. In total, 40 data items per observer were obtained (three concentrations and one blank). Agreement among observers was measured by the intraclass correlation coefficient (ICC) by using the calculator in https://www.statstodo.com/ICC_Exp.php (Haber et al., 2005; Landis and Koch, 1977). This agreement quota was established for observations made by three different laboratory technicians for 10 repetitions. The individual results of the model that assumes that the same raters perform the evaluations in all cases were interpreted as follows: 0–0.2 indicates poor agreement: 0.3–0.4 indicates fair agreement; 0.5–0.6 indicates moderate agreement; 0.7–0.8 indicates strong agreement; and >0.8 indicates almost perfect agreement.

2.6. Immunofluorescence detection of Giardia cysts

The Aqua-Glo™ kit (Waterborne Inc., USA) was used to detect *Giardia* cysts by utilizing the principle of direct immunofluorescence as described previously (Robertson and Gjerde, 2000). This reagent consists of fluorescein-labeled mouse genus-specific monoclonal antibodies made to cyst wall antigenic sites of *Giardia duodenalis* and DAPI (4',6-diamidino-2-phenylindole) prepared at 2 mg/mL in methanol. The reagent shows varying degrees of cross-reaction with cysts from other *Giardia* species. The cysts appear bright apple green when viewed under a fluorescence microscope using appropriate filters for fluorescein. This antibody cross-reacts with some algae species. The DAPI binds to DNA, fluorescing blue using a UV filter setting. The sample was screened at 400X magnification and *Giardia* cysts were enumerated. Reading of slides was done was performed by two microscopy operators with an Evos FL Auto cells System microscope (Life Technologies, USA). Criteria to identify *Giardia* cysts included brilliant apple-green fluorescing round to ovoid objects (8–18 μm long by 5–15 μm wide) with brightly highlighted edges at 40X and after switching to the UV filter block for DAPI at 100X, the object should exhibit one or more of the following characteristics: (a) light-blue internal staining (no distinct nuclei) and a green rim; (b) intense blue internal staining: (c) up to four distinct, sky-blue nuclei. If atypical structures were not observed, then each object meeting the criteria was defined as a positive result. Using 100X total magnification, the shape, measurements (to the nearest 0.5 μm), and number of nuclei and presence of median body or axonemes (if applicable) for each apple-green fluorescing object meeting the size and shape characteristics were recorded.

2.7. DNA extraction method and PCR for Giardia

The Wizard Genomic DNA Purification Kit (Promega, USA) with mechanical lysis with zirconium bead was applied for DNA extraction in vegetable eluates. This method previously showed the best recovery performance (Triviño-Valencia et al., 2016). We centrifuged 500 μl of sediment eluates at 13,500 g for 6 min at 4 °C and re-suspended pellet in 20 μl of isoamyl alcohol and 600 μl of DNAazol lysis buffer and incubated at room temperature for 5 min. Then, we added 0.3 g of 0.5-mm zirconium beads (BioSpec product Inc, USA) and followed by shaking in a mini-bead beater (Stratech UK) for 1 min and placed such in ice for 1 min; we repeated this step five times. After centrifugation at 13,500 g for 6 min, we transferred the supernatant to a new tube and mixed it with 200 μl of nuclear lysis buffer, vortexed it for 20 s and maintained it at 37 °C for 30 min. Then, we added 100 μl of protein precipitation lysis, vortexed for 20 s and placed the tube in ice for 5 min. Subsequently, we centrifuged the sample at 13,500 g for 1 min and transferred the supernatant to another tube with 200 μl of ethanol at 75% v/v, mixed gently 4 times and centrifuged at 13,500 g for 2 min and discarded the supernatant and dissolved the pellet in 80 μl of Tris-EDTA buffer. The DNA from reference strain used as control for PCR reactions was *Giardia* isolate H3 assemblage B (http://www.ncbi.nlm.nih.gov/nuccore/AY258616) donated by Dr. Karen Shapiro (University of California, Davis).

Giardia was detected by amplifying specific sequences for the DNA coding SSU rRNAr. Sequence of primers for first PCR were: Forward RH11 5'-CATCCGGTCGATCCTGCC-3' and reverse RH4 5'-AGTCGAACCCTGATTCTCCGCC-3' that amplifies a product of 292 bp (Hopkins et al., 1997). For second PCR, primers were Forward GiarF 5'-GACGCTCTCCCCAAGGAC-3' and reverse GiarR 5'-CTGCGT CACGCTGCTCG-3' that amplified a fragment of 175 pb (Read et al., 2002). The PCR mix in the first PCR consisted of 12.5 μl of GoTaq Green Master Mix (Promega, USA) containing deoxyribonucleoside triphosphates 0.2 mM, MgCL 25 mM, Taq polymerase, 1.5 μl of primers and 3 μl of DNA in a final volume of 25 μl. For the second PCR, the mix concentration of reagents was the same and the DNA was 1 μl of the amplification product of the first PCR. Cycle conditions were: denaturation at 94 °C for 5 min; then 40 cycles comprising 1 min at 94 °C, annealing at 61 °C for 1 min and 1 min at 72 °C; followed by a final extension of 10 min at 72 °C. The second PCR cycles were: denaturation at 94 °C for 5 min; then 14 cycles comprising 1 min at 94 °C, annealing at 61 °C for 1 min and extension step 1 min at 72 °C; followed by a final extension of 10 min at 72 °C. We also included control for contamination during DNA extraction to control carryover contamination and consisted it in a tube without template but containing all reagents for DNA extraction. To avoid contamination, we took several measures, such as separate space to set up PCRs (pre-PCR room) separate filter tips and pipettes, and various negative controls (no DNA and DNA extraction contamination controls). The Veriti Thermal Cycler (Thermo Fisher Scientific, USA) performed all PCR amplifications.

To test the specificity of the PCR amplification, DNA was extracted from *Entamoeba coli*, *Entamoeba histolytica*, and *Trichuris trichiura* isolates provided by Professor Fidel Angel Nuñez from "Instituto Pedro Kouri" in Havana, Cuba. In addition, DNA was obtained from *Escherichia coli, Candida albicans*, *Toxoplasma gondii*, *Cryptosporidium parvum*, and *Blastocystis hominis* isolates at our laboratory to examine if cross-reaction existed with this PCR assay.

2.8. Effect of bovine serum albumin (BSA) on inhibition of PCR detection of Giardia

In other series of experiments to test the inhibition of the PCR assay by the vegetable matrix, we inoculated 50 ml of saline solution at 0.9% containing 100 *Giardia* cysts in the polypropylene bags containing 35 g of lettuce or cabbage leaves and DNA extraction was performed. In this group of samples, we evaluated the effect of adding three different concentrations of bovine serum albumin (BSA, Eurobio, France) to the PCR mix. After this, the BSA optimal concentration to reduce PCR inhibition was used to evaluate the reproducibility, in three separate experiments, with cabbage and lettuce samples inoculated with 10 *Giardia* cysts.

2.9. Field application on retail salad produce

In order to test our method on vegetables sold for public consumption, we purchased each of the vegetables (salad and cabbage) at three supermarkets, three local retail markets, and three street vendors in Armenia, Quindío (central western Colombia). These are the sites (street vendors, local retail market, and supermarkets) where most people in Colombia purchase vegetables. From each vegetable retail site, three

cabbages and three lettuces, intended to be consumed raw, were purchased and were processed at the Universidad del Quindío Laboratory.

2.10. Sequencing and alignment analysis of Giardia PCR products

For sequencing, PCR products were gel-purified from low-melt agarose gels, followed by recovery using the Wizard PCR SV and PCR clean up system kit (Promega, WI). Sequencing was done under BigDye® terminator cycling conditions by using the normal automatic service by Macrogen (Korea) in 3730XL DNA sequencer with the same primers as the PCR amplifications. Sequences were aligned with Clustal W in Molecular Evolutionary Genetics Analysis (MEGA) software, Version 5.05 (available at: http://www.megasoftware.net/).

3. Results

3.1. Microscopy evaluation of protocols for Giardia isolation in salad products

Given that immunofluorescence and PCR are expensive methods to detect *Giardia spp.* in food, we decided, in controlled laboratory conditions, to use artificially spiked samples to test all the possible conditions for the method protocol by using optic microscopy. This approach reveals that each vegetable had a different protocol, which obtained the best percentage recoveries. The best protocol for cabbage combined the extractant solution of sulfamic acid solution plus stirring with stomacher, obtaining recovery of 47.7% ± 7.5 (Table 1). For lettuce samples, the best method was glycine solution with stomacher stirring, obtaining a recovery of 46.6% ± 5.3 (Table 2).

Performance of the method in cabbage was established by a calibration curve (Table 3) with three different concentrations of *Giardia* cysts. The LOD was of 2.58 (~3 cysts) and the LOQ was 8.6 (~9 cysts). Agreement among three observers (intraclass correlation coefficient) was 0.99, indicating a near perfect agreement for the evaluation in cabbage samples with a slope of 0.74 and $R^2 = : 0.9982$.

3.2. Application of the method with detection by immunofluorescence and PCR in artificially spiked cabbage and lettuce samples

By using the best protocol for the formalin/ether isolation method established by microscopic detection for cabbage and lettuce, we analyzed the sensitivity by using immunofluorescence or PCR detection. For this, we first evaluated the recovery percentage with three different numbers of cysts on cabbage by using immunofluorescence detection. For 10 cysts, the mean recovery percentage ±standard deviation on three assays was: 26 ± 5; for 50 cysts, it was 86 ± 11, and for 100 cysts, it was 80 ± 17. Fig. 1 shows a typical result of immunofluorescence analysis on artificially spiked cabbage samples.

Then, we established the conditions to limit inhibition of PCR detection by adding 1 ml of cabbage or lettuce concentrated eluates to 100 *Giardia duodenalis* cysts diluted in saline solution. After DNA extraction, the PCR assay was performed in presence or not of three concentrations of BSA on the PCR mix (200, 600, and 800 ng). We found that it was necessary to use 800 ng of BSA to prevent inhibition of detection by cabbage or lettuce eluates (Fig. 2). No cross-reaction of the PCR assay was found when assaying DNA from *Toxoplasma gondii*, *Entamoeba coli*, *Entamoeba histolytica*, *Trichuris trichiura*, *Escherichia coli*, *Candida albicans*, *Cryptosporidium parvum*, or *Blastocystis hominis*. Afterwards, we inoculated artificially 10 cysts on cabbage and lettuce leaves and conduct PCR detection. After seven repetitions, we found that on cabbage two of seven assays were positive (29%; 95%CI: 0–62) and on lettuce the PCR was positive on three of seven assays (42%; 95%CI: 6–78).

Table 1
Percent recovery (**R**) of 10 *Giardia* cysts inoculated in samples of *Brassica oleracea* (cabbage) by using three extractant solutions: **M1** (Glycine 1 M); **M2** (Sulfamic acid, Tween 80 0.1%) and **M3** (Chloride saline solution 0.9%) and two methods of stirring: **S1** (stomacher) or **S2** (mechanical shaking). The greater R is shown in bold.

Methods	Mean R	% Lower	% Upper
M1, S1	11.1	4.6	17.6
M1, S2	17.7	11.2	24.2
M2, S1	**47.7**	**41.2**	**54.2**
M2, S2	20.0	13.4	26.5
M3, S1	11.1	4.6	17.6
M3, S2	20.0	13.3	19.6

Table 2
Percent recovery (**R**) of 10 *Giardia* cysts inoculated in samples of *Lactuca sativa* (lettuce). by using three extractant solutions: **M1** (Glycine 1 M); **M2** (Sulfamic acid Tween 80 0.1%) and **M3** (Sodium chloride 0.9%) and two methods of stirring: **S1** (stomacher) or **S2** (mechanical shaking). The greater recovery percentage is shown in bold.

Methods	Mean R	% Lower	% Upper
M1, S1	**46.6**	**41.4**	**51.8**
M1, S2	17.7	12.5	23.0
M2, S1	17.7	12.5	23.0
M2, S2	17.7	12.5	23.0
M3, S1	11.1	5.8	16.3
M3, S2	12.2	7.9	16.4

3.3. Application of the method in retail products and results of alignment of PCR product sequences

In total, 54 products (cabbage n = 27 and lettuce n = 27) were obtained from supermarkets and street vendors. Each vegetable was divided into six pieces of leaves of 35 g and placed in individual polystyrene bags; then, each piece of leaf was submitted to the recovery process that was made for pieces of cabbage with sulfamic acid solution plus stirring with stomacher, and for pieces of lettuce (n = 27) with glycine solution plus stirring with stomacher. After this, all the six elution from each vegetable were collected in a 50-ml tube and submitted to concentration with formalin/ether method and PCR amplification, as described in the Materials and Methods section. We found that two samples of lettuce (7.4%) and one from cabbage (3.7%) were positive for *Giardia* through the PCR assay. The three positive samples were purchased from the same street vendor. After Clustal alignment of the three DNA sequences (Fig. 3A), a Maximum Likelihood phylogenetic tree showed that one sequence from lettuce and one from cabbage were grouped with reference strains of assemblage B and one of lettuce to assemblage E (Fig. 3B).

4. Discussion

Cabbage and lettuce are two of the most consumed vegetables in Colombia (Fao and MinSalud, 2013). Monitoring methods in salad products for *Giardia* and other protozoa is urgently need. We evaluated an alternative method that showed similar performance to the previously reported method based on immunoseparation (Cook et al., 2007; Utaaker et al., 2015). *Giardia* isolation from cabbage was challenging, given the abundance of debris and solid material that hindered the separation process. We found that cabbage needs a different method from that used with lettuce; this is a similar finding to that previously described for different salad produce with the immunoseparation method (Cook et al., 2007). Cabbage is particularly rich in polysaccharides, its different composition can explain why elution agents act differently (Hanschen et al., 2014). Sulfamic acid is used as an acidic cleaning agent, whereas glycine is used as a buffering agent ("The Merck Index Online - chemicals, drugs and biologicals," n.d.). No previous reports have been made about the performance of detection methods for *Giardia* on cabbage. The recovery percentage of the selected methods (46% with glycine for lettuce and 47% with sulfamic acid for cabbage) was excellent compared to that established as acceptable, of at least of 30% from lettuce, with the

Table 3
Calibration curve with 100, 50, 10 and 0 *Giardia sp* cysts spiked in samples of cabbage (*Brassica oleracea)* and detected by microscopic observation. Data are the mean ± standard deviation of the observations made by three different observers, for 100 and 50 cysts there was one sample by each observer and for 10 cysts there were 10 repetitions by three observers.

Number of *Giardia* cysts spiked	Observer 1	Observer 2	Observer 3	Mean	SD	Relative SD	% Recovery
100	78	81	83	80.6	2.5	3.11	80.66
50	38	32	42	37.3	5.0	13.48	74.66
10	7.3	7.5	7.7	7.5	0.2	2.66	75.0
0	0	0	0	0	0	0	0

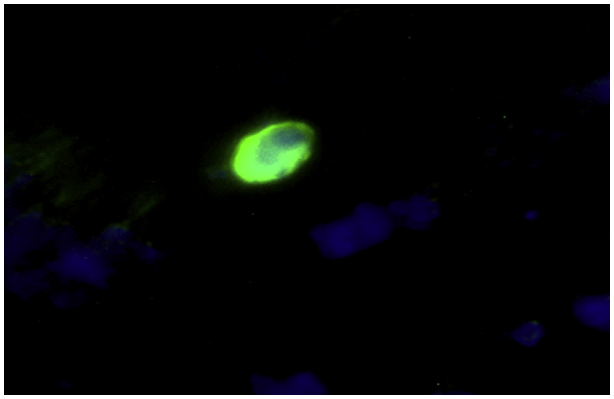


Fig. 1. Immunofluorescence detection of *Giardia* cysts with anti-*Giardia duodenalis* fluorescein labelled monoclonal antibody, after spiking 10 cysts of *G. duodenalis* from human origin, on cabbage leaves and recovered by formalin/ether method. The characteristic green fluorescent ovoid object with DAPI positive stain (inner blue fluorescence corresponding to DNA nuclei content) is observed in the center of the image (100X).

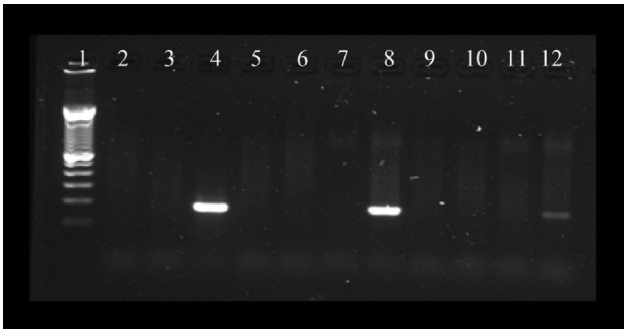


Fig. 2. Agarose 1.5% gel image of the products of the *Giardia* SSU PCR nested amplification assay (product size of 175 bp). Lane 1: Molecular weight markers 100 bp DNA ladder, Invitrogen, USA (upper band: 2,000 bp, brighter intermediate band: 600 bp, lower band 100 bp). Lane 2: Negative control (PCR mix without DNA). Lane 3: Negative control of extraction (PCR mix plus product of extraction of tube without DNA template). Lane 4: Positive control from *Giardia duodenalis* isolate H3 assemblage B. Lane 5: Lettuce sample inoculated with saline solution 0,9% without *Giardia* cysts (blank control). Lane 6. Lettuce sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 200 ng of BSA. Lane 7. Lettuce sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 600 ng of BSA. Lane 8: Lettuce sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 800 ng of BSA. Lane 9: Cabbage sample inoculated with saline solution 0,9% without *Giardia* cysts (blank control). Lane 10: Cabbage sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 200 ng of BSA. Lane 11. Cabbage sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 600 ng of BSA. Lane 12. Cabbage sample inoculated with saline solution 0,9% containing 100 *Giardia* cysts and 800 ng of BSA.

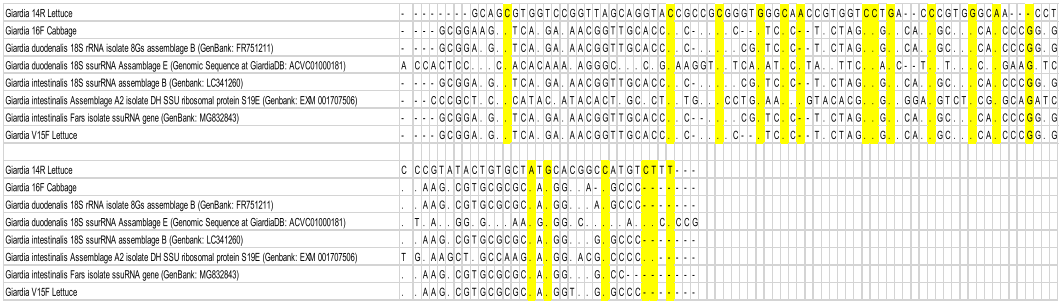
standardized method (Utaaker et al., 2015). Results of recovery and detection via microscopy or PCR for 10 cysts were similar (26% vs. 29%, respectively). In the future, the method proposed should be submitted to inter-laboratory comparisons to establish the variation in performance beyond the ideal conditions, as was evaluated in this work.

We also evaluated the PCR method for the specific amplification of *Giardia* after applying the isolation formalin-ether methodology in lettuce and cabbage and found that it was necessary to add a large amount of bovine serum albumin to the PCR mix to counteract the inhibition by salad produce natural components. When applied, the PCR method to detect *Giardia* cysts on vegetables is more sensitive than microscopic or immunofluorescence methods (Tiyo et al., 2016; Utaaker et al., 2017). This indicates higher sensitivity; however, it is also well-known that large amounts of PCR inhibitors exist in vegetables (Utaaker et al., 2017). For this reason, it was essential to establish what conditions were optimal to amplify DNA from *Giardia* obtained from vegetable material under our conditions.

Herein, we described a method that can detect, with sufficient sensitivity, contamination by *Giardia spp*. on salad produce. This method obtained cysts from salad produce that can be analyzed by immunofluorescence or PCR. The method was able to detect fresh retail produce contaminated with *Giardia* that were sequenced and the assemblage group identified. Though PCR detection does not determine viability or infectivity, none of the techniques currently available appear to be entirely suitable for reliable assessment of human exposure to infective protozoa in food or for routine verifications of control measures (Rousseau et al., 2018). For this reason, it has been recommended to determine initial levels of contamination by using DNA-based assays because they are sensitive enough to detect low quantities of parasites and are accessible for routine analyses (Rousseau et al., 2018). Although these methods overestimate exposure to infective parasites by detecting all populations of protozoa (live and infectious, live and non-infectious, or dead) they offer information of the maximum occurrence and about the level of food contamination. Additionally, it is possible – after sequencing PCR products for *Cryptosporidium* and *Giardia* – to establish the species and genetic assemblages, respectively (Rousseau et al., 2018). Although both *in vitro* and *in vivo* methods have been developed to evaluate the viability and infectivity of the cysts of these parasites; currently, none of these seem to be suitable for routine application in the water and food industries. The gold standard is the animal infectivity assay, but this is prohibitive for routine analysis (Rousseau et al., 2018). Consequently, we propose that identification via PCR is a technique of choice because it can discriminate the species. Food should be free from the presence of protozoa and any contamination (by viable or non-viable protozoa) indicates failure in good agricultural practices. As a proof of principle, we could identify that salad produce sold in the street was contaminated. Thus, we were able, with our method, to detect salad produce infected; this can be sufficient information to take public health measures.

When the formalin-ether and the PCR detection for *Giardia* was applied on retail products by using the optimal conditions established at the laboratory, our method was able to identify two lettuces and one cabbage obtained from street vendors as positive. The percent of vegetables detected by the PCR is similar to that reported in India (Utaaker et al., 2017). Previous work by using microscopic detection reported 2% of *Giardia* cysts in produce from Norway (Robertson and Gjerde, 2001). Our findings are also similar to those reported in Jordan, where lettuce from street vendors had more *Giardia* than lettuce from supermarkets (Ismail, 2016). In addition, we could demonstrate that two of them were

A.



B.

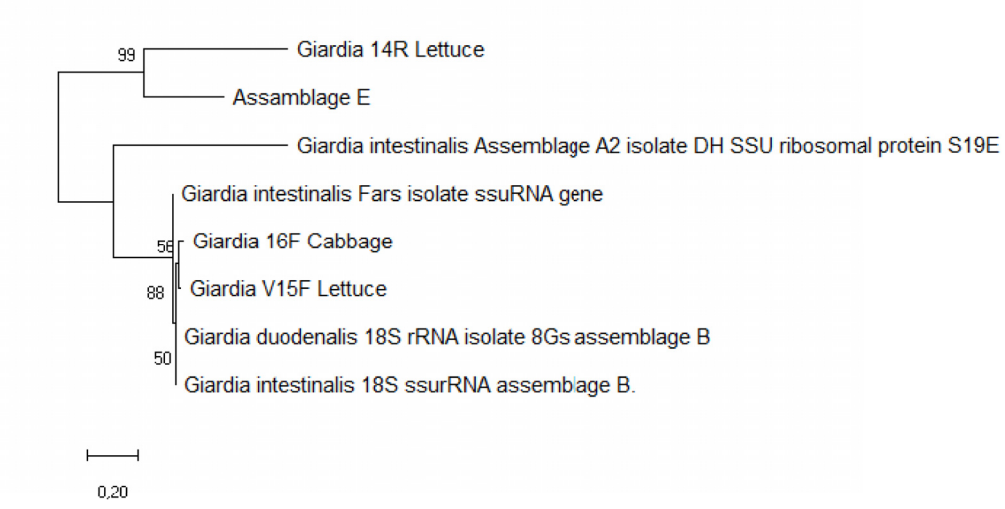


Fig. 3. A. Clustal alignment for polymorphic sites for *Giardia duodenalis* SSU rRNAr of three sequences obtained in street vendors of salad produce (Giardia14R Lettuce; Giardia 16F; Giardia v15 F) and reference sequences obtained at Genbank and Giardia DB databases, the number of sequences in databases are showed in parenthesis, conserved sites are highlighted. Periods (.) denote agreement with the consensus sequence. Dashes (–) denote insertions and deletions (INDELS) in the nucleotide sequence. B. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-484,28) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 87 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

of B assemblage, which is pathogenic to man and another one to assemblage E, not found in humans (Xiao and Fayer, 2008).

5. Conclusion

We report the laboratory validation of a new *Giardia* isolation method from salad produce that is cheap, reproducible and can identify *Giardia* on cabbage and lettuce. This work concentrated efforts to identify the best conditions for isolation and for PCR detection of *Giardia*. Future work with this method should establish the costs effectiveness and inter-laboratory reproducibility and also it should be analyzed if it can be used for other protozoa, like *Cryptosporidium* and *Cyclospora*.

Declarations

Author contribution statement

Natalia Hernández-Arango, Valeria Pinto, Deicy Muñoz-Sanchez: Conceived and designed the experiments; Performed the experiments.

Fabiana Lora Suarez: Conceived and designed the experiments.

Jorge Enrique Gómez-M: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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