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ScienceDirect
Journal of Microbiological Methods 70 (2007) 312–318
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

# Development of a method for the detection of waterborne microsporidia

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Received 19 January 2007; received in revised form 1 May 2007; accepted 9 May 2007
Available online 21 May 2007

**Abstract**

Microsporidia are obligate intracellular pathogens capable of infecting humans. There is credible evidence to suggest that microsporidial infections may be transmitted through consumption of spores in contaminated water; however, methods to detect this pathogen have not been standardized and microsporidia occurrence studies have not been conducted. Concentration of spores by continuous flow centrifugation (CFC), purification using immunomagnetic separation (IMS), and detection by either microscopy or real-time polymerase chain reaction (PCR) were evaluated for detection of *Encephalitozoon intestinalis* spores in seeded water samples. Recovery efficiency of CFC using microscopic detection ranged from 38.7–75.5% in filtered tap water. Using an indirect IMS method, 78.8–90.2% of seeded spores were recovered in ultrapure water (18 MΩ); however, the lack of a specific monoclonal antibody and the presence of other particulates interfered with the IMS assay in some turbid samples. Despite low recovery efficiencies and the detectable presence of PCR inhibitors in each of the samples, a combination of CFC concentration, indirect IMS, and real-time PCR produced a positive test result in six of ten natural water samples (turbidity 0.1–28.9 NTU) at a seeding level of 50 spores/L.


*Keywords:* Continuous flow centrifugation; Encephalitozoon; Enterocytozoon; Microsporidia

## 1. Introduction

Microsporidia are a group of obligate intracellular pathogens capable of initiating disease in a plethora of vertebrate and invertebrate hosts (Wittner and Weiss, 1999). Most human infections are found in immunocompromised populations and produce a variety of systemic and non-systemic diseases (Didier et al., 1998; Garcia et al., 1994; Kotler and Orenstein, 1998). The genera most often responsible for these infections include *Encephalitozoon* (*E. cuniculi*, *E. hellem*, *E. intestinalis*) and *Enterocytozoon* (*E. bienusi*). These organisms produce small (1–2 μm) environmentally-resilient spores, that initiate infection after oral ingestion of spores through contaminated food, water or fomites.

Detection methods for waterborne protozoa typically involve the combination of three components: 1) collection of large volume water samples (1–100 L) using filtration, flocculation, or centrifugation; 2) selective purification of the target organism from the filter eluate, floc, or pelleted material; and 3) detection of the target organism using microscopy or molecular methods. Unlike other protozoa such as *Cryptosporidium* and *Giardia*, there is no standardized method for detection of microsporidia in water samples. Using an assortment of experimental methods, several groups have documented the presence of microsporidia in surface and recreational water (Dowd et al., 1998, 2003; Fournier et al., 2000; Coupe et al., 2006; Fournier et al., 2002). As a result of these findings, microsporidia were placed on both the 1998 and 2005 U.S. EPA Contaminant Candidate List for drinking water (CCL-1, CCL-2) (Federal Register, 1998, 2005). The Federal Register comments specifically note gaps in detection methods, treatment data, and occurrence studies for these organisms. A few studies have attempted to recover microsporidia from water samples (Dowd et al., 1998; Li et al., 2003; Stine et al., 2005); each report describes relatively cumbersome methods and all have met with

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doi:10.1016/j.mimet.2007.05.005

limited success, suggesting that detection of microsporidia in environmental samples may prove even more difficult than *Cryptosporidium* and its presence may be significantly underestimated. It will not be possible to assess the magnitude of the risk attributable to waterborne microsporidia until there is a validated, reliable method for detecting this microorganism in water.

This work evaluates individual components that may be useful for comprehensive method development. The specific objectives of this study were to 1) optimize and evaluate continuous flow centrifugation (CFC) for concentration of microsporidia from water; 2) develop and validate a method for IMS capture of microsporidia from concentrated water samples; 3) evaluate the combined approach of CFC, IMS, and real-time PCR to detect microsporidia in seeded water samples.

## 2. Materials and methods

### 2.1. Spore production

Spores of *E. intestinalis* (ATCC 50603), were propagated in rabbit kidney cells (RK-13) according to published methods (Wolk et al., 2000). Spores were recovered from spent culture medium by centrifugation and purified from cell debris using a differential density gradient centrifugation. Spore-containing pellets were washed with sterile NANOpure water and stored in PBS or sterile NANOpure water at 4 °C. For all seeding experiments, precisely enumerated spore standards were prepared in 1.5 mL eppendorf tubes using flow cytometry with cell sorting (FCCS) according to methods published for production of *Cryptosporidium* and *Giardia* standards (Hoffman et al., 2003). Spore standards and controls were prepared using either unlabeled spores or spores that had previously been labeled with a FITC-conjugated polyclonal antibody.

### 2.2. Polyclonal antibody

Antibody labeling was performed using an affinity-purified, FITC-conjugated polyclonal rabbit anti-*Encephalitozoon* antibody generated against UV-treated *E. cuniculi*, *E. hellem*, and *E. intestinalis* spores and conjugated to FITC (Bethyl Corp, Montgomery, TX).

### 2.3. Continuous flow centrifugation (CFC)

A channel-type continuous flow centrifuge (Amicus Separator, Baxter Corporation, Round Lake, IL) was used to concentrate water samples as described previously (Borchardt and Spencer, 2002) with the modifications described below. Before centrifuging a sample, the flexible plastic, belt-like separation and input/output tubing were primed with phosphate buffered saline (PBS) containing 0.1% Tween 80 (Sigma, St. Louis, MO). Standards containing either 100, or 1000 *E. intestinalis* spores, previously labeled with FITC-conjugated polyclonal antibody, were prepared using flow cytometry and the spores were seeded into either 10 L of filtered tap water or natural water samples. All samples were processed at a feed rate of 70 mL/min and a relative gravitational force of approximately 900 ×*g*. Particulates were retained in the separation chamber and the supernatant was pushed out of the belt via the output line (Fig. 1). Following centrifugation, the contents of the separation chamber were drained into a beaker. The separation chamber was rinsed three times with 30 mL PBS-Tween 80 (0.05% v/v) solution to dislodge any spores that may have adhered to the inner chamber walls. Rinse-volumes and separation chamber contents were poured into four 50 mL polycarbonate tubes and centrifuged at 31,500 ×*g* for 50 min. The supernatant was aspirated and the four pellets and rinse-volumes were combined resulting in a 2 mL volume in a single tube. The sample was further concentrated by centrifugation (14,500 ×*g*, 7 min) to a final volume of 400 µL. The sample was transferred to welled slides and allowed to dry completely before mounting with antifade mounting media (MeriFluor mounting media, Meridian Bioscience, Cincinnati, OH). The effectiveness of spore recovery for the CFC procedure is reported as percent recovery (i.e. total number of spores added to the water sample divided by the number recovered multiplied by 100). For initial evaluation, organisms that had been previously labeled with FITC-conjugated polyclonal anti-*Encephalitozoon* antibody were used to allow the research teams to more accurately assess percent recovery and efficiency of the instrumentation.

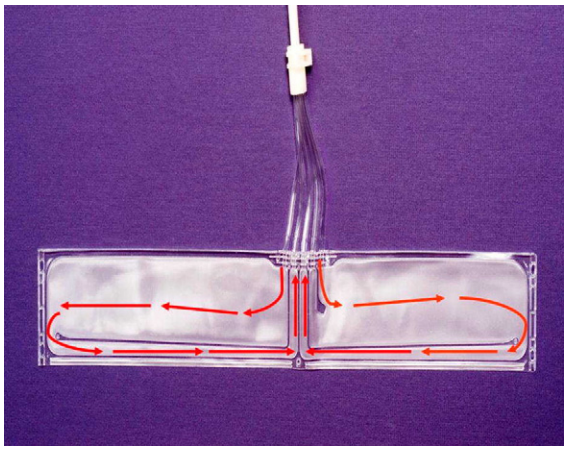

Fig. 1. Plastic disposable channel apparatus of the Amicus® continuous flow centrifuge. Each apparatus consists of two channels allowing two samples to be independently centrifuged at the same time. Arrows indicate the water flow paths.

### 2.4. Indirect immunomagnetic separation (IMS)

#### 2.4.1. Captivate™ ferrofluid

Spore standards containing either 943 (SD 3.2, $n=3$) or 500 (SD 8.0, $n=5$) *E. intestinalis* spores, previously labeled with FITC-conjugated polyclonal anti-*Encephalitozoon*, were prepared in 0.5 mL reagent water and 0.5 mL PBS with 0.01% Tween 20 and 0.1% BSA. Twenty µL Captivate™ ferrofluid goat anti-rabbit immunoglobulin (Ig) supermagnetic particles (Molecular Probes, Eugene, OR) were added and the suspensions were rocked for 60 min at room temperature (20–25 °C). The tubes were placed in a magnetic particle concentrator and