

## Transport of microsporidium *Encephalitozoon intestinales* spores in sandy porous media

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### Abstract

The retention and transport of microsporidium *Encephalitozoon intestinales* spores in two water-saturated sandy porous media was investigated in this study. The initial breakthrough of the spores in the column effluent occurred essentially simultaneously with that of a non-reactive tracer, indicating no significant velocity enhancement. A large fraction (45–73%) of the spores injected into the columns was not recovered in the effluent, indicating removal from solution through colloid retention processes of attachment and/or straining. The relative significance of attachment and straining to total retention was evaluated in additional experiments. An experiment was conducted with a sieved coarse fraction of porous media for which straining is unlikely to be of significance based on the relative diameters of the spores and porous-medium pores. The spore recovery for this experiment was similar to the recoveries obtained for microsporidia transport in the un-sieved parent porous medium. An additional experiment was conducted with a subsample of the coarse fraction that was acid-washed to reduce potential surface attachment sites. Spore recovery was complete for this experiment. These results suggest surface deposition was the primary removal mechanism in our system. This conclusion is supported by the results of an experiment wherein deionized water was flushed through a column that was previously flushed with electrolyte solution. The effluent spore concentrations were observed to increase upon injection of deionized water, indicating re-mobilization of spores upon a change in water chemistry. The measured data were successfully simulated using a mathematical model incorporating colloid filtration. The results of this study suggest that the transport of microsporidia in sandy porous media is governed by established colloid-transport processes.

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### 1. Introduction

The protozoan microsporidia, which comprise greater than 1000 species spanning more than 140 genera, can

infect all classes of vertebrates and most classes of invertebrates (Wittner and Weiss, 1999). Currently there are seven genera that are known to cause human infection (*Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Pleistophora*, *Vittaforma*, *Trachipleistophora*, and *Brachiola*), as well as several as yet unclassified species (Garcia, 2002). Most human infections by microsporidia to date have occurred

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in immuno-compromised individuals, such as those with auto-immune disorders or those undergoing immune-system suppression treatment for cancer or organ transplantation (Weber et al., 1994; Kotler and Orenstein, 1999). Microsporidiosis is usually an enteric disease, with the small intestine as the most common site of human infection. Infection is the result of ingesting the environmentally resistant spore. Only in this stage are microsporidia infectious and believed to be able to survive outside a host cell (Vavra and Larsson, 1999). The primary source of microsporidium spores is the feces of infected hosts (Bornay-Llinares et al., 1998; Fayer et al., 2003).

Recent research indicates that typical water treatment methods may not be completely effective for microsporidia removal (Slifko et al., 2000; Wolk et al., 2000; Gerba et al., 2003). Thus, concern exists with regard to the potential for microsporidia to contaminate potable water supplies. Human pathogenic microsporidia have been found in water samples collected from tertiary wastewater effluent, irrigation water, surface water, and groundwater (Sparfel et al., 1997; Dowd et al., 1998; Enriquez et al., 1998; Thurston-Enriquez et al., 2002). A waterborne outbreak of microsporidiosis occurred in France in the summer of 1995, with 200 people becoming infected (Cotte et al., 1999). These reports illustrate the potential of microsporidia to contaminate water supplies and cause waterborne illness.

The transport behavior of microorganisms in porous media is of interest with regard to the fate of pathogens associated with wastewater recharge, riverbank filtration, septic systems, feedlots, and land application of biosolids. Factors affecting the transport and fate of viruses and bacteria in the subsurface have been widely investigated (e.g., Yates and Yates, 1988; Schijven and Hassanizadeh, 2000; Ginn et al., 2002). Conversely, the subsurface transport and fate of protozoans has been studied to a much lesser extent. The transport of *Cryptosporidium parvum* oocysts has recently begun to receive attention (e.g., Mawdsley et al., 1996; Brush et al., 1999; Harter et al., 2000; Hsu et al., 2001; Logan et al., 2001; Darnault et al., 2003; Tufenkji et al., 2004). The results of this research indicate that transport of cryptosporidia is mediated by standard colloid filtration processes (surface deposition) and by straining. However, there appears to be no published work regarding the transport behavior of microsporidia in porous media. The objective of this study was to investigate the transport behavior of microsporidium *Encephalitozoon intestinales* spores in water-saturated sandy porous media.

## 2. Materials and methods

### 2.1. Materials

Microsporidium *E. intestinales* was selected for use because it is one of the most common human pathogenic

microsporidia and is relatively easy to grow in cell culture. Spores were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were grown on RK13 rabbit kidney cells (line number CC1-37, ATCC, Manassas VA) and Vero (EG) green monkey kidney cells (CCL-81, ATCC). Maintenance and production of the cell culture and spores are detailed in John et al. (2003). Briefly, *E. intestinalis* spores were withdrawn from the cell-culture media and concentrated by centrifugation. Percoll (Sigma-Aldrich) was added to promote purification by separation. The final pellet was washed with 0.01 M, pH 7 phosphate buffer solution (0.54 g/L NaHPO<sub>4</sub> and 0.88 g/L KH<sub>2</sub>PO<sub>4</sub>) and centrifuged for 15 min at 2150 rpm (1500g). Purified spores were enumerated by hemacytometer counting under phase-contrast microscopy at 400× magnification.

Microsporidia spores are oval and generally range between 1 and 5 μm in diameter, although a few species have been known to produce spores up to 40 μm in diameter (Larsson and Lebbad, 2003). The diameter of the spores used in this study were measured using a calibrated fluorescent microscope. Digital images were collected using a camera (Nikon D70) attached to the microscope. The images were processed with ImageJ software (National Institutes of Health), which was used to determine mean lengths of the major and minor axes. These were 2.4 (±0.6) and 1.2 (±0.5) μm, respectively.

Two sandy porous media were used for the experiments. The first is a commercially available, well-sorted (20/30 mesh) natural sand (Accusand, Le Sueur, MN). The second is a sandy soil (Vinton) collected from Tucson, AZ. The properties of these two porous media are listed in Table 1. Two additional experiments were conducted after specific treatments of the Vinton soil to further investigate retention mechanisms. First, an experiment was conducted using a coarse sieved fraction of the soil for which straining is unlikely to be of significance based on the relative diameters of the spores and porous-medium pores. The median and range of grain diameters for this sieved fraction are 3.0 and 1.4–4.0 mm, respectively. An additional experiment was conducted with a subsample of the coarse fraction that was treated with 5N nitric acid to reduce potential surface attachment sites. Analysis of the parent, sieved, and acid-treated Vinton media using X-ray diffraction and energy dispersive X-ray spectroscopy indicates the only primary apparent difference is a significantly reduced calcium carbonate content for the acid-treated media.

Solutions were made with autoclaved, filtered (0.22 μm), deionized water with NaCl (0.01 M) as the electrolyte (ionic strength = 0.01 M). Pentafluorobenzoic acid (PFBA) was used as a conservative, non-reactive tracer to characterize the hydrodynamic properties of the packed columns. The PFBA samples were

Table 1  
Properties of porous media

Porous medium	Sand (%)	Silt (%)	Clay (%)	TOC <sup>a</sup> (%)	Median grain diameter (μm)	Pore diameter <sup>b</sup> < 1 μm (%)	Pore diameter 1–50 μm (%)	Pore diameter > 50 μm (%)
Sand	100	0	0	0.04	677	0.03	16.0	84.0
Vinton	97	1.8	1.2	0.01	234	8.9	63.3	27.8

<sup>a</sup>TOC = total soil organic carbon, measured by Soil, Water and Plant Analysis Laboratory, University of Arizona.

<sup>b</sup>Determined as described by Danielson and Sutherland (1986).

Table 2  
Microsporidium experiment conditions, recoveries, and optimized rate coefficients

Experiment	Porous medium	C <sub>0</sub> <sup>a</sup>	Input pulse	Recovery (%)	k <sub>a</sub> (h <sup>-1</sup> ) <sup>b</sup>
1	Sand	9.0 × 10 <sup>5</sup>	4.6	50	1.3 (1.2–1.4)
2	Sand	1.8 × 10 <sup>5</sup>	4.9	30	1.9 (1.3–2.4)
3	Sand	5.1 × 10 <sup>5</sup>	3.7	55	0.8 (0.7–0.9)
4 <sup>c</sup>	Sand	3.0 × 10 <sup>6</sup>	4.5	45	1.5 (0.9–2.1)
5	Vinton	5.2 × 10 <sup>5</sup>	3.5	27	2.0 (1.9–2.2)
6	Vinton-Coarse	1.9 × 10 <sup>5</sup>	3.0	25	1.8 (1.6–2.0)
7 <sup>d</sup>	Vinton-Coarse Acid washed	3.5 × 10 <sup>5</sup>	3.4	~100	N.A.

<sup>a</sup>C<sub>0</sub> is injection concentration (spores/mL).

<sup>b</sup>k<sub>a</sub> is attachment rate coefficient optimized from model calibration to the measured data; values in parentheses represent 95% confidence intervals.

<sup>c</sup>Experiment conducted with viable spores.

<sup>d</sup>Mean of two experiments.

analyzed using a UV–VIS spectrophotometer (Shimadzu UV-1601, Columbia, MD) at λ = 260.

## 2.2. Methods

Stainless steel columns (7 cm long and 2.1 cm in diameter; Alltech, Deerfield, IL) and tubing (0.32 cm diameter) were used for all experiments. Water flow was generated using a single-piston high-pressure liquid chromatography pump (Accuflo, Fisher Scientific, Incorporated; Pittsburgh, PA). Preparation for each experiment began with the uniform packing of the columns with dry porous media (porosity = 0.36 ± 0.03; bulk density = 1.73 ± 0.04 g/cm<sup>3</sup>). Each packed column was then flushed with the electrolyte solution to achieve uniform saturation. The spores were inactivated prior to use by exposure to UV light for 30 min. An experiment was also conducted with viable spores. This experiment was conducted in a biohazard flow hood (Labconco Corporation; Kansas City, Missouri). All samples from this latter experiment were treated with ultraviolet light prior to analysis. Three to five pore volumes of the spore solution were injected into the columns, followed by several pore volumes of electrolyte solution (no spores present). In an additional experiment, several pore volumes of deionized water was flushed through the

column after the electrolyte flush to evaluate the impact of a change in water chemistry on spore retention. The flow rate for all experiments was equivalent to a pore-water velocity of 9–10 cm/h. Effluent samples (2 mL) were collected continuously in polypropylene 15 mL screw-top sterile test tubes using a fraction collector (RediFrac, Pharmacia LKB, Uppsala, Sweden). The specific details for each experiment are provided in Table 2.

The samples were prepared for analysis in the following manner. The effluent samples were vacuum-filtered through 0.22 μm pore diameter cellulose acetate membranes (Sartorius Ag, Germany) treated with phosphate buffer solution. The spores were then stained with 0.5 mL of Microspor-a-glo antibody staining solution (Waterborne, Inc.; New Orleans, LA) for 40 min. This solution contains monoclonal antibodies specific for *E. intestinalis*. Slides were prepared with DABCO-Glycerol Mounting Medium (2% 1,4 diazabicyclo(2.2.2) octane) (DABCO). The filters were washed twice with PBS and placed on the DABCO-coated slide, covered with more DABCO, and incubated. The cover slip was then placed on the slide and secured using clear nail polish.

The spores were enumerated using a UV-light microscope (Olympus BH2-RFCA; Melville, NY). The

antibody staining solution bound to the spore walls fluoresced upon excitation with the UV lamp. Fifty randomly selected fields of view per sample (of 16000 total) were counted at 1000× magnification to calculate the concentration of the spores in the effluent samples. The effluent concentrations were normalized by the injection concentration and plotted against pore volumes eluted to produce breakthrough curves. The breakthrough curves were subjected to standard temporal moment analysis to calculate equivalent recoveries (zeroth moment) and retardation factors (first moment).

### 2.3. Mathematical modeling

A one-dimensional mathematical model incorporating advection, dispersion, and retention is used to simulate transport under conditions of steady-state flow in a homogeneous porous medium. Colloid retention is described using standard colloid filtration theory (e.g., Schijven and Hassanizadeh, 2000; Ginn et al., 2002), in which irreversible surface deposition (attachment) of the colloids is expressed in terms of collision efficiency ( $\eta$ ) and sticking efficiency ( $\alpha$ ):

$$\frac{\partial C}{\partial t} = -\frac{3(1-\theta)}{2d_p} \eta \alpha v C = -k_a C,$$

where  $C$  is aqueous-phase colloid concentration,  $\theta$  is porosity,  $\rho_b$  is the bulk density,  $d_p$  is the porous-medium grain diameter,  $v$  is average pore-water velocity, and  $k_a$  is the first-order attachment rate coefficient.

A finite-element numerical scheme is used to solve the equations under appropriate initial and boundary conditions. Operator splitting is performed to solve the coupled differential equations separately. A non-linear least squares method is employed to determine optimized parameters by calibration of the model to the measured breakthrough curves.

### 3. Results and discussion

The breakthrough curves for the conservative, non-reactive tracer pentafluorobenzoic acid, used to characterize the hydrodynamic properties of the packed columns, are sharp and symmetrical (data not shown). Breakthrough of pentafluorobenzoic acid in the column effluent occurs at approximately one pore volume, indicating no sorption and associated retardation. Mass recoveries of pentafluorobenzoic acid, determined by calculating the zeroth temporal moments, are greater than 99% for all experiments. These results indicate ideal hydrodynamic behavior for the packed-column systems.

Breakthrough curves obtained for the microsporidium spores are shown in Fig. 1 for the various porous media. Initial breakthrough of the spores in the column effluent

occurs at approximately one pore volume, similarly to PFBA. This is supported by the results of the moment analysis, which produced equivalent retardation factors ranging from 0.9 to 1.3. This indicates no measurable velocity enhancement was observed for microsporidium. This is most likely a function of the relative sizes of the microsporidium spores and the porous-medium pores.

Effluent recoveries for microsporidium ranged from 27% to 55% for the experiments conducted with untreated porous media (experiments 1–5, Table 2). The effluent recovery for the experiment conducted with viable spores was similar to the recoveries observed for the inactivated-spore experiments (Table 2). The reduced recoveries for microsporidium likely reflect removal of spores from solution via processes such as surface deposition (attachment) and straining. The level of removal observed for microsporidium is in the same general range as results reported for cryptosporidium transport in sandy porous media (Brush et al., 1999; Harter et al., 2000; Tufenkji et al., 2004).

The potential contribution of straining to retention of colloids during transport in porous media has received relatively limited attention. Initial analyses of conditions for which straining would be significant focused on

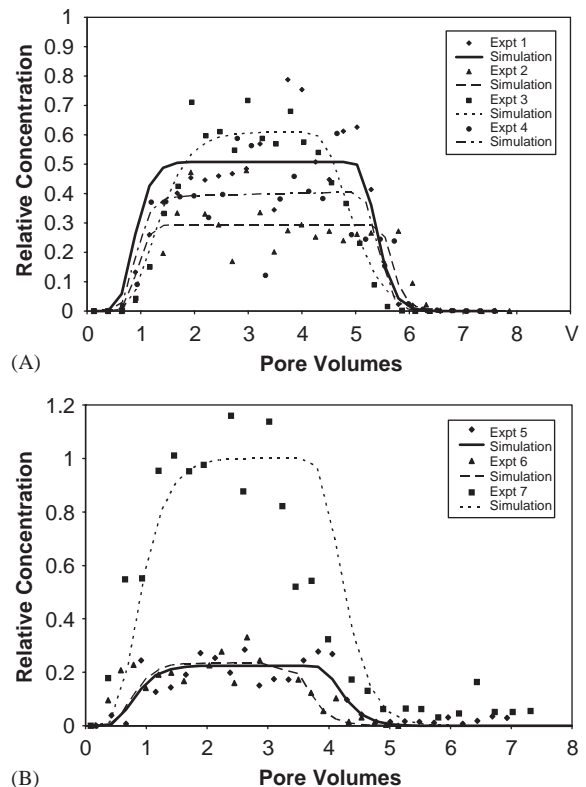


Fig. 1. Breakthrough curves for microsporidium spores in NaCl solution (0.01 M): (A) for the 20/30-mesh sand; (B) for the Vinton soil.

relationships comparing porous-medium grain size ( $d_i$ , the  $i$ th % by mass finer than) to colloid size ( $d_c$ ). Sakthivadivel (1969) reported straining to be insignificant for  $d_{50}/d_c > 20$ , based on experiments. Herzig et al. (1970) posited little straining would be expected for  $d_{50}/d_c > 12$ , based on geometric analysis. A ratio of  $d_{15}/d_c > 9$  is obtained from the results of penetration experiments conducted using finer and coarser sands (Sherard et al., 1984). A similar ratio,  $d_{10}/d_c > 6$ , is obtained from the geometric-based analysis reported by Matthess and Pekdeger (1985). More recent experiment-based work incorporating well-sorted subsurface porous media has produced a more stringent ratio of  $d_{50}/d_c > 200$  (Bradford et al., 2002, 2004; Tufenkji et al., 2004). The calculated ratios for the sand and Vinton soil are 400 and 138, respectively, using a value of  $1.7 \mu\text{m}$  for the microsporidium spores. Based on these values, it appears that straining may potentially be of some significance for transport in the Vinton soil, but most likely not for the sand.

The potential significance of straining can be more directly evaluated by comparing spore size to pore dimensions. The results of recent micromodel experiments showed that colloids did not enter pore throats that were less than 1.5 times larger than the colloid (Sirivithayapakorn and Keller, 2003). For the sand used in our study, essentially all pores have estimated diameters greater than  $1 \mu\text{m}$  (Table 1). Given the measured diameters of the microsporidium spores used herein, it is unlikely that straining contributed significantly to retention for the experiments conducted with sand. Conversely, approximately 9% of the pores are estimated to be smaller than  $1 \mu\text{m}$  in diameter for the Vinton soil. Thus, straining may be of more significance for this porous medium.

The relative significance of attachment and straining to total retention of microsporidium by the Vinton soil was evaluated in additional experiments. An experiment was conducted with a sieved coarse fraction of Vinton soil for which the median grain diameter is approximately ten times larger than that of the unsieved Vinton soil. Straining is unlikely to be of significance for this sieved fraction considering the relative diameters of the spores compared to the porous-medium grains ( $d_{50}/d_c > 1700$ ). The spore recovery for this experiment was similar to the recovery obtained for microsporidia transport in the un-sieved Vinton soil (experiments 5 and 6, Table 2). An additional experiment was conducted with a subsample of the coarse fraction that was acid-washed to reduce potential surface attachment sites. Spore recovery was complete for this experiment (experiment 7, Table 2). These results suggest surface deposition was the primary removal mechanism for microsporidium transport in Vinton soil.

The role of a surface-deposition process in retention of microsporidium is further supported by the results

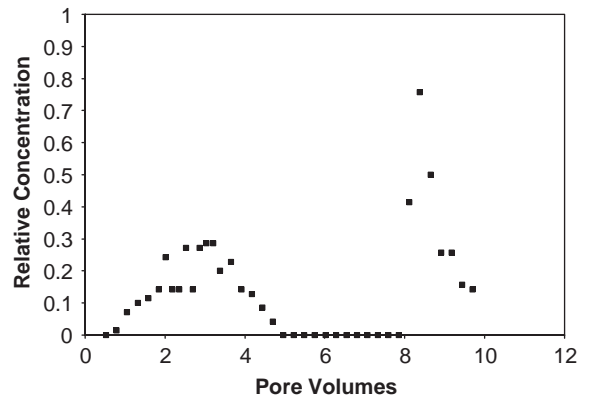


Fig. 2. Results of an experiment conducted with NaCl electrolyte solution, with subsequent deionized water flush, for the 20/30-mesh sand. Recovery of spores in the effluent doubled from 16% to 32% upon flushing with deionized water.

obtained when deionized water was flushed through a column that was previously flushed with electrolyte solution. For this experiment, effluent spore concentrations increased upon injection of deionized water, indicating re-mobilization of spores upon a change in water chemistry (Fig. 2). The introduction of water with a reduced ionic strength increased recovery of the spores, indicating an electrostatic-adsorption process (e.g., Gerba, 1984; Elimelech and O'Melia, 1990; Hsu et al., 2001; Tufenkji et al., 2004) for which the degree of reversibility is dependent upon extant conditions. Such behavior has been observed for other biocolloids (e.g., Bales et al., 1991, 1993).

The simulations produced with a mathematical model incorporating standard colloid filtration provided reasonable fits to the measured breakthrough curves (see Fig. 1). Values for optimized rate coefficients obtained from the model calibrations are presented in Table 2. The rate coefficients range from  $0.8$  to  $2 \text{ h}^{-1}$  for all experiments, with the exception of the experiment conducted with acid-washed Vinton soil that exhibited essentially no retention of spores.

#### 4. Summary

This study provided an initial investigation of the transport of microsporidium *E. intestinales* spores in two water-saturated sandy porous media. A large fraction of the spores injected into the columns was not recovered in the effluent as a result of colloid filtration processes. Spore recovery was influenced by changes in water and soil-surface chemistry, suggesting removal from solution through surface deposition. While apparently insignificant for these experiments, straining is likely to be of more importance for porous media with larger fractions

of fines. The measured data was successfully simulated using a mathematical model incorporating colloid filtration. The results of this study suggest that the transport of microsporidium *E. intestinalis* in sandy porous media is governed by established colloid-transport processes.

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