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 fluorescent, 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-
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 Pastelette. 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 for 2 h prior to analysis so that the surface moisture of the samples was identical to that used for

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-diamino-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 (150 mM; pH 7.2).

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.

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 MAb. 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.

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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 (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 we obtained with the assay for Giardia 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 reporter 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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REFERENCES