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Development of a Method for Detection of *Giardia duodenalis* Cysts on Lettuce and for Simultaneous Analysis of Salad Products for the Presence of *Giardia* Cysts and *Cryptosporidium* Oocysts[▽]

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We report a method for detecting *Giardia duodenalis* cysts on lettuce, which we subsequently use to examine salad products for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. The method is based on four basic steps: extraction of cysts from the foodstuffs, concentration of the extract and separation of the cysts from food materials, staining of the cysts to allow their visualization, and identification of cysts by microscopy. The concentration and separation steps are performed by centrifugation, followed by immunomagnetic separation using proprietary kits. Cyst staining is also performed using proprietary reagents. The method recovered 46.0% ± 19.0% (n = 30) of artificially contaminating cysts in 30 g of lettuce. We tested the method on a variety of commercially available natural foods, which we also seeded with a commercially available internal control, immediately prior to concentration of the extract. Recoveries of the Texas Red-stained *Giardia* cyst and *Cryptosporidium* oocyst internal controls were 36.5% ± 14.3% and 36.2% ± 19.7% (n = 20), respectively. One natural food sample of organic watercress, spinach, and rocket salad contained one *Giardia* cyst 50 g^{-1} of sample as an indigenous surface contaminant.

With the increasing concern about transmission of pathogenic microorganisms by foods, there is a need to control the entire food chain, from primary producer to consumer. This can be accomplished through screening and certification programs, which apply highly sensitive and cost-effective methods for detecting food-borne pathogens. These will require the use of detection methods which are robust, reproducible, and practical.

The food industry is becoming increasingly aware of the potential for contamination of foodstuffs with the transmissive stages of *Giardia duodenalis* (9, 15, 17, 20). Fresh produce, in particular, as it is consumed with minimal preparation, is a potential vehicle of transmission, and *G. duodenalis* cysts have been detected on produce in several countries (1, 12, 13, 14). Contaminated irrigation water, especially, appears to constitute a major route of contamination of fresh produce (3, 21). Practical and reliable detection methods for monitoring foodstuffs will aid the prevention of parasitic disease outbreaks associated with contaminated food (8).

The procedure developed could be used by analytical laboratories that may be asked to analyze lettuce or salad products for more than one parasite. The simultaneous elution and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts from the same sample using a single extractant is the most convenient option, given that combination immunomagnetic separation (IMS) kits for both *Cryptosporidium* and *Giardia* are available commercially. Previously published methods that use IMS (12, 13, 14) used commercial kits that were developed for concentrating cysts and oocysts from water concentrates, not food matrices. These do not maximize cyst and oocyst recoveries from foods. In order to maximize cyst and oocyst recoveries from foods, the choice of pH is critical not only for extracting them from the food matrix but also for concentrating them by IMS (19). For *Cryptosporidium* oocysts, both the choice of extractant and method of extraction from foodstuffs influence their recoveries (4). The method of Cook et al. (4) developed for detecting *Cryptosporidium* oocyst contamination on lettuce (based on elution of oocysts with 1 M glycine [pH 5.5] followed by IMS and analysis by microscopy) is the only published, validated (5) method available. Lessons learned from developing methods for detecting *Cryptosporidium* oocysts as surface contaminants on fresh produce (19) underpin this study.

We report a method to detect *G. duodenalis* in lettuce, which was developed with a view to providing an analytical tool that would be suitable for routine adoption and future proposal as a standard. We used this method to determine whether commercially available, natural food samples contained *Giardia* cysts and *Cryptosporidium* oocysts as indigenous surface contaminants. In order to increase confidence in the method developed, we seeded each sample with a commercially available fluorogenic, internal control (Texas Red-stained *Giardia* cysts and *Cryptosporidium* oocysts) and then extracted each sample.

MATERIALS AND METHODS

Parasite. Human-derived *G. duodenalis* cysts were purified from fecal samples sent to the Scottish Parasite Diagnostic Laboratory (SPDL) for routine examination. Cysts were purified according to previously published methods within 3 days of submission. Briefly, stool samples were defatted, and cysts were concentrated by water-ether sedimentation (2), and the concentrated cysts in the water-ether pellets were further purified by sucrose flotation (specific gravity, 1.06) (16). Purified samples were suspended in reverse osmosis water and enumerated by hemocytometer, and the density of cysts was adjusted so that the final sus-

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pension applied to each sample contained ~100 cysts in 50 μl of phosphate-buffered saline (150 mM; pH 7.2).

Sample procurement and inoculation of cysts. Webb's lettuce was obtained from local wholesalers in York, United Kingdom. The outer leaves were discarded, and the remaining leaves were picked off and mixed prior to inoculation. Leaves were weighed onto plastic weighing boats, and each sample contained 30 g of lettuce. Each sample was artificially contaminated with approximately 100 cysts, by pipetting five 10-μl volumes of suspension at dispersed points on the surface of the topmost leaves. Once inoculated, samples were kept at room temperature for 2 h prior to analysis so that the surface moisture of the samples appeared as similar as possible.

Extraction of *Giardia* cysts. Extraction of cysts was performed according to the method of Cook et al. (4). The sample was placed in a filtered stomacher bag (Seward, London, United Kingdom). Two hundred milliliters of 1 M glycine was added to the bag, and the sample was stomached for 30 s to elute cysts from lettuce surfaces. The filter bag was pulled upward to remove the sample from the extractant and squeezed by hand to remove as much of the extractant as possible. The filter bag containing the lettuce sample was then discarded. The extractant was centrifuged at 2,500 × *g* for 10 min, the supernatant was decanted into a clean beaker, and the pellet (or pellets, if more than one tube was used for centrifugation) was collated into a glass Leighton tube (Dynal, United Kingdom) using a plastic pastette. The pellet was then resuspended in 10 ml of the supernatant prior to IMS.

IMS. IMS was performed to separate cysts from residual food materials by using Dynabeads GC Combo IMS test kits (Dynal, United Kingdom), according to the manufacturer's instructions. The final suspension (50 μl) was pipetted onto a well of a four-well microscope slide (C. A. Hendley, Loughton, Essex, United Kingdom) and air dried at room temperature.

Fluorescence labeling. Cysts were stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) that recognizes exposed epitopes on *Giardia* cysts (Giardia-a-Glo; Waterborne Inc., New Orleans, LA), according to the manufacturer's instructions. Oocysts were stained with FITC-conjugated MAb that recognizes exposed epitopes on *Cryptosporidium* oocysts (Crypt-a-Glo; Waterborne Inc., New Orleans, LA). Trophozoite and sporozoite nuclei were stained with the fluorogenic DNA intercalator 4′,6′-diamidino-2-phenylindole (DAPI) according to the method of Grimason et al. (7) as modified by Smith et al. (18). Samples were mounted in 60:40 glycerol:phosphate-buffered saline containing 2% (wt/vol) of the antifade chemical 1,4-diazabicyclo(2,2,2)octane, and then each microscope slide was covered by a glass coverslip, which was sealed onto the slide using clear nail varnish, and viewed within 30 min of preparation.

Microscopy. Microscopy was performed on an Olympus BH2 fluorescence microscope (40× and 100× objectives; 12.5× eyepieces), equipped with Nomarski differential interference contrast optics. A blue filter (excitation, 480 nm; emission, 520 nm) was used for the detection of FITC-conjugated MAb-labeled (oo)cysts and a UV filter block for DAPI (excitation, 350 nm; emission, 450 nm). A green filter block (excitation, 535 nm; emission, >590 nm) was used to visualize Texas Red staining. All evaluations for the presence of fluorescent nuclei and internal morphology were undertaken at magnifications of both ×500 and ×1,250.

Method development: determination of the mean percentage recovery. The percentage of cysts recovered by the method was determined by comparing the number of cysts applied to each lettuce sample and the number recovered from the same sample. To determine the number of cysts applied to each sample from the cyst suspension, 10 50-μl volumes of suspension were dispensed onto 10 separate wells of four-well microscope slides. The same suspension was used to inoculate lettuce samples, which were then analyzed. Three samples were analyzed at a time. The mean number of cysts recovered from each batch of three samples was compared with the mean number of cysts in 50 μl of the suspension used to inoculate them, and the percentage of recovery was calculated. The mean percent recovery from 10 batches of three samples was then determined.

Analysis of fresh produce for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. Two batches, each of 10 separate salad products intended to be consumed raw, were collected from local retail outlets in York, United Kingdom (see Table 2) and were extracted at the Central Science Laboratory (CSL), York. Due to the variability in surface moisture of the ready-to-eat salad samples, it was not possible to obtain a similar level of surface moisture residue, visually, after drying the samples for a fixed (~2 h) time period. Therefore, each sample was seeded with a commercially produced, quality-assured, known dose of reporter *Giardia* cysts and *Cryptosporidium* oocysts (BTF ColorSeed, batch number CS-CG100-48) and extracted within 1 h of inoculation. This product contains 100 fluorescence-activated cell-sorted, Texas Red-stained *Cryptosporidium* oocysts and 100 similarly stained and sorted *Giardia* cysts which were used as internal controls to determine the recovery efficiency of the method used for recovering

TABLE 1. pH optimization of 1 M glycine for extracting *G. duodenalis* cysts from lettuce samples

pH	% Recovery[a]
3.0	5.4 (± 0.9)
3.5	3.9 (± 3.1)
4.0	3.0 (± 0)
4.5	25.1 (± 8.2)
5.0	39.3 (± 17.0)
5.5	34.9 (± 22.6)
6.0	36.9 (± 15.6)
6.5	24.1 (± 2.3)
7.0	25.1 (± 13.1)

[a] Values are means (± standard deviations) ($n = 3$).

Giardia cysts and *Cryptosporidium* oocysts from the specified foodstuffs (see Table 2). Extracts from samples 1 to 10 were prepared on 8 March 2004 at CSL and sent to SPDL by courier that day. Extracts from samples 11 to 20 were prepared on 16 March 2004 at CSL, stored at 4°C, and then sent to SPDL by courier on 17 March 2004.

SPDL received coded extracts (1 to 10) on the late afternoon of 9 March 2004, and the samples were kept in the dark at 4°C until the following day (10 March 2004) when they were concentrated by IMS. Coded samples (11 to 20) were received on 18 March 2004 and were concentrated on the same day. Immediately after IMS, the concentrates were placed on welled microscope slides, air dried, and fixed in absolute methanol. Slides were stained just prior to microscopy. Microscopical identification was performed by two individuals, each with a minimum of 5 years of training in the identification of both *Giardia* cysts and *Cryptosporidium* oocysts.

The percentage of *Giardia* cysts and *Cryptosporidium* oocysts recovered by the method was determined by comparing the number of Texas Red-stained cysts and oocysts recovered from each sample to the number of cysts and oocysts applied to the same sample. All cysts and oocysts present on the microscope slide were stained with FITC-conjugated MAbs. These were then enumerated under the FITC filter set. However, only the Texas Red-stained reporter cysts and oocysts could be visualized and enumerated under the Texas Red filter of the epifluorescence microscope. By subtracting the number of Texas Red (red)-stained cysts and oocysts from the total number of FITC (green)-stained cysts and oocysts, the number of cysts and oocysts naturally contaminating the product could be calculated.

All procedures performed at the SPDL, with the exception of those developed specifically for this study, were conducted in compliance with standard operating procedures accredited by Clinical Pathology Accreditation Ltd. (United Kingdom) and with United Kingdom Drinking Water Inspectorate Regulatory *Cryptosporidium* standard operating procedures.

RESULTS AND DISCUSSION

The procedure used for extracting *Giardia* cysts from lettuce is identical to that used for *Cryptosporidium* oocysts (4) so that analytical laboratories which may be asked to analyze lettuce or salad products for more than one parasite can simultaneously elute and enumerate *Cryptosporidium* oocysts and *Giardia* cysts from the same sample.

The mean (± standard deviation) number of cysts applied onto each lettuce sample from the cyst suspension, following analysis of 10 separate wells of four-well microscope slides, was 87.1 ± 18.4. A pH range of between 4.5 and 7.0 generated similar cyst recoveries (24.1% to 39.3%) (Table 1), which were higher than those obtained using glycine buffers ranging from pH 3.0 to 4.0 (3.0% to 5.4%) (Table 1). Recoveries using glycine between pH 5.0 and 6.0 generated the highest recoveries (Table 1) but had large standard deviations. In keeping with the buffer used for recovering *Cryptosporidium* oocysts from lettuce (4), 1 M glycine (pH 5.5) was chosen to recover *Giardia* cysts from lettuce samples.

TABLE 2. Description of natural samples analyzed and outcome of analysis of foodstuff samples for the presence of *Cryptosporidium* oocysts and *Giardia* cysts

Sample no.	Product	Amt analyzed	% Recovery with ColorSeed		Natural contamination (no. of cysts per 50 g of sample)		Slide background[a]
			Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*	
1	Romaine hearts	150 g	13	38	None	None	H
2	Mixed chilies	All contents of packet	20	21	None	None	L
3	Continental four-leaf salad	100 g	26	38	None	None	H
4	Crispy green salad	100 g	25	39	None	None	H
5	Organic gem lettuce	1 Head	3	31	None	None	H
6	Lettuce sticks	5 Sticks	25	23	None	None	M
7	Sliced, peeled carrots	All contents of packet	46	34	None	None	M
8	Fusion salad	100 g	15	44	None	None	H
9	Mange-touts	100 g	18	20	None	None	M
10	Cantonese vegetable stir-fry	100 g	44	20	None	None	M
11	Organic herb salad	50 g	70	57	None	None	H
12	Curly parsley	30 g	59	43	None	None	L
13	Spring onions	3 Pieces	46	38	None	None	H
14	Organic iceberg lettuce	70 g	49	65	None	None	H
15	Sliced, peeled carrots	50 g	26	28	None	None	H
16	Birds Eye chillies	14 g	16	5	None	None	M
17	Organic watercress, spinach, and rocket salad	50 g	62	53	None	1	M
18	Organic bay leaf salad	50 g	60	49	None	None	H
19	Pak choi	1 Piece	57	44	None	None	M
20	Baby sweet corn and asparagus	100 g	45	41	None	None	M

[a] Background contamination of slides was rated as high (H), medium (M), or low (L).

Cook et al. (4) determined that 1 M glycine (pH 5.5) extracted the highest percentage of *Cryptosporidium parvum* oocysts from artificially seeded lettuce (59.0% ± 12.0%; $n = 30$). In a preliminary experiment, a pH range of between 4.5 and 7.0 generated the highest *Giardia* cyst recoveries (24.1% to 39.3%) (Table 1). Recoveries using 1 M glycine at pH 5.0 (39.3% ± 17.0%), pH 5.5 (34.9% ± 22.6%), and pH 6.0 (36.9% ± 15.6%) were similar. Unlike *Cryptosporidium* oocyst recovery from lettuce, where two peaks in recovery (pH 3.0 and 5.5) (4, 19) were noted following elution with 1 M glycine, cyst recoveries plateau between pH 5.0 and 6.0 (Table 1). This is suggestive of the fact that the noncovalent interactions between *G. duodenalis* cysts and lettuce differ from those between *C. parvum* oocysts and lettuce, but at a pH of around 5.5 the release of both *G. duodenalis* cysts and *C. parvum* oocysts from lettuce can be maximized. Thus, in keeping with the buffer used for *Cryptosporidium* oocyst recovery from lettuce, 1 M glycine (pH 5.5) was also chosen to recover cysts from lettuce.

The percentage of cysts recovered from 30 samples of 30 g of lettuce each inoculated with ~100 cysts was 46.0% ± 19.0%. As the dispatch of samples from CSL and their receipt at SPDL could take more than 1 day, we assessed the stability of the seeded samples over a 24-h period, which we judged satisfactory, with similar recoveries achieved on days 0 and 1 (50.3% ± 13.1% and 42.2% ± 17.6%, respectively).

The infectious dose of human-derived *G. duodenalis* ranges from 25 to 100 cysts, but 10 cysts caused infection in two human volunteers (10, 11); therefore, the recovery efficiency of this method will allow identification of lettuce samples contaminated with sufficient viable *Giardia* cysts to pose a risk of infection upon consumption.

The results of the analysis of fresh produce sold at retail are presented in Table 2. No *Cryptosporidium* oocysts were detected as natural contaminants. One sample (sample 17, a fresh salad mixture of organic watercress, spinach, and rocket salad) contained one *Giardia* cyst 50 g^{-1} of sample as an indigenous surface contaminant. The morphology of this cyst indicated that it was not viable: the cytoplasm of the trophozoite was contracted from the cyst wall and had condensed into the center of the cyst; therefore, it would not have presented a risk had it been consumed.

Using the Texas Red-stained *Giardia* cyst and *Cryptosporidium* oocyst reporter system, *Giardia* cyst recoveries were 36.5% ± 14.3%, and *Cryptosporidium* oocyst recoveries were 36.2% ± 19.7% ($n = 20$). In many instances, cyst and oocyst recoveries differed considerably for individual matrices (Table 2). The recoveries using the Texas Red-stained *Giardia* cyst and *Cryptosporidium* oocyst reporter system for fresh produce differed from those we obtained with the assay for *Giardia* on lettuce (36.5% ± 14.3% [$n = 20$] versus 46.0% ± 19.0% [$n = 30$], respectively), and this difference is probably due to the variability of the noncovalent interactions between cyst and oocyst surfaces and surfaces of the various fresh produce types we tested.

Furthermore, in many instances cyst and oocyst recoveries differed considerably for individual matrices (Table 2), indicating that the noncovalent interactions responsible for *Giardia* cyst and *Cryptosporidium* oocyst attachment onto these matrices differ and that 1 M glycine (pH 5.5) may not optimize cyst and oocyst release from all salad vegetables. This, we suspect, will be true for the extractants used in other published methods. This finding has two consequences: first, that previously

published analyses may well be sizeable underestimates, depending on the food matrices used and, second, that it is imperative that a reporter system is incorporated into this analysis, particularly when a variety of matrices are tested. We recommend the Texas Red-stained *Cryptosporidium* oocyst and *Giardia* cyst reporters for such analyses.

In conclusion, we have demonstrated the effectiveness of our method for analysis of food samples for the presence of *Giardia* (and, by inference since the same extraction procedure is used, for *Cryptosporidium*). Furthermore, as the size of the lettuce samples analyzed was determined by the quantity of the foodstuff which would normally constitute a portion size (6), the developed method should be useful in exposure assessment studies for any microbiological risk assessment for *Giardia* (and *Cryptosporidium*) on lettuce. Incorporation of the fluorogenic oocyst and cyst reporter system increased quality assurance and identified the importance of the food matrix effect in various commercial salad products.

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