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Towards standard methods for the detection of *Cryptosporidium parvum* on lettuce and raspberries. Part 1: Development and optimization of methods

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Abstract

No standard method is available for detecting protozoan parasites on foods such as soft fruit and salad vegetables. We report on optimizing methods for detecting *Cryptosporidium parvum* on lettuce and raspberries. These methods are based on four basic stages: extraction of oocysts from the foodstuffs, concentration of the extract and separation of the oocysts from food materials, staining of the oocysts to allow their visualization, and identification of oocysts by microscopy. The concentration and separation steps are performed by centrifugation, followed by immunomagnetic separation using proprietary kits. Oocyst staining is also performed using proprietary reagents. The performance parameters of the extraction steps were extensively optimized, using artificially contaminated samples. The fully developed methods were tested several times to determine their reliability. The method to detect *C. parvum* on lettuce recovered 59.0±12.0% (n=30) of artificially contaminated oocysts. The method to detect *C. parvum* on raspberries recovered 41.0±13.0% (n=30) of artificially contaminated oocysts.
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1. Introduction

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 detection of foodborne pathogens. These will require the use of standardized detection methods, which are robust and reproducible. Standard methods are also necessary for informing food hazard management systems, supporting enforcing authorities, and facilitating tracing of illness-causing microorganisms.

There are no standardised methods for detecting the transmissive stages of protozoan parasites on/in foods, although the potential for contamination of foodstuffs with the transmissive stages of *Cryptosporidium parvum* is gaining increasing attention (Girdwood and Smith, 1999; Rose and Slifko, 1999; Nichols and Smith, 2001). Fresh produce in particular, as it is consumed with minimal preparation, is a potential vehicle of transmission, and *C. parvum* has been detected on produce in several countries (De Oliveira and Germano, 1992; Monge and Arias, 1996; Ortega et al., 1997; Robertson and Gjerde, 2000, 2001a; Robertson et al., 2002) using reagents and methods developed for detecting *Cryptosporidium* oocysts in water concentrates. Practical and reliable detection methods for monitoring foodstuffs will aid prevention of parasitic disease outbreaks associated with contaminated food (Jaykus, 1997).

We report methods to detect *C. parvum* in lettuce and raspberries, which were developed with a view to providing analytical tools which would be suitable for routine adoption and future proposal as standards.

2. Materials and methods

2.1. Source of *C. parvum* oocysts

Recently excreted, purified *C. parvum* oocysts (Iowa isolate, previously known as *C. parvum* genotype 2) were purchased

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from Pleasant Hill Farm (PHF), Troy, Idaho, USA. Purification was performed by ether sedimentation and sucrose flotation at PHF and oocysts were dispatched by courier to the SPDL. Oocyst viability, determined by the methods of Campbell et al. (1992, 1993) and Robertson et al. (1993), remained above 85% throughout the course of the trials. Purified oocyst suspensions were prepared in phosphate-buffered saline (PBS, 150 mM; pH 7.2) and enumerated by haemocytometer. Oocyst suspensions, used for assessing optimized extractant formulation for lettuce and raspberries, compatibility testing for IMS and artificially contaminating lettuce and raspberries, contained approximately 100 oocysts in 50 µl PBS, unless otherwise stated. Ten replicate counts were determined for each oocyst suspension, prior to use.

2.2. Food samples

Webb's lettuce were obtained from local wholesalers. Each sample consisted of 30 g lettuce leaves. Outer leaves were discarded, and the remaining leaves removed individually and mixed prior to artificial contamination.

Fresh raspberries were obtained from local wholesalers, and each sample consisted of 60 g berries.

2.3. Artificial contamination of samples

Individual (30 g) lettuce leaf samples were weighed in plastic, disposable weighing boats and placed, with leaves partially overlapping, in a shallow container. Five × 10 µl volumes of oocyst suspension were pipetted onto five separate areas on the surface of the topmost leaves of each sample, which were then left at room temperature for approximately two hours until the suspending fluid was almost dry, following which the extraction procedure was undertaken.

Individual (60 g) raspberry samples were weighed in plastic, disposable weighing boats. Five 10 µl volumes of oocyst suspension were pipetted onto separate berries in each sample. The samples were then left at room temperature for approximately two hours until the suspending fluid was almost dry, then the extraction procedure was applied.

2.4. Extraction of oocysts

This involved addition of a liquid extractant to the sample, followed by stomaching, pulsification, orbital shaking or rolling to facilitate removal of the oocysts from the food surface. The extract was then concentrated by centrifugation, before immunomagnetic separation (IMS) of the oocysts from the residual food materials. Stomaching, pulsification and orbital shaking were evaluated for use with lettuce. As we found that stomaching and pulsification damaged raspberry surfaces excessively, only orbital shaking and rolling were evaluated for use with raspberries.

2.4.1. Extractants

These included mild buffers and detergents. Their formulations appear in Table 1.

Table 1
Evaluation of extractants for compatibility with the Crypto-Scan® IMS test kit (ImmuCell Corporation, Portland, Maine): recovery efficiencies after extraction of oocysts from the artificially contaminated extractant

Extractant	Percentage oocysts recovered [a]
0.1 M HEPES, pH 5.5	89.4 (±5.1)
1 M sodium bicarbonate, pH 6.0	88.2 (±9.8)
1 M glycine, pH 5.5	105.3 (±18.4)
1 M bicine, pH 5.6	81.1 (±12.5)
1% lauryl sulfate	90.2 (±5.5)
EB [b]	5.9 (±4.5)
0.1 M tricine, pH 5.4	9.8 (±2.5)
PBS, pH 7.2	11.4 (±5.7)

[a] Values are the means of three replicate tests. Figures in parentheses are standard errors.

[b] This is the buffer recommended in the US EPA's method 1623 ("*Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA") for the extraction of oocysts from capsule filters. Its composition is: 0.01 M Tris pH 7.4, 0.1% lauryl sulfate, 0.005 M EDTA, 150 ppm antifoam A.

2.4.2. Stomaching

Each sample was placed in a filtered Stomacher® bag (Seward, UK), 200 ml extractant was added, then the sample was stomached for 1 min. The filter was pulled upwards in the stomacher bag to remove the lettuce sample from the extractant, then squeezed by hand to remove as much of the fluid as possible. The filter bag containing the lettuce sample was then discarded.

2.4.3. Pulsification

Pulsification is an alternative method to stomaching; through rapid beating of a sample, it can release microorganisms from food surfaces into suspension. Each sample was placed in a filtered Stomacher® bag (Seward, UK), 200 ml extractant was added, and the sample was pulsified in a Pulsifier™ (Microgen Bio Products Ltd., UK) for 1 min. The filter was pulled upwards in the stomacher bag to remove the lettuce sample from the extractant, then squeezed by hand to remove as much of the fluid as possible. The filter bag containing the lettuce sample was then discarded.

2.4.4. Orbital shaking

Each sample was placed in a clean 250 ml glass beaker, then 150 ml extractant was added. The beaker was placed on an orbital shaker and shaken at low speed (80 rpm) for 1 min. The contents of the beaker were then strained through a filtered stomacher bag into another clean 250 ml beaker. A further 50 ml extractant was used to rinse out the beaker, which was poured over the raspberries suspended in the filter, into the beaker containing with the original extract.

2.4.5. Rolling

Each sample was placed in a clean 500 ml plastic centrifuge pot (Nalgene®), 150 ml extractant was added then the pot was capped. The pot was placed on a spiral roller and rolled for 1 min (Spirimax 5, Denley, UK). The contents of the pot were then strained through a filtered stomacher bag into a clean 250 ml glass beaker. A further 50 ml extractant were used to rinse out the pot, which was poured over the raspberries

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