Development of Detection Methods for Waterborne Microsporidia

Project Scope

Microsporidia are a diverse group (1,200 species belonging to 143 genera) of obligate intracellular protozoa that are pathogenic to a broad range of vertebrate and invertebrate hosts. At least 13 microsporidia, including members of the genera *Encephalitozoon* and *Enterocytozoon*, cause disease (e.g., gastroenteritis, encephalitis) in humans—especially among those with compromised immune systems. The infectious form of microsporidia is a small (~2 µm diameter) spore which is resistant to environmental conditions and conventional water treatment practices.

Recognizing the potential of microsporidia to be transmitted through drinking water, EPA placed microsporidia species on the first (1998) and most recent (2005) Contaminant Candidate List (CCL). Notably, the 1998 CCL included microsporidia on a list of contaminants for which improved analytical methods were necessary and constituted a research priority. This project aimed to develop a sensitive and reliable recovery method for microsporidia spores using combined molecular biotechnologies. The main objectives of this research were to:

- Generate purified spores for development of analytic methods;
- Develop/optimize an efficient sample collection method;
- Develop methods for sample concentration/purification by flow cytometry;
- Conduct diagnostic assay/viability testing; and
- Validate a finished method in natural waters.

To carry out these objectives, the researchers conducted a series of experiments using spores in the attempt to develop a finished method that could be used to assess the fate, ecology, and

Grant Title and Principal Investigators

Development of Detection and Viability Methods for Waterborne Microsporidia (EPA Grant #R828041)

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Key Findings and Implications

Analytical Accomplishments:

- Demonstrated that existing cell culture propagation methods for three common strains of microsporidia produce two types of spores that differ in infectivity.
- Showed that continuous flow centrifugation and real-time polymerase chain reaction (PCR) can effectively concentrate and detect waterborne microsporidia, respectively; however, the reliability of the immunomagnetic separation (IMS) element for both approaches is questionable.

Implications of Research and Impacts of Results:

- The presence of smaller, noninfective spores should be considered when designing water disinfection studies because they comprised the majority (~80 percent) of the spore suspensions prepared in this project; thus, failure to account for the large number of noninfectious spores in any given dose could result in a significant overestimation of viability reduction.
- IMS recovery could be significantly improved if a new, highly specific monoclonal antibody were developed and incorporated into the microsporidia assay developed in this project.
- Without the ability to effectively purify spores from sample concentrates, method development efforts for microsporidia will likely stall, making it extremely difficult to study the fate, ecology, and distribution of these pathogens in the environment and in drinking water.
- Reverse transcriptase PCR cell culture assays were successful in determining spore viability; however, the PCR component appeared to be specific for different genes for each species tested, meaning a nested primer or multiplex approach would be necessary to identify all three *Encephalitozoon* species using one assay.

Publications include 1 peer reviewed journal article and 6 conference/symposium presentations.

Project Period: August 2000 to October 2003

Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes directly to the second of three Long-term Goals for drinking water research: (2) By 2010, develop new data, innovative tools and improved technologies to support decision making by the Office of Water on the Contaminant Candidate List and other regulatory issues, and implementation of rules by states, local authorities, and water utilities.

The inclusion of microsporidia on the first CCL and its categorization as requiring improved analytical methods for its detection was the impetus for this project. The continued inclusion of microsporidia on the current 2005 CCL shows that there is a continuing need to conduct research, including methods-related research, on this diverse group of microorganisms. The technologies assessed in this project included flow cytometry/cell sorting and continuous flow centrifugation for the recovery of microsporidia from large sample volumes, and DNA amplification/hybridization for diagnostic assay of purified water concentrates. The performance of the detection method developed in this project was assessed in field trials conducted across a variety of natural waters. Despite many inroads made on the detection of waterborne microsporidia, the authors concluded that without the ability to effectively purify spores from sample concentrates, they could not develop a complete method that would detect microsporidia in natural waters. Efforts to determine spore viability met with mixed success, successfully identifying different assays to determine viability in three *Encephalitozoon* species. Based on their findings, however, the researchers were able to recommend areas of future research that could improve the analytical detection approaches developed during this project.

distribution of infectious microsporidia in the aquatic environment.

Project Results and Implications

Identification of Variation Within Cell Culture-Propagated Spore Suspensions: The investigators propagated spores of *Encephalitozoon cuniculi, E. hellem,* and an intestinal isolate of *E. intestinalis i*n a rabbit kidney cell line which were subsequently purified using density gradient separation. Flow cytometric analyses of purified spore suspensions produced over a 1-year period showed that freshly harvested suspensions produced using these spore propagation methods were comprised of two subpopulations differing in physical size, permeability to vital dyes, antibody staining, and infectivity in cell culture assays. The morphological identity of the smaller spore type was not identified; it is possible they are an artifact of the tissue culture propagation methods used by the research team in this project. This finding has significant implications for previous and future disinfection studies because these smaller, noninfectious spores comprised the majority (nearly 80 percent) of the purified spore suspension. Thus, failure to account for the large number of noninfectious spores in any given dose could result in a significant overestimation of viability of 1 log unit (90 percent deactivation) or greater. Consequently, the methods development work summarized below was performed using infectious, large subpopulation microsporidia spores that comprised nearly 16 percent of the spore total.

Development of Polyclonal Antibody to *Encephalitozoon* and Characterization of an Existing Antimicrosporidia Antibody: Several antibodies were evaluated for use in concentration/purification methods for microsporidia. Monoclonal antibody 7G7 exhibited a variable staining pattern, brightly staining the small spore subpopulation but only dimly staining the larger, infectious spore subpopulation. Rabbit polyclonal antibody produced as part of this study stained only *E. cuniculi*. However, an additional rabbit polyclonal antibody generated against ultraviolet-treated *Encephalitozoon* spores was highly reactive with all three *Encephalitozoon* species utilized in this project and was used in the analytical methods development studies that are summarized below.

Evaluation of Flow Cytometry With Cell Sorting (FCCS) for Isolation of Microsporidia From Reagent Water and Prepared Turbid Water Concentrates: Microsporidia standards purified using flow cytometry containing either 100 spores prelabeled with fluorescein isothiocyanate (FITC)-conjugated polyclonal

antibody or 100 unlabeled spores were sorted from nontarget particles using FCCS. Recovery of prelabeled microsporidia averaged 93.1 percent while recovery of the non-prelabeled spores (subsequently labeled in solution) averaged 82.2 percent. Recovery of microsporidia spores spiked into a prepared turbidity matrix ranged from 63.9 to 79.4 percent.

Evaluation of Continuous Flow Centrifugation (CFC) for Concentration of Microsporidia Spores From Water Samples: The continuous flow centrifuge evaluated in this study was a modified blood aphersis (blood component separating) unit that concentrates particles in a flexible plastic belt. Standards of precisely enumerated, fluorescently-labeled *E. intestinalis* spores were added to 10 L filtered tap water, natural water samples, or 2 Nephelometric Turbidity Unit (NTU; a measure of turbidity) water. Following sample concentration, particulates were eluted from the belt, concentrated using multiple, high-speed centrifugations, and examined microscopically. Recovery efficiencies from spiked tap water are summarized in Table 1.

Table 1. Spore Recovery from 10 L Spiked Water Samples Using CFC Concentration and Microscopic Examination

Spore Concentration (per L)	Mean (%)	Range (%)
10	61.5	38.7 - 75.5
100	63.8	52.0 - 75.5

Development of Immunomagnetic Separation (IMS) Methods for Purification of *E. Intestinalis* Spores From Water: The spore capture efficiency of eight IMS constructs was evaluated in this study. The highest recoveries were achieved using indirect IMS methods, in which water concentrates were labeled with polyclonal anti-microsporidia antibody and the antibody-labeled spores were subsequently purified using IMS particles coated with anti-rabbit immunoglobulin G. Two commercially available IMS products, Dynabeads® (2.5 µm) and Captivate™ ferrofluid (200 nm), successfully purified spores from reagent water. The Dynabeads® product recovered an average of 90.2 percent of 1,000 spores seeded into reagent water while the Captivate™ product recovered an average of 79 percent at both the 500 and 1,000 spore level. The Captivate™ product also was tested using dilute pond water and recovered 67 percent of 500 spores seeded into the sample. However, retrieval of the smaller Captivate™ magnetic particles from samples with higher turbidity was not successful. The research team speculated that particulate matter from the sample concentrate coats the magnetic particles and reduces their attraction to the magnet prior to recovery. For this reason, further IMS studies were performed using the larger Dynabeads®. Additional evaluation of the IMS method using quantitative, real-time polymerase chain reaction (PCR; summarized below) to assess relative recovery of microsporidia spores in natural water samples showed that IMS using polyclonal antibodies may not offer the sensitivity and specificity needed for reliable detection of these protozoa. Although, the relative recovery of microsporidia spores averaged 46.1 percent in reagent water, the IMS-real-time PCR method failed to detect microsporidial nucleic acid in seven natural water concentrates seeded with 500 spores. Internal positive controls identified inhibitors in all samples, and therefore the perceived loss may not be completely attributed to lost spores. Based on the results of this study, additional work is needed to develop monoclonal antibodies to use with IMS methods for the purification of microsporidial spores.

Evaluation of PCR Detection of *E. Intestinalis* Seeded in Natural Water Samples: PCR detection of seeded *E. intestinalis* spores using 16S rRNA primers was conducted in this study. Although detection of 25 spores in reagent grade water was achieved in some experiments, detection of even 100 spores seeded into natural water samples yielded variable results. Three different commercially available DNA extraction kits were evaluated; however, the test results appear to be influenced by both the extraction

method and water matrix. Most DNA extraction kits have been designed for use with clinical samples that tend to be more homogeneous and free from interfering substances; thus, matrix composition and pH must be considered carefully when selecting DNA extraction methods if molecular analyses for microsporidia are to be successful.

Evaluation of Reverse Transcriptase PCR Cell Culture (RT-PCRCC) Assay as a Means to Assess Microsporidia Spore Viability: RT-PCRCC assays were performed on *E. intestinalis* spores using primers targeted to the 16S rRNA, hsp70, or β-tubulin genes to determine whether viable spores could be differentiated from nonviable spores. These assays were also performed with *E. cuniculi* and *E. hellem* spores to test whether these primers could be used to detect multiple *Encephalitozoon* species in water. Microsporidial spores were heat-inactivated and viability determined by both RT-PCRCC assays and standard *in vitro* cell culture assays. PCR assays using primers designed to 16S rRNA performed on *E. intestinalis*, *E. cuniculi*, and *E. hellem* spores were able to amplify all three species but did not differentiate viable and nonviable spores. Hsp 70 gene amplification compared favorably with standard cell culture infectivity results and was able to differentiate between live and dead *E. cuniculi* and *E. hellem* spores; however, this primer was not able to differentiate live and heat-killed *E. intestinalis* spores. Assays performed using β-tubulin primers correlated with standard cell culture infectivity data and were able to differentiate between live and dead *E. intestinalis* and *E. hellem* spores, but failed to differentiate live and heat-killed *E. cuniculi* spores. Thus, in order to use a RT-PCR assay for detection of viable microsporidia in water samples, it may be necessary to use a nested primer approach, a multiplex approach, or develop additional primer sets that would identify all three species with one assay.

Evaluation of Quantitative, Real-Time PCR for Detection of Microsporidia in Water Samples: During the third and last year of this project, through a partnership with the Southern Arizona Veterans Administration (VA) Health Care System in Tucson, AZ, real-time PCR detection methods for waterborne microsporidia spores were assessed in both reagent water and natural waters. Spore standards were prepared using FCCS in either reagent water or in post-IMS water concentrates. Nucleic acid was extracted and subjected to real-time PCR using primers directed to the 16S rRNA gene. Quantitative standards were prepared for each real-time PCR run using 10, 50, 100, and 500 spores/sample. Data obtained by plotting the PCR cycle thresholds versus the corresponding quantitative standard concentrations were used to develop a standard curve from which the unknown samples could be quantified. Microsporidia spores were successfully detected at low levels in both reagent water and in post-IMS water concentrates.

Recovery Determination of the CFC-IMS-Quantitative PCR Method Using Seeded Natural Water Samples: Ten source water samples (20 L each) taken from eight lakes and rivers located throughout the United States were collected and shipped on ice for use in this last study. All samples were split into two-10 L aliquots. One 10 L sample was spiked with *E. intestinalis* spores prior to concentration by CFC as described previously, while the remaining 10 L was processed as an unspiked background control. Concentrated samples were shipped to the Wisconsin State Laboratory of Hygiene, where IMS was conducted by the research team using Dynabeads. These IMS beads were subsequently shipped to the Southern Arizona VA Health Care System laboratory for quantitative, real-time PCR analysis. Although relative recovery percentages were quite low (0.01 to 0.11 percent), six of the 10 samples tested positive at 50 spores/L. The automated, real-time PCR assay reported in this study successfully identified low levels of microsporidia in several natural water samples. Like most other environmental molecular-based assays however, nucleic acid amplification in other natural water samples was subject to inhibition. Further research should focus on identification of PCR inhibitors and the development of strategies to overcome them.

Summary: Although CFC and real-time PCR have been shown to effectively concentrate and detect waterborne microsporidia, respectively, the reliability of the IMS portion of the method is uncertain. The

research team concluded however, that IMS recovery could be significantly improved if a new, highly specific monoclonal antibody were developed that could effectively purify microsporidia from sample concentrates. Reverse transcriptase PCRCC assays were successful in determining spore viability, however the PCR component appeared to be specific for different genes for each species tested, meaning a nested primer or multiplex approach would be necessary to identify all three *Encephalitozoon* species using one assay.

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For More Information

NCER Project Abstract and Reports:

http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/825/report/0

Peer Reviewed Publications

Hoffman, R.M., Marshall, M.M,, Polchert, D.M., and Jost, B.H. 2003. Identification and characterization of multiple subpopulations of Encephalitozoon intestinalis. Applied and Environmental Microbiology 69(8):4966-4970.