

***ENCEPHALITOOZON CUNICULI:***  
**DIAGNOSTIC TEST AND METHODS OF INACTIVATION**

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# ***ENCEPHALITOOZON CUNICULI*: DIAGNOSTIC TEST AND METHODS OF INACTIVATION**

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**(ABSTRACT)**

*Encephalitozoon cuniculi* is a zoonotic protozoan parasite in the phylum Microspora that has been shown to naturally infect several host species, including humans, rabbits and dogs. Currently, serological diagnosis of infection is made using the immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA). Although these methods are sensitive and reliable, there are several drawbacks to both tests. Cross-reactivity between other *Encephalitozoon* species is common, and specialized equipment is required for IFA and ELISA. Most wildlife species are unable to be tested using these methods, because species-specific antibodies are required. One goal of this work was to develop a new serological test for diagnosing *E. cuniculi* infection that would be more practical for use in small veterinary and medical clinics. The effectiveness of the agglutination test was examined in CD-1 and C3H/He mice infected with *E. cuniculi* or one of 2 other *Encephalitozoon* species. The results indicate that the agglutination test is 86% sensitive and 98% specific for *E. cuniculi*, with limited cross-reactivity to *E. intestinalis*. The test is fast and easy to conduct, and requires no specialized equipment or species-specific antibodies.

Recent reports of microsporidial DNA in crop irrigation waters suggest that unpasteurized juice products may be contaminated with *E. cuniculi*. High pressure processing (HPP) is an effective means of eliminating bacteria and extending the shelf life of products while maintaining the sensory features of food and beverages. The effect of HPP on the *in vitro* infectivity of *E. cuniculi* spores was examined. Spores were exposed to between 140 and 550 MPa for 1 min, and then spores were loaded onto cell culture flasks or were kept for examination by transmission electron microscopy (TEM). Spores treated with between 200 and 275 MPa showed reduction in infectivity. Following treatment of 345 MPa or more, spores were unable to infect host cells. No morphologic changes were observed in pressure-treated spores using TEM.

The effect of disinfectants on *in vitro* infectivity of *E. cuniculi* spores was also examined. Spores of *E. cuniculi* were exposed to several dilutions of commercial bleach, HiTor and Roccal, and 70% ethanol for 10 minutes and then loaded onto Hs68 cells. The results of this study showed that all concentrations of disinfectants tested were lethal to *E. cuniculi* spores. *Encephalitozoon cuniculi* spores are more sensitive to disinfectants than are coccidian oocysts and other parasite cysts.

## **Dedication**

I dedicate this thesis to my father, Monty Wetch, for inspiring me to study disease and for showing me that miracles happen every day. I love you.

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# CHAPTER 1

## INTRODUCTION

*Encephalitozoon cuniculi* is an important zoonotic parasite infecting a wide range of host species. In humans, symptoms are typically observed only in immunocompromised individuals, such as AIDS patients, organ transplant recipients and cancer patients undergoing chemotherapy. *Encephalitozoon cuniculi* primarily infects the brain, eyes and urinary tract, causing encephalitis, cataracts, kidney failure and death. Symptomatic human infections with *E. cuniculi* are rare, but infection with other microsporidia species is more common. It is estimated that between 8-10% of the AIDS population in the United States is clinically infected with *E. intestinalis* or *Enterocytozoon bieneusi* (Deplazes et al., 2000). These microsporidia species primarily infect intestinal enterocytes, causing fatty diarrhea, nausea and weight loss that may last several months. Drugs are available to treat microsporidia infections in humans, but these typically only relieve symptoms temporarily, with frequent relapses following cessation of treatment. One goal of this study was to examine the activity of a newly approved anti-parasitic drug on *E. cuniculi* spores *in vitro*.

*Encephalitozoon cuniculi* is also an important parasite of domestic animals, including dogs, cats, rabbits, cows, sheep and horses. This parasite was first identified in a colony of laboratory rabbits in 1922 as a cause of vestibular disease (Wright and Craighead, 1922), and it is still a common pathogen of purpose-bred and pet rabbits (Harcourt-Brown and Holloway, 2003). Rabbits are the only host in which clinical signs

are observed in otherwise healthy adult animals. *Encephalitozoon cuniculi* infects the eyes, kidneys and brain of rabbits, causing clinical signs such as cataracts, head tilt, paralysis and death. In dogs, disease is only seen in puppies. Clinical signs of *E. cuniculi* infection in pups are almost entirely neurological, and include blindness, ataxia and seizures. Another goal of this research was to examine several commercially available disinfectants for their effects on *E. cuniculi* spores in order to identify useful methods of sterilizing animal facilities and housing.

*Encephalitozoon cuniculi* is the only microsporidian parasite that is considered to be zoonotic, though some evidence suggests that other species may also be zoonotic. Three strains of *E. cuniculi* have been characterized: Type 1 from dogs, type 2 from rodents, type 3 from rabbits (Didier et al., 1995). In areas where cohabitation with dogs is common, humans are primarily infected with type 1, and the same is true for type 3 from rabbits.

The most common methods of serological diagnosis are the immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA). Although these methods are sensitive and reliable for detecting antibodies to *E. cuniculi*, there are several drawbacks to both tests. Cross-reactivity between other *Encephalitozoon* species is common, and specialized equipment is required for IFA and ELISA. Most wildlife species are unable to be tested using these methods, because species-specific antibodies are required. One goal of this study was to develop a simple test for detecting antibodies to *E. cuniculi*.

Aside from zoonotic transmission, it is likely that humans are ingesting microsporidia spores from contaminated food and water. A recent report by Thurston-Enriquez et al. (2002) found DNA from human microsporidia species in crop irrigation

waters from throughout North and Central America. Microsporidia spores are extremely resistant to environmental factors and could remain infective long after crops were harvested. A final goal of this research was to examine the effects of high pressure processing on spores of *E. cuniculi* as a method for sterilizing juice products.

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## **CHAPTER 2**

### **REVIEW OF RELATED LITERATURE**

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## Introduction

Microsporidia are obligate intracellular parasites of the phylum Microspora. There are over 1200 species of microsporidia, and natural infections have been documented in arthropods, reptiles, amphibians, birds, mammals, and other protozoa (Weiss, 2001). The infective spore stage is characterized by the presence of a polar tube that is used to infect host cells (Weiss, 2001). Hosts are infected by ingestion or inhalation of spores passed in the urine or feces (Figure 1) (Bigliardi and Luciano, 2001). Transplacental transmission has been shown to occur in dogs, rabbits, horses and cows, but not in humans (McInnes and Stewart, 1991; Harcourt-Brown, 2003; Van Rensburg, 1991; Reetz, 1995). Infection with microsporidia is usually asymptomatic, except in young or immunocompromised hosts (Wasson and Peper, 2000). Human and animal infections with microsporidia were recognized prior to the AIDS pandemic, but overall awareness of the Phylum as important parasites of warm-blooded animals came about only after the advent of a large immunocompromised human population associated with HIV infection, transplantation or chemotherapy.

## Pathology of microsporidiosis in humans

There are four species of microsporidia of interest in human and veterinary medicine: *Encephalitozoon cuniculi*, *E. intestinalis*, *E. hellem*, and *Enterocytozoon bieneusi*. Among human cases of microsporidia infection, *E. intestinalis* and *Ent. bieneusi* are most frequent (Franzen and Muller, 2001). Studies conducted among AIDS patients across the United States have shown that approximately 10% of patients sampled were infected with *Ent. bieneusi*, and about 8% were infected with *E.*

*intestinalis* (Deplazes et al., 2000). *Encephalitozoon hellem* is the third most common human microsporidian, and *E. cuniculi* has been reported in a small number of human cases (Franzen and Muller, 2001).

Intestinal pathology is the most common symptom of human microsporidia infection, and is usually caused by *Ent. bienewisi* or *E. intestinalis* (Snowden, 2004). Intestinal enterocytes are the primary cells infected, and fatty diarrhea due to malabsorption is the most common symptom. Patients may experience diarrhea and nausea intermittently for several months, accompanied by progressive weight loss (Franzen and Muller, 2001).

*Encephalitozoon* species are more widespread in their pathology, with all three species able to cause severe disseminated infection. Keratoconjunctivitis is common among *Encephalitozoon* species, and is usually caused by *E. hellem* (Franzen and Muller, 2001). This condition may be asymptomatic, but conjunctival inflammation and loss of visual acuity are common, with corneal ulcers developing in a few cases (Franzen and Muller, 2001). Sinusitis is another common manifestation of *E. hellem*, and rhinitis and nasal polyps occur in some cases (Franzen and Muller, 2001)(Wasson and Peper, 2000). Urinary tract infections are rarely seen in patients infected with *E. cuniculi* or *E. intestinalis*. Some urinary tract infections are asymptomatic, but severe disease can occur. Hematuria and cystitis are common symptoms, and severe infection can lead to renal failure (Franzen and Muller, 2001; Wasson and Peper, 2000).

### Microsporidia infection in domestic animals

*Encephalitozoon cuniculi* was first identified in 1922 in a colony of research rabbits, and today it is a common pathogen infecting domestic and purpose-bred rabbits

(Snowden, 2004; Wasson and Peper, 2000; Wright and Craighead, 1922). There have not been any large-scale studies of encephalitozoonosis in pet rabbits in the U.S., but surveys conducted in Europe have shown high rates of infection in asymptomatic pet rabbits (7-42%) and in those with neurological symptoms (40-85%)(Table 1).

Many rabbits infected with *E. cuniculi* are asymptomatic, or they demonstrate only minor clinical signs, such as slower reactions to stimuli (Harcourt-Brown and Holloway, 2003). Clinical signs of *E. cuniculi* infection in rabbits are almost entirely neurological, with some cases developing ocular lesions and cataracts (Wasson and Peper, 2000; Harcourt-Brown and Holloway, 2003; Snowden and Shadduck, 1999). Lethargy and head tilt are usually the first signs, and ataxia, paresis and hind limb paralysis follow (Wasson and Peper, 2000; Harcourt-Brown and Holloway, 2003). This presentation has often been confused with a diagnosis of vestibular disease caused by bacterial infection. Full paralysis and death may occur in severe cases. Infection of the kidneys leads to polydypsia and polyuria, and gross examination reveals a characteristic pitted appearance of the shrunken fibrotic kidneys (Harcourt-Brown and Holloway, 2003). Although gross lesions are not usually visible, meningoencephalitis can be observed upon histological examination (Wasson and Peper, 2000; Harcourt-Brown and Holloway, 2003).

Dogs may be naturally infected by a few species of microsporidia, but infection with *Encephalitozoon cuniculi* is most common. Serologic studies for *E. cuniculi* have been conducted worldwide, and the prevalence of dogs with specific antibodies ranges from 8% to 38% (Table 2).

*Encephalitozoon cuniculi* is usually asymptomatic in adult dogs but can be fatal to pups born to infected dams (Szabo and Shadduck, 1987a; McInnes and Stewart, 1991;

Stewart et al., 1979). The most common signs of *E. cuniculi* infection in dogs are neurological, and they include depression, incoordination, ataxia, blindness and seizures (McInnes and Stewart, 1991; Stewart et al., 1979; Didier et al., 1998). Histological examination of tissues typically shows multifocal or disseminated intracellular parasites in the brain with varying amounts of inflammation and vasculitis. Nephritis and hepatitis are also common, with parasites primarily localized in renal cortical tubular epithelium and sometimes in the glomerular epithelium (Szabo and Shadduck, 1987b; Botha, et al., 1986; Shadduck et al., 1978).

*Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* are primarily human parasites, but infections have been documented to occur naturally in dogs (Bornay-Llinares et al., 1998; Mathis et al., 1999a; Lores et al., 2002). Limited serologic studies have been conducted in parts of Europe that show the prevalence of antibodies to *Ent. bieneusi* may be as high as 11% (Table 2). No clinical data is available about the pathology of these infections in dogs.

*Encephalitozoon cuniculi* and *Ent. bieneusi* have been shown to infect domestic cats, though these infections are rare and poorly studied (Mathis et al., 1999a; Pang and Shadduck, 1985). Limited serologic surveys in Europe have been conducted to detect antibodies against *E. cuniculi* and *Ent. bieneusi*. Up to 8% of cats tested positive for *Ent. bieneusi* and up to 23% of cats exhibited antibodies to *E. cuniculi* (Table 2).

One case of Encephalitozoonosis in a naturally infected Siamese kitten has been documented (Rensburg and Plessis, 1971). Clinical signs included severe muscle spasms and depression (Rensburg and Plessis, 1971). Upon histopathological examination of tissues, meningoencephalitis and interstitial nephritis were evident (Rensburg and Plessis, 1971). A single attempt at experimental infection has been reported in young



cats (Pang and Shadduck, 1985). The animals were generally asymptomatic, although they were thin and had rough coats (Pang and Shadduck, 1985). Upon examination of tissues, interstitial nephritis was present in all cats, and a few exhibited meningoencephalitis (Pang and Shadduck, 1985).

In a limited number of reports, microsporidia have been shown to naturally infect horses, sheep, goats, pigs and cows, but very little is known about the true prevalence or the pathogenicity of infection in these hosts. Serological surveys have been conducted reporting infection of livestock species with *E. cuniculi* and *Ent. bienewsi* (Table 3).

*Encephalitozoon intestinalis* and *Ent. bienewsi* have been shown to occur naturally in pigs (Bornay-Llinares et al., 1998; Deplazes et al., 1996a; Dengjel et al., 2001; Buckholt et al., 2002). A recent survey of pigs at various slaughterhouses throughout the New England states found that 31% of over 200 pigs were positive for *Ent. bienewsi* by PCR (Buckholt et al., 2002). No clinical signs were reported, but parasites were observed in the liver of one of the pigs (Buckholt et al., 2002).

Natural infections by *E. cuniculi*, *E. intestinalis* and *Ent. bienewsi* have been documented to occur in cattle (Halanova et al., 1999; Reetz, 1995). Clinical signs of infected cows have not been described, but abortion has resulted in a few cases. Examination of aborted fetuses has demonstrated *E. cuniculi* in the placenta, brain, liver, kidneys, myocardia, and lungs (Reetz, 1995).

A group of researchers in Israel discovered that horses are frequently infected with *E. cuniculi*. The study examined 102 horses from 3 private riding farms across the country. The horses were examined for clinical manifestations of *E. cuniculi* infection, and serum was drawn from each animal. Among the 73 asymptomatic horses, 43 (60%) were seropositive, and 25 of 30 (80%) symptomatic horses were seropositive by

immunofluorescent assay (IFA)(Levkutova, et al., 2004). Symptomatic horses had a variety of clinical signs, mainly cholic (11) and neurological signs (6)(Levkutova, et al., 2004). A few animals exhibited cholic, reproductive disturbances, lameness, sinusitis or pruritis (Levkutova, et al., 2004).

Two cases of abortion resulting from infection of asymptomatic mares with *E. cuniculi* have been documented (Van Rensburg et al., 1991; Patterson-Kane et al., 2003). In one case, *E. cuniculi* was found only in the placenta, causing necrosis of chorionic villi (Patterson-Kane et al., 2003). The second case found *E. cuniculi* in the kidneys of the aborted fetus (Van Rensburg et al., 1991). Severe lymphoplasmacytic interstitial nephritis was noted, but no other organs were affected, and no parasites were seen in the placenta (Van Rensburg et al., 1991).

### Zoonotic potential of microsporidia

Microsporidia are widespread throughout the animal kingdom, but only recently have they begun appearing in humans. Although there is still no direct evidence of animal-human transmission, it is likely that some microsporidia are zoonotic. Recent scientific information supports the concept that *E. intestinalis* is a parasite primarily found in humans while *E. cuniculi* and *E. hellem* are primarily found in animal hosts. Currently, only *E. cuniculi* is considered a zoonotic parasite. Three strains of *E. cuniculi* have been described (Didier et al., 1995a). Strain I has been isolated from rabbits (Desplazes et al., 1996b; Mathis et al., 1997), strain II is generally associated with rodents, and strain III from dogs (Snowden et al., 1999; Didier et al., 1996). Strain I is found in humans in Europe (Mathis et al., 1997; Rossi et al., 1998), while strain III is the human isolate found in the Americas (Didier et al., 1995a). This is certainly interesting,

considering that rabbits are more commonly kept as pets or for food in Europe, and dogs are the most popular pet in the Western hemisphere.

*Encephalitozoon hellem* also has three genotypes, with type I being the most common strain found in humans (Mathis et al., 1999b). Ribosomal RNA gene sequencing of human and avian isolates has been conducted to examine its zoonotic potential. The results showed 100% homology between the avian and human isolates (Snowden et al., 2000).

A recent report described the first discovery of *E. intestinalis* in dogs, pigs and cows using molecular analysis of fecal samples (Bornay-Llinares et al., 1998). Another study sequenced the SSUrRNA gene of *E. intestinalis* found in fecal samples obtained from gorillas and the humans who share their habitat and found them to be identical (Graczyk et al., 2002).

## **Diagnosis of microsporidia infection**

### Staining methods

There are several staining techniques available for identification of microsporidia spores. However, staining methods can, at best, identify microsporidia, but cannot distinguish between species. Current staining methods are also hindered by the fact that microsporidia are often mistaken for bacteria or yeasts.

Calcofluor white M2R is a chemofluorescent stain that is commonly used for identifying microsporidia in fecal smears, urine sediments, and respiratory lavages (Didier et al., 1995b). This is the most sensitive method for staining microsporidia, and the procedure can be completed in only 15 minutes (Didier et al., 1995b). However, this

stain is not specific for microsporidia, and spores may be confused with yeasts or other particulate matter in the sample (Garcia, 2002). A compound microscope with a UV light source is needed for this staining method, so it is used primarily in diagnostic laboratories rather than in clinical settings.

Chromotrope-based (modified trichrome) staining methods are also used for identifying microsporidia in feces, urine sediment or respiratory secretion samples. Chromotrope stains give spores a pinkish red color, and quick-hot Gram-chromotrope stains color spores a dark violet (del Aguila et al., 1998). Characteristic morphological features of microsporidia that are visible using these stains are a belt-like stripe, a vacuole and Gram-positive granules. Again, spores may be confused for bacteria or yeasts.

Giemsa and Gram stains are not recommended for fecal smears because they do not differentiate between microsporidia and bacteria or yeast that may be present in the sample. However, Giemsa stains may be useful in urine or tissue samples, and spores stain light blue (Garcia, 2002).

#### Molecular methods

Polymerase chain reaction (PCR) is a much more reliable alternative to staining for diagnosing microsporidia infection. PCR can be used to positively identify species of microsporidia from feces, urine sediment or tissue samples. Specific primers and assays are available for *E. cuniculi*, *E. intestinalis* and *E. hellem* on an experimental basis or through medical diagnostic laboratories on a limited basis (del Aguila et al., 1998). Based on expense of equipment and availability of primers, this is not a practical solution for identifying microsporidia infection in medical or veterinary laboratories.

### *Morphological methods*

Transmission electron microscopy is another way to identify and differentiate between species of microsporidia. *Enterocytozoon bienersi* is slightly smaller than *Encephalitozoon* species; *Ent. bienersi* is 0.5 x 1.5 µm in size, while the *Encephalitozoon* species range from 1.0-1.5 x 2.0-2.5 µm (Weiss, 2001). The number of polar tube coils is useful in differentiating between species of microsporidia (Table 4). *Encephalitozoon intestinalis* can be distinguished from all other *Encephalitozoon* species by examining the parasitophorous vacuole (PV) within an infected host cell (Figure 2). This is the only species of microsporidia known to exist in a septated PV that isolates each developing spore (Chu and West, 1996). *Enterocytozoon bienersi* does not create a PV; instead it develops in direct contact with the host cell cytoplasm (Wasson and Peper, 2000). Although TEM is a reliable way of diagnosis, it is obviously the least practical and most expensive method for use in medical and veterinary practices.

### *Serological methods*

Monoclonal and polyclonal antibodies have been developed for *E. cuniculi*, *E. intestinalis*, *E. hellem* and *Ent. bienersi* for use in IFA tests on an experimental basis and in some diagnostic laboratories (Alfa Cisse et al., 2002; Aldras et al., 1994; Enriquez et al., 1997). IFA is the gold standard for serological diagnosis of microsporidia infection. IFA titers greater than 1:20 are considered positive (Didier et al., 1998).

Western blotting is another technique that can identify *E. cuniculi* antibodies in serum. Recently, a group of researchers used Western blotting to examine proteins of the polar tube and exospore of *E. cuniculi* (van Gool et al., 2004). Serum from an HIV-negative laboratory worker who accidentally infected himself with *E. cuniculi* was

collected at 1 and 20 months post-infection and tested against spores of *E. cuniculi* and *E. intestinalis*. When testing the serum samples against *E. cuniculi* spores, they found that at 1 month (PI) serum exhibited only one strong band at 28 kDa (van Gool et al., 2004). By 20 months PI, strong bands were formed at 17, 20, 28, 30, 32, 34-38 and 47 kDa (van Gool et al., 2004). Weak bands were present at 27, 44, 55, 70 and 150 kDa (van Gool et al., 2004). When the serum was tested against *E. intestinalis* spores, only one band was present at 26 kDa (van Gool et al., 2004).

Another group experimentally infected rabbits with a human isolate of *E. cuniculi* for testing by Western blot. When sera from infected rabbits was tested against *E. cuniculi* spores, seven bands were observed. Five bands were found between 18 and 45 kDa, with approximate weights of 19, 23, 28, 35 and 36 kDa (Croppo et al., 1997). Dark bands were seen at 90 and 190 kDa, and a dark doublet was observed immediately below the 45 kDa marker (Croppo et al., 1997).

The enzyme-linked immunosorbent assay (ELISA) is another method for diagnosing microsporidia infection serologically. Two groups of researchers have evaluated the ELISA for diagnosis of *E. cuniculi* by comparing the results with IFA. Boot et al. (2000) examined 135 purpose-bred rabbits diagnosed with infection by histology and 75 rabbits without a history of illness. A few samples were positive by only one method, but no statistically significant differences were calculated between the sensitivities and specificities of the two assays (Boot et al., 2000).

Another study examined sera from rabbits, dogs, mice and squirrel monkeys by the dot-ELISA test and compared the results to the IFA. A few samples were positive by ELISA and negative by IFA, but none were positive by IFA and not ELISA (Beckwith et al., 1988). The agreement between the two tests was calculated using a Kappa value.

The Kappa values were 1.0 for dogs, 0.77 for rabbits and monkeys and 0.74 for mice (Beckwith et al., 1988). The researchers found the dot-ELISA to be more sensitive than IFA to *E. cuniculi* antibodies (Beckwith et al., 1988).

Although IFAs and ELISAs are sensitive methods for diagnosing infection with *E. cuniculi*, neither is practical for all laboratories. IFA tests require a compound microscope with a fluorescent light source, and ELISA tests require a plate reader to interpret results. Also, serological screening of many wildlife species is not yet possible due to the requirement for species-specific antibodies. One main goal of this research is to develop a method of serological diagnosis that will address each of these problems.

### High pressure processing

High pressure processing (HPP) is used commercially as a non-thermal means to extend the shelf life of foods and beverages by eliminating pathogens and denaturing destructive enzymes (Tewari et al., 1999). HPP acts primarily by disrupting hydrogen bonds and three-dimensional configuration of protein molecules. It has a number of advantages over traditional thermal processing, including shorter processing time, minimal heat damage problems, no adverse changes due to ice-phase forms during pressure-shift freezing, minimal modifications to functionality, and preservation of flavor, texture, color and vitamin C (Tewari et al., 1999).

Recent reports have proposed the possibility that microsporidia may be present in juice products (Slifko et al., 2000a). A recent study examined water sources in North and Central America for the presence of protozoan parasites (Thurston-Enriquez et al., 2002). The water sources sampled were used for irrigation of various fruit and vegetable crops. Thurston-Enriquez et al. (2002) discovered that DNA from human

pathogenic microsporidia was present in all water sources examined. Microsporidia in irrigation water could adhere to the surface of fruits and vegetables. Since spores are extremely resistant to damage, they may remain viable on the produce for weeks or months. These facts also suggest that microsporidia may be present in juice products.

Studies have shown that HPP is useful in eliminating bacteria and protozoa from juices without altering taste or appearance. Slifko et al. (2000b) examined the effects of HPP on *Cryptosporidium parvum* oocysts in apple and orange juice. It was determined that 550 MPa for 60 sec decreases the *in vitro* infectivity of *C. parvum* oocysts. Lindsay et al. (in press) show that exposure of *Toxoplasma gondii* oocysts to 340 MPa for 60 sec reduces viability of oocysts *in vivo*. A major goal of this research is to determine whether HPP will be useful for inactivating spores of *E. cuniculi*.



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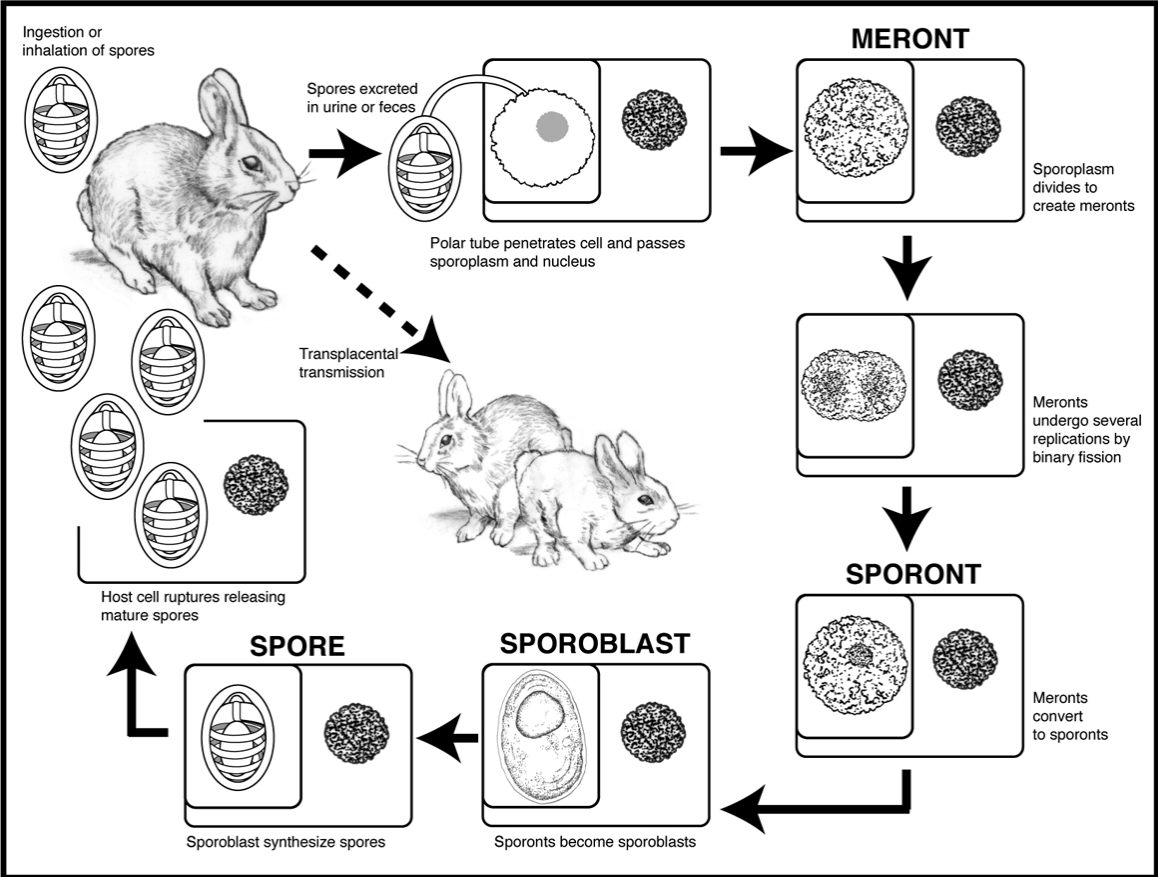
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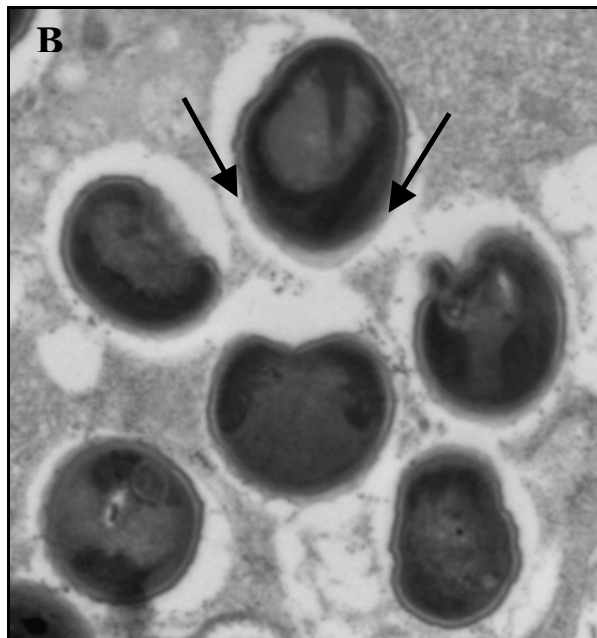
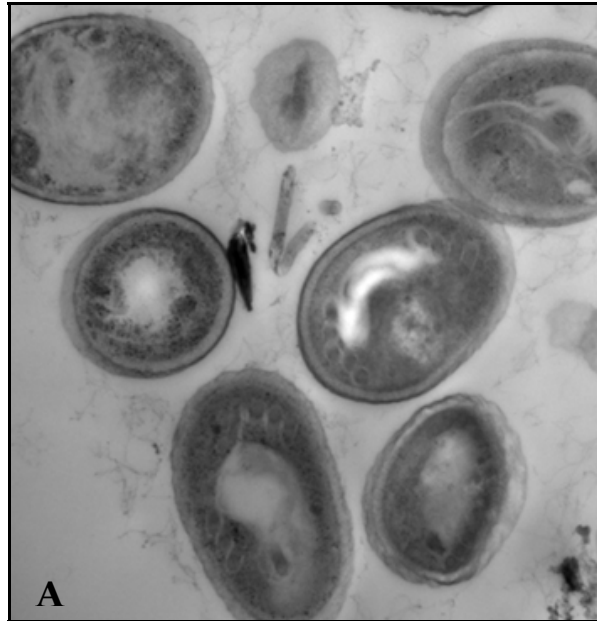
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**Figure 1:** General life cycle of microsporidia



**Figure 2:** Transmission electron micrographs showing spores of *E. cuniculi* (A) and *E. intestinalis* (B) developing within CV-1 cells. Arrows point to septa isolating spores of *E. intestinalis*





**Table 1:** Prevalence of *Encephalitozoon cuniculi* in pet rabbits as determined by serologic surveys

Location	Health	No. tested	No. positive	% positive	Reference
Slovakia	n/a	571	238	41.7%	Halanova, et al.
Switzerland	asymptomatic	292	22	7.5%	Deplazes, et al.
	neurologic symptoms	72	61	84.7%	Deplazes, et al.
W. Yorkshire	asymptomatic	38	14	36.8%	Deplazes, et al.
	neurologic symptoms	58	23	39.7%	Harcourt-Brown and Holloway
	ocular lesions	8	8	100.0%	Harcourt-Brown and Holloway
Spain	n/a	22	2	9.1%	Lores, et al.

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**Table 2:** Prevalence of microsporidia in cats and dogs as determined by serologic surveys

	No. tested	No. positive	% positive	Parasite	Reference
<b>Cats</b>					
Slovakia	72	17	23.6%	<i>E. cuniculi</i>	Halanova, et al.
Switzerland	45	1	2.2%	<i>E. cuniculi</i>	Deplazes, et al.
Switzerland	12	1	8.3%	<i>Ent. bienewisi</i>	Mathis, et al.
Germany	60	3	5.0%	<i>Ent. bienewisi</i>	Dengjel, et al.
<b>Dogs</b>					
Slovakia	193	73	37.8%	<i>E. cuniculi</i>	Halanova, et al.
London	248	33	13.3%	<i>E. cuniculi</i>	Hollister, et al.
Africa	270	75	27.8%	<i>Encephalitozoon sp.</i>	Stewart, et al.
Switzerland	36	3	8.3%	<i>Ent. bienewisi</i>	Mathis, et al.
Spain	17	2	11.8%	<i>Ent. bienewisi</i>	Lores, et al.

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**Table 3:** Prevalence of microsporidia in livestock as determined by identification of parasites in fecal samples or by IFA

Location	Animal	No. tested	No. positive	% positive	Parasite	Reference
Israel	Horses	102	68	66.7%		Levkutova, et al.
Switzerland	Pigs	109	38	34.9%	<i>Ent. bienewisi</i>	Breitenmoser, et al.
USA	Pigs	202	64	31.7%	<i>Ent. bienewisi</i>	Rinder, et al.
USA	Cows	413	13	3.1%	<i>Ent. bienewisi</i>	Fayer, et al.
USA	Cows	413	70	16.9%	microsporidia sp.	Fayer, et al.
USA	Cows	338	29	8.6%	<i>Ent. bienewisi</i>	Sulaiman, et al.
Portugal	Cows	48	3	6.3%		Sulaiman, et al.
Slovakia	Cows	55	20	36.4%	<i>E. cuniculi</i>	Deplazes, et al.
Slovakia	Sheep	59	8	13.6%		Halánová, et al.
Slovakia	Goats	48	2	4.1%	<i>E. cuniculi</i>	Cisláková, et al.
Spain	Goats	7	1	14.3%	<i>Ent. bienewisi</i>	Lores, et al.

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**Table 4:** Morphological characteristics of microsporidia

	<i>E. cuniculi</i>	<i>E. intestinalis</i>	<i>E. hellem</i>	<i>Ent. bieneusi</i>
Spore dimensions (µm)	1.5 x 2.5	1.2 x 2.0	1.0 x 2.5	0.5 x 1.5
Number of polar tube coils	5-7 (single row)	4-7 (single row)	4-9 (single row)	4-5 (double row)
Development in host cell	PV	PV	Septated PV	Direct contact with cytoplasm

PV: parasitophorous vacuole

## CHAPTER 3

### **DIRECT AGGLUTINATION TEST FOR *ENCEPHALITOOZON CUNICULI***

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## **ABSTRACT**

*Encephalitozoon cuniculi* is a small protozoan parasite in the phylum Microspora. It has been shown to naturally infect several host species, including humans. Infection with microsporidia is usually asymptomatic, except in young or immunocompromised hosts. Currently, serological diagnosis of infection is made using the indirect immuno fluorescent antibody assay (IFA) or enzyme-linked immunosorbent assay (ELISA). Although these methods are sensitive and reliable, there are several drawbacks to the IFA and ELISA tests. Cross-reactivity between other *Encephalitozoon* species is common, and specialized equipment is required to conduct these tests. This paper reports the development of a direct agglutination test for detecting IgG antibodies to *E. cuniculi*. The utility of the agglutination test was examined in CD-1 and C3H/He mice infected with *E. cuniculi* or one of 2 other *Encephalitozoon* species. The results indicate that the agglutination test is 86% sensitive and 98% specific for *E. cuniculi*, with limited cross-reactivity to *E. intestinalis*. No cross reactivity to *E. hellem* was observed. The test is fast and easy to conduct, and species-specific antibodies are not required.

Key words: *Encephalitozoon cuniculi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, agglutination test, immunofluorescent antibody assay, western blot

## 1. INTRODUCTION

Microsporidia are small protozoan parasites characterized by the use of a polar tube for host cell invasion (Weiss, 2001). There are several species of microsporidia that infect humans and domestic animals. *Encephalitozoon cuniculi* is a common microsporidian that has an extremely diverse host range. It was first identified as a cause of vestibular disease in laboratory rabbits, and remains a common pathogen of purpose-bred and pet rabbits today (Snowden, 2004; Wasson and Peper, 2000; Wright and Craighead, 1922). Rabbits are the only host species in which clinical signs are commonly observed in immunocompetent adult animals (Snowden, 2004). Head tilt is usually observed first, and disease may progress to full or partial paralysis and death (Harcourt-Brown and Holloway, 2003; Wasson and Peper, 2000). In other host species, such as dogs, clinical signs are limited to young or immunocompromised animals, and include depression, blindness and seizures (Didier et al., 1998; McInnes and Stewart 1991; Pang and Shaddock, 1985; Rensburg and Plessis, 1971).

In humans, symptoms are observed primarily in immunocompromised populations, such as AIDS patients and individuals receiving immunosuppressant drugs. Although this disease is rare in humans, it is often fatal. *Encephalitozoon cuniculi* can cause severe disseminated infection in humans, but it primarily infects the eyes, sinuses, urinary tract and brain (Franzen and Muller, 2001). Infection of the brain and meninges, leading to edema, encephalitis and lesions in the brain, is usually fatal (Franzen and Muller, 2001).

Several species of microsporidia are suspected zoonoses, but *E. cuniculi* is the only species that is currently widely accepted to be a zoonotic parasite (Didier et al., 1995). For this reason, it is important that veterinarians and human physicians have

access to reliable methods for detecting antibodies to microsporidia infection. Currently, infections are most often identified by enzyme-linked immunosorbent assays (ELISA) or indirect immunofluorescent antibody assay (IFA), the gold standard for serological diagnosis of microsporidia infection (Enriquez et al., 1997; Boot et al., 2000; Beckwith et al., 1988). IFA titers greater than 1:20 and ELISA titers greater than 1:800 are considered to be indicative of exposure (Didier et al., 1998). The purpose of the present study was to develop a direct agglutination test that is based on agglutination of *E. cuniculi* spores by antibodies present in serum.



## 2. MATERIALS AND METHODS

Spores of *E. cuniculi* and *E. intestinalis* were obtained from the American Type Culture Collection, Manassas, VA [ATCC# 50502 (*E. cuniculi*) and 50506 (*E. intestinalis*)]. Spores of *E. hellem* were obtained from Dr. Ron Fayer, USDA/ARS, Environmental Microbial Safety Laboratory, Beltsville, MD. Human foreskin fibroblasts (Hs68) (ATCC #CRL-1635) were cultured in 75 cm<sup>2</sup> cell culture flasks in a CO<sub>2</sub> incubator at 37°C in RPMI 1640 media containing 10% fetal bovine serum and antibiotics. Microsporidia spores were inoculated onto confluent Hs68 cells and allowed to grow for several weeks. *Encephalitozoon cuniculi* spores were harvested from Hs68 cell cultures by removing the supernatant from infected flasks and passing it through a 3.0 µm filter. CD-1 or C3H/He mice were inoculated either orally or subcutaneously with 10 x 10<sup>6</sup> spores of *E. cuniculi*, *E. intestinalis* or *E. hellem*. Serum was collected weekly from at least 2 mice per group via retro-orbital sinus. Two interferon-γ (IFN-γ) gene knock-out BALB/c mice were also infected with *E. cuniculi*, and serum was collected at 4 weeks post-infection (PI).

Spores of *E. cuniculi* were collected from infected cell cultures and fixed in 2 ml of 37% formaldehyde solution for 10 to 15 sec in a 15 ml conical centrifuge tube and then diluted with phosphate buffered saline (PBS, pH 7.4) up to 15 ml and stored at 4°C. Prior to use in the agglutination test, the spores were washed twice in PBS and resuspended in alkaline buffer-eosin solution (7.02 g NaCl; 3.09 g H<sub>3</sub>BO<sub>3</sub>; 24 ml 1N NaOH; 4 g horse serum albumin factor V; 50 mg eosin Y; 1.0 g sodium aside; distilled H<sub>2</sub>O to make 1 L; pH 8.7). The eosin aids in the visualization of the agglutination reaction. Next, 0.5 ml of 0.2 M 2-mercaptoethanol was added to each 1 ml of the spore

buffer solution to destroy IgM antibodies that may be present in the test serum, and to prevent non-specific agglutination caused by IgM molecules.

The direct agglutination test was conducted in 96 well round bottom plates. Test sera were diluted with PBS and 25  $\mu$ l of serial test dilutions were combined with 75  $\mu$ l of antigen solution and mixed thoroughly by pipetting up and down several times. The plates were covered with parafilm and incubated overnight at 37 °C in a CO<sub>2</sub> incubator. Positive and negative control mouse sera were separately examined on each plate. The agglutination reactions were read the next morning. Diffuse opacity across the entire diameter of the well was considered a positive agglutination reaction. A central discrete opaque dot or button was considered a negative reaction. Several concentrations of spores (13, 67, 100, 133 and 200 x 10<sup>6</sup> spores/ml) were tested to determine which gave the most definitive results. Serum harvested from *E. cuniculi*, *E. hellem* and *E. intestinalis*-infected mice was tested using this method, along with samples from mice infected with *Toxoplasma gondii* or *Sarcocystis neurona*. Serum from 2 dogs naturally infected with *E. cuniculi* was also examined (NEED REF from Dr. Snowden). Serum samples from beavers and raccoons were also screened using the agglutination test.

The sensitivity and specificity of the antibody agglutination test was calculated using standard formulas. Any samples that were falsely positive or negative by the agglutination test were compared to normal samples using Western blot to identify any differences in serum proteins that could cause false results. IFA testing was also performed on 6 positive samples from 7 weeks PI to confirm the results of the agglutination test.

Cross-reactivity between *E. cuniculi* and *E. intestinalis* was examined using Western blotting and IFA. For Western blotting, serum from *E. cuniculi*-infected CD-1 mice was tested against both *E. cuniculi* and *E. intestinalis* spores.  $5 \times 10^6$  spores were loaded per well of a 10% polyacrylamide gel, and the serum was diluted to 1:100 in TBS. Peroxidase-labeled goat anti-mouse antibodies diluted to 1:500 in tris-buffered saline (TBS)(pH 7.4) served as secondary antibodies. The reaction was visualized by exposing peroxidase-labeled nitrocellulose strips to the developing solution (60 mg 4-chloro-1-naphthol, 10 ml cold methanol, 100 ml TBS, 0.6 ml H<sub>2</sub>O<sub>2</sub>) until bands appeared. IFA testing was conducted using serum from *E. cuniculi*-infected mice and spores of *E. cuniculi* and *E. intestinalis*. Serum dilutions of 1:20 and 1:40 were tested using fluorescein-labeled goat anti-mouse antibodies diluted to 1:50 in phosphate-buffered saline (PBS) (pH 8.4)

### 3. RESULTS

The results of this study indicate that the agglutination test is effective for detecting antibodies of *Encephalitozoon cuniculi* in mice and dogs (Table 1). No cross reactivity occurred to *E. hellem* and only minor cross reactivity to *E. intestinalis* was present (Table 1). Of 20 samples from *E. cuniculi*-infected mice, only 3 were falsely negative. However, 1 of the 3 mice was only sampled up to 4 weeks PI, and 5 weeks was required for some mice to seroconvert in the agglutination test. Both samples from naturally infected dogs were positive by the agglutination test. Of 44 samples from mice not infected with *E. cuniculi*, only 1 was positive by the agglutination test, an *E. intestinalis*-infected mouse. The sensitivity of the agglutination test was 86% and the specificity to be 98%. The positive predictive value for this test is 95%.

Of the three falsely negative samples, 2 came from C3H/He mice, and one came from a BALB/c INF-g knock-out mouse. There was not sufficient serum remaining for Western blot analysis of the BALB/c sample, but the test was conducted for the C3H/He mice. The samples from these 2 mice were compared against another mouse from the same experimental group. The results of Western blotting showed that both falsely negative samples were lacking a 32 kDa protein that was present in the truly positive sample. One falsely positive sample was also missing proteins of 30 and 75 kDa. All 3 samples were examined using IFA, and all samples were positive, although the falsely negative samples had lower titers.

The falsely positive sample was from a mouse infected with *E. intestinalis*, indicating that there is still a possibility of cross-reactivity with other *Encephalitozoon* species using this test. Western blot analysis was performed on the falsely positive sample and another sample from the same group of *E. intestinalis*-infected mice. The

results showed the presence of a single band approximately 35 kDa in size in the falsely positive sample. No bands were observed in the truly negative sample.

Western blot analysis was performed using both *E. cuniculi* and *E. intestinalis* spores against serum from *E. cuniculi*-infected mice. Spores of *E. cuniculi* reacted with proteins at 120, 75, 42, 32, 30, 19 and 15 kDa. A large band was observed from 45 to 58 kDa. Western blot analysis of spores from *E. intestinalis* against serum from *E. cuniculi*-infected mice revealed several cross-reactive bands. Thin bands were observed at 120, 75, 58, 40, 38 and 30 kDa; heavy bands were present at 35 and 50 kDa.

Cross-reactivity with the IFA test was observed when serum from *E. cuniculi*-infected mice was tested against *E. intestinalis* spores. A much weaker reaction was observed against *E. intestinalis* than *E. cuniculi* spores.

Serum from 42 raccoons (Hancock et al., 2005) and 62 beavers (Jordan et al., 2005) were also examined using the agglutination test. Antibodies to *E. cuniculi* were present in 1 of 42 raccoons and 0 of 62 beavers using the agglutination test.

#### 4. DISCUSSION

*Encephalitozoon cuniculi* is an important parasite in human and veterinary medicine. Rabbits and dogs are the most commonly infected domestic animals, and research suggests that the parasite is transmitted from these animals to their human owners. Currently, ELISA and IFA testing are the 2 serologic tests commonly used in research and hospital laboratories. While these methods are reliable for diagnosing infection, they have distinct disadvantages being that each method requires the use of specialized equipment. ELISA requires a special plate reader, and IFA requires a compound microscope with a fluorescent light source. Also, much cross-reactivity occurs between *Encephalitozoon* species using IFA, and genetic analysis is often required to confirm the infective species.

One advantage of the direct agglutination test is that no species-specific reagents are needed. This is helpful in examining exotic and wildlife species for serological evidence of exposure. For example, we examined sera from raccoons and beavers using our agglutination test. Commercial anti-sera is available only for raccoon, therefore without our agglutination test we would not have been able to test the beavers.

Our study describes the development of a new serologic test based on agglutination of *E. cuniculi* spores with serum antibodies. This test is highly sensitive and specific for antibodies to *E. cuniculi*, and cross-reactivity is reduced as compared to IFA. No specialized equipment is required in order to conduct this test, and results are available within 24 hours. Since species-specific antibodies are not required, this assay can be used to screen many animal species.

## **ACKNOWLEDGMENTS**

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**Table 1:** Results of the agglutination test for *Encephalitozoon cuniculi*

Sample	Infection	Ag Test	Ag Titer	IFA Titer	Proteins observed by Western blot
Dog	<i>E. cuniculi</i>	+	ND	ND	ND
Dog	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	-	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	800	800	ND
CD-1	<i>E. cuniculi</i>	+	200	400	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. cuniculi</i>	+	ND	ND	ND
C3H	<i>E. cuniculi</i>	-	-	50	10, 30, 35, 48, 57, 75, 225 kDa
C3H	<i>E. cuniculi</i>	-	-	100	10, 35, 48, 57, 225 kDa
C3H	<i>E. cuniculi</i>	+	100	400	10, 30, 32, 35, 48, 57, 75, 225 kDa
C3H	<i>E. cuniculi</i>	+	100	400	ND
C3H	<i>E. cuniculi</i>	+	50	200	ND
C3H	<i>E. cuniculi</i>	+	200	800	ND
C3H	<i>E. cuniculi</i>	+	ND	ND	ND
C3H	<i>E. cuniculi</i>	+	ND	ND	ND
C3H	<i>E. cuniculi</i>	+	ND	ND	ND
CD-1	<i>E. intestinalis</i>	+	-	50	35 kDa
CD-1	<i>E. intestinalis</i>	-	ND	ND	No reaction
CD-1	<i>E. intestinalis</i>	-	ND	ND	ND
CD-1	<i>E. intestinalis</i>	-	ND	ND	ND
C3H	<i>E. intestinalis</i>	-	ND	ND	ND
C3H	<i>E. intestinalis</i>	-	ND	ND	ND
C3H	<i>E. intestinalis</i>	-	ND	ND	ND
CD-1	<i>E. hellem</i>	-	ND	ND	ND
CD-1	<i>E. hellem</i>	-	ND	ND	ND
CD-1	<i>E. hellem</i>	-	ND	ND	ND
CD-1	<i>E. hellem</i>	-	ND	ND	ND
C3H	<i>E. hellem</i>	-	ND	ND	ND
C3H	<i>E. hellem</i>	-	ND	ND	ND
C3H	<i>E. hellem</i>	-	ND	ND	ND
C3H	<i>E. hellem</i>	-	ND	ND	ND

CD-1	None	-	ND	ND	ND
CD-1	None	-	ND	ND	ND
CD-1	None	-	ND	ND	ND
CD-1	<i>T. gondii</i>	-	ND	ND	ND
KO	<i>S. neurona</i>	-	ND	ND	ND

- ND = not done
- Titers reported for samples from 7 weeks PI.
- 23 additional *S. neurona* positive mouse samples also tested negative using the agglutination test.

## **CHAPTER 4**

# **EFFECTS OF HIGH PRESSURE PROCESSING ON *IN VITRO* INFECTIVITY OF *ENCEPHALITOZON CUNICULI***

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## **ABSTRACT**

High pressure processing (HPP) has been shown to be an effective means of eliminating bacteria and destructive enzymes from a variety of food products. HPP extends the shelf life of products while maintaining the sensory features of food and beverages. The present study examined the effects of HPP on the infectivity of *Encephalitozoon cuniculi* spores in vitro. Spores were exposed to between 140 and 550 MPa for 1 min in a commercial HPP unit. Following treatment the spores were loaded onto cell culture flasks or were kept for examination by transmission electron microscopy. No effect was observed on the infectivity of spores treated with 140 MPa. Spores treated with between 200 and 275 MPa showed reduction in infectivity. Following treatment of 345 MPa or more, spores were unable to infect host cells. No morphologic changes were observed in pressure-treated spores using transmission electron microscopy.

## INTRODUCTION

*Encephalitozoon cuniculi* is a small protist parasite in the phylum Microspora. There are over 1200 species of microsporidia, and the phylum is characterized by a unique organelle used to infect host cells, the polar tube. Hosts are infected by ingestion or inhalation of spores passed in the urine or feces, or by transplacental transmission in some animals. Infection with *E. cuniculi* is usually asymptomatic, except in young or immunocompromised hosts. Diarrhea is the most common symptom of infection in immunocompromised individuals, but disseminated infections can occur, causing conjunctivitis, sinusitis, nephritis and encephalitis (Franzen and Muller, 2001). Microsporidia infection can be fatal in immunocompromised patients. Human and animal infections with *E. cuniculi* were recognized prior to the AIDS pandemic, but overall awareness of the Phylum as important parasites of warm-blooded animals came about only after the advent of a large immunocompromised population.

High pressure processing (HPP) is used commercially as a non-thermal means to extend the shelf life of foods and beverages by eliminating pathogens and denaturing destructive enzymes (Tewari et al., 1999). It has a number of advantages over traditional thermal processing, including shorter processing time, minimal heat damage problems, no adverse changes due to ice-phase forms during pressure-shift freezing, minimal modifications to functionality, and preservation of flavor, texture, color and vitamin C (Tewari et al., 1999).

The present study was conducted in response to recent reports discussing the possibility that microsporidia may be present in juice products (Slifko et al., 2000a). A recent study examined water sources in North and Central America for the presence of protozoan parasites (Thurston-Enriquez et al., 2002). The water sources sampled were

used for irrigation of various fruit and vegetable crops. Thurston-Enriquez et al. (2002) discovered that DNA from human pathogenic microsporidia was present in all water sources examined. Microsporidia in irrigation water could adhere to the surface of fruits and vegetables. Since spores are extremely resistant to damage, they may remain viable on the produce for weeks or months. These facts also suggest that microsporidia may be present in juice products.

Studies have shown that HPP is useful in eliminating bacteria and protozoa from juices without altering taste or appearance. Slifko et al. (2000b) examined the effects of HPP on *Cryptosporidium parvum* oocysts in apple and orange juice. It was determined that 550 MPa for 60 sec decreases the *in vitro* infectivity of *C. parvum* oocysts. Lindsay et al. (2004) show that exposure of *Toxoplasma gondii* oocysts to 340 MPa for 60 sec reduces viability of oocysts *in vivo*. Another study found that subjecting orange juice to pressures of 400 MPa for 60 sec significantly reduced the viability of *E. coli* O157:H7, but some organisms were able to survive the treatment (Linton et al., 1999). The present study was conducted to determine the effect of HPP on the infectivity of *E. cuniculi* spores in cell culture.

## MATERIALS AND METHODS

*Encephalitozoon cuniculi* (American Type Culture Collection, #50502) spores were harvested from HS68 cell cultures (see below) by removing the supernatant from infected flasks and passing it through a 2  $\mu\text{m}$  filter. In trials 1 and 2, spores were suspended in Hank's balanced salt solution (HBSS). In trial 3, spores were suspended in pasteurized apple cider. Spores were sealed in leak proof bags at a concentration of  $20 \times 10^6$  spores/ml. These bags were placed in additional bags containing a 10% bleach solution to protect against contamination of the processor. The spores were exposed to pressures ranging from 140 to 550 MPa and held at the desired pressure for one minute. Three trials were conducted for in vitro infectivity studies, and each measurement was made in triplicate.

Human foreskin fibroblast cells (American Type Culture Collection, Hs68, no. CRL-1635) were grown to confluence in 25  $\text{cm}^2$  cell culture flasks. They were grown in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% sodium pyruvate added. One milliliter of the spore suspension was removed from each bag and loaded into individual flasks of Hs68 cell cultures. The cultures were examined at 30 days, and each flask was evaluated for percent of host cells infected.

One additional study was conducted for examination by transmission electron microscopy (TEM). Spores were treated with 410 MPa or 275 MPa and compared to untreated control spores for changes in morphology. After pressure treatment, 1 ml from each bag was removed and centrifuged to pellet the spores. The spores were fixed in 3% (v/v) glutaraldehyde in PBS (pH 7.4). Spore pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanols, passed through 2 changes of propylene oxide, and embedded in Poly/Bed 812 resin



(Polysciences Inc., Warrington, Pennsylvania). Spores were examined with a Zeiss 10CA transmission electron microscope operating at 60 kV, and digital photos were taken using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, Massachusetts). Eleven TEM pictures were taken of spores treated with 410 MPa, and 3 were taken of spores treated with 275 MPa. These photos were compared to 13 TEM photos of untreated *E. cuniculi* spores.

## **RESULTS**

No effect was observed on the infectivity of spores treated with 140 MPa. Spores treated with pressures between 275 and 310 MPa showed reduction in infectivity, but this result was variable. Following treatment of 345 MPa or more, spores were unable to infect host cells (Table 1). Examination by TEM revealed no morphologic changes in the pressure treated spores as compared to controls (Figure 1). This is not unexpected, since HPP acts primarily by disrupting hydrogen bonds and three-dimensional configuration of protein molecules.

## **DISCUSSION**

This study was conducted to examine the possibility of using high pressure processing to sterilize juice products that may contain microsporidia. There is at least one report that DNA from human pathogenic microsporidia has been found in water sources used for irrigation of produce crops (Thurston-Enriquez et al., 2002). Since microsporidia are resistant to environmental conditions, spores that adhere to produce may still be viable when crops are processed for juice. Some companies are hesitant to pasteurize juice products for fear of weakening the flavor. It is unknown whether pasteurization kills microsporidia, but non-pasteurized products could certainly contain viable spores. High pressure processing is a viable alternative for producers who do not wish to pasteurize juices. Pathogenic bacteria and protozoa can be eliminated, and the processing time required for HPP is shorter than for pasteurization.

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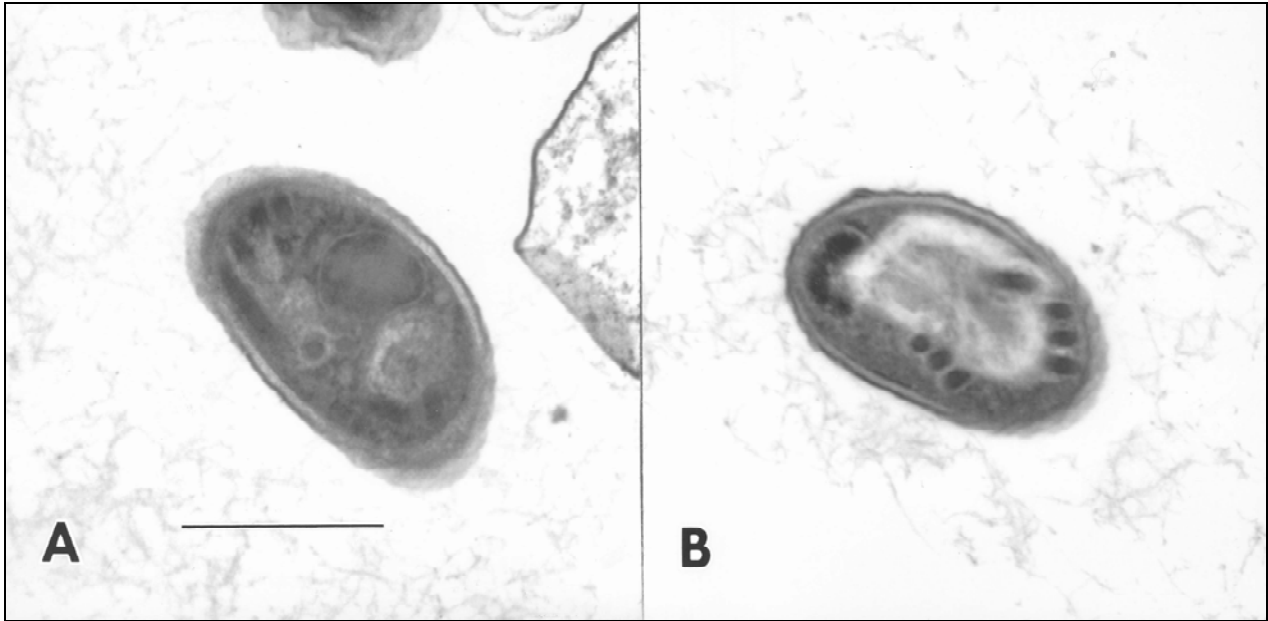
**Table 1:** Infectivity of *E. cuniculi* spores in cell culture following high pressure processing

<b>Trial</b>	<b>Days PE*</b>	<b>Pressure (MPa)</b>	<b>Infective</b>	<b>% CPE<sup>†</sup></b>
<b>1</b>	30	0	Yes	90
		140	Yes	90
		275	Yes	10
		410	No	0
		550	No	0
<b>2</b>	33	0	Yes	70
		200	Yes	30
		275	Yes	0
		310	No	0
		345	No	0
		380	No	0
		410	No	0
		480	No	0
<b>3</b>	30	0	Yes	50
		200	Yes	10
		240	Yes	22
		275	Yes	5
		310	Yes	> 1

\* Day PE = Day post exposure cytopathic effect was measured

<sup>†</sup> % CPE = % cytopathic effect

**Figure 1:** Transmission electron micrographs demonstrating the structure of a control spore **(A)** and a 480 MPa pressure-treated spore **(B)** of *Encephalitozoon cuniculi*. Bar = 1  $\mu$ M.



## CHAPTER 5

# ACTIVITY OF DISINFECTANTS AGAINST SPORES OF *ENCEPHALITOOZON CUNICULI*

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## **ABSTRACT**

*Encephalitozoon cuniculi* is a small protist parasite in the phylum Microspora. Hosts are infected by ingestion or inhalation of spores passed in the urine or feces. Infection with *E. cuniculi* is usually asymptomatic, except in young or immunocompromised hosts. This study examined the effects of various disinfectants on *in vitro* infectivity of *E. cuniculi* spores. Fifty million spores of *E. cuniculi* were exposed to several dilutions of commercial bleach, HiTor™ and Roccal™, and 70% ethanol for 10 minutes and then loaded onto Hs68 cells. The results of this study found that all concentrations of disinfectants tested were lethal to *E. cuniculi* spores. Spores of *E. cuniculi* are more sensitive to disinfectants than are coccidial oocysts and other parasite cysts.

**Keywords:** *Encephalitozoon cuniculi*, microsporidia, disinfectants, sterilization



## 1. INTRODUCTION

Microsporidia are important parasites of humans and a wide variety of animal species. *Encephalitozoon cuniculi* is a common pathogen of rabbits and other domestic animals, and occasionally causes disease in immunocompromised humans. Serologic surveys conducted throughout Europe report rates of infection as high as 42% in asymptomatic rabbits (Halanova et al., 2003) and 85% in rabbits exhibiting neurological symptoms (Deplazes et al., 1996). Clinical signs of infection in rabbits are almost entirely neurological, with some cases developing ocular lesions and cataracts. Lethargy and head tilt are usually the first signs, and ataxia, paresis and hind limb paralysis follow (Harcourt-Brown and Holloway, 2003). In humans, *E. cuniculi* primarily infects the eyes, sinuses, urinary tract and brain, but severe disseminated infections have been observed in severely immunosuppressed individuals (Franzen and Muller, 2001).

The present study examined the effectiveness of various disinfectants for inactivating spores of *E. cuniculi*. Few reports are available on the effects of chemical disinfectants on the viability of microsporidia. Waller (1979) found that 30 minutes of exposure to 70% ethanol kills *E. cuniculi* spores. A more recent study examined several chemicals for their effect on *E. intestinalis* spores. It was reported that exposure to 70% ethanol for 30 seconds resulted in 100% reduction in infectivity as compared to controls (Santillana-Hayat et al., 2002). Exposure to bleach (37.8 g/L NaOCl) for 5 minutes resulted in complete destruction of the spores (Santillana-Hayat et al., 2002). The present study examines the effect of ethanol, bleach, and 2 commercial products on the *in vitro* infectivity of spores of *E. cuniculi* in order to identify products that may be useful for sterilizing rabbit cages.

## **2. MATERIALS AND METHODS**

### **2.1. *Encephalitozoon cuniculi* and cell culture**

*Encephalitozoon cuniculi* spores (American Type Culture Collection, Manassas, Virginia, ATCC # 50502) were grown and harvested from human fibroblast (Hs68, American Type Culture Collection, ATCC CRL-1635) cells as previously described (Jordan et al., 2005). The Hs68 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics as previously described and grown to confluence in 75 cm<sup>2</sup> flasks. Spores were collected from the supernatant, filtered through a 3 µM filter to remove host cell debris, and counted in a hemocytometer prior to use in the present study. Monolayers of HS68 cell grown in 25 cm<sup>2</sup> flasks were used to determine the efficacy of disinfection treatments on *E. cuniculi* spores.

### **2.2. Efficacy of disinfectants**

Several dilutions of commercial bleach (The Clorox Company, Oakland, California) (Table 1) and 70% ethanol were prepared in distilled water. Commercially-available disinfectants Roccal™ (Pharmacia & Upjohn Company, Kalamazoo, Michigan) and HiTor™ (Huntington Professional Products, St. Paul, Minnesota) were diluted according to manufacturer's directions (0.39%) and at a concentration of 0.1% in distilled water. Active components of all disinfectants are listed in Table 2. All test solutions were chilled at 4°C to prevent any temperature increase that could result due to exothermic reactions. Tubes containing 50 x 10<sup>6</sup> spores of *E. cuniculi* were washed in Hank's Balanced Salt Solution (HBSS) and centrifuged to pellet spores. Spores were resuspended in 10 ml of the desired solution, with one tube suspended in HBSS to serve

as a no treatment control. Tubes were held at 4°C for 10 minutes, and then centrifuged at 1500 rpm for 10 minutes to pellet spores. Disinfectant solutions were discarded, and spores were washed twice in HBSS by centrifugation. Spores were resuspended in 10% cell culture growth media and added to Hs68 cell cultures. A small amount of each suspension was examined using a compound microscope. Cell cultures were examined daily for evidence of *E. cuniculi* infection.

### **3. RESULTS & DISCUSSION**

All dilutions of bleach, HiTor™ and Roccal™, and 70% ethanol were lethal to spores of *E. cuniculi* after 10 minutes exposure at 4°C. The HBSS treated control spores were infectious for Hs68 cells. Examination of the spore suspensions found that *E. cuniculi* spores were completely destroyed by all concentrations of bleach. No spores were visible under the microscope; only cellular debris was observed. HiTor is sold as a germicidal detergent designed to disinfect, clean and deodorize in one step. Roccal is labeled as a veterinary and animal care disinfectant that is safe for use in animal breeding and housing facilities. Both dilutions of HiTor™ and Roccal™ rendered *E. cuniculi* non-infective for cell culture, but spores appeared normal when viewed using light microscopy. Seventy percent ethanol was also lethal to spores, rendering them non-infective. Spores treated with ethanol were normal when examined using light microscopy. All disinfectants examined should be effective for the killing of *E. cuniculi* spores in rabbit cages and in other areas of facilities that house rabbits.

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**Table 1:** Concentrations of bleach stock and hypochlorite tested

<b>Bleach</b>	0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 10.0%
<b>Sodium hypochlorite</b>	0.00525%, 0.0105%, 0.01575%, 0.021%, 0.02625%, 0.0525%, 0.105%, 0.1575%, 0.21%, 0.2625%, 0.525%

**Table 2:** Concentrations of active compounds in disinfectants

<b>Disinfectant</b>	<b>Active Compound</b>	<b>Stock Concentration</b>
Bleach	NaOCl	5.25%
HiTor™	C <sub>22</sub> H <sub>48</sub> ClN	9.70%
	n-Alkyl (C <sub>14</sub> , 50%; C <sub>12</sub> , 40%; C <sub>16</sub> , 10%) dimethyl benzyl ammonium chloride	6.46%
Roccal™	C <sub>22</sub> H <sub>48</sub> ClN	9.20%
	Alkyl (C <sub>12</sub> , 61%; C <sub>14</sub> , 23%; C <sub>16</sub> , 11%, C <sub>18</sub> , 2.5%, C <sub>8</sub> & C <sub>10</sub> , 2.5%) dimethyl benzyl ammonium chloride	9.20%
	Alkyl (C <sub>12</sub> , 40%; C <sub>14</sub> , 50%; C <sub>16</sub> , 10%) dimethyl benzyl ammonium chloride	4.60%
	bis-n-tributyltin oxide	1.00%
Ethanol	(CH <sub>3</sub> ) <sub>2</sub> OH	100%

## CHAPTER 6

### GENERAL CONCLUSIONS

*Encephalitozoon cuniculi* is an important parasite in human and veterinary medicine. Rabbits and dogs are the most commonly infected domestic animals, and research suggests that the parasite may be transmitted from these animals to their human owners. Because *E. cuniculi* is a zoonotic pathogen, it is important that veterinarians are able to accurately diagnose infection. Currently, ELISA and IFA testing are the 2 serologic tests commonly used in research and hospital laboratories, however, these methods each require the use of specialized equipment that may not be present in private veterinary practices.

Chapter 3 describes the development of a new serologic diagnostic test for the presence of agglutinating antibodies to *E. cuniculi* in serum. The results indicate that this test is highly sensitive and specific to *E. cuniculi* with limited cross-reactivity to other *Encephalitozoon* species. Of 20 samples from *E. cuniculi*-infected mice, only 3 were falsely negative. However, 1 of the 3 mice was only sampled up to 4 weeks PI, and 5 weeks was required for some mice to give positive results. Western blot analysis performed on 2 of the 3 false negative samples showed no bands in these samples, but a truly positive sample exhibited bands approximately 62 and 69 kDa in size. All 3 samples were examined using IFA, and all samples were positive, although the falsely negative samples had significantly lower titers. Samples from 2 naturally infected dogs were positive by the agglutination test.



Of 44 samples from mice not infected with *E. cuniculi*, only 1 was positive by the agglutination test, an *E. intestinalis*-infected mouse. The falsely positive sample was from a mouse infected with *E. intestinalis*, indicating that there is still a possibility of cross-reactivity with other *Encephalitozoon* species using this test. Western blot analysis was performed on this sample and another from the same group of mice. The results showed the presence of a single band approximately 35 kDa in the falsely positive sample. No bands were observed in the truly negative sample.

Cross-reactivity between *E. cuniculi* and *E. intestinalis* was examined using Western blot analysis. When serum from *E. cuniculi*-infected mice used as the primary antibody, spores of *E. cuniculi* reacted with proteins at 120, 75, 42, 32, 30, 19 and 15 kDa. A large smear was observed from 45 to 58 kDa. Western blot analysis of spores from *E. intestinalis* against serum from *E. cuniculi*-infected mice revealed several cross-reactive bands. Thin bands were observed at 120, 75, 58, 40, 38 and 30 kDa; heavy bands reacted at 35 and 50 kDa. Cross-reactivity with the IFA test was observed when serum from *E. cuniculi*-infected mice was tested against *E. intestinalis* spores, however a much weaker reaction was observed against *E. intestinalis* than *E. cuniculi* spores.

Statistical analysis of the agglutination test found the sensitivity to be 86% and the specificity to be 98%. The agglutination test would be more practical than the IFA or ELISA for use in a small veterinary clinic. The test is easy to conduct and requires only basic equipment that is likely already present in most clinics. The test can be completed in under 10 minutes, and results are available within 24 hours. Because no species-specific antibodies are required, this test would be useful for serological screening of exotic and wildlife species.

Another study was conducted to examine the possibility of using high pressure processing to sterilize juice products that may contain microsporidia. There is at least one report that DNA from human pathogenic microsporidia has been found in water sources used for irrigation of produce crops. Since microsporidia are resistant to environmental conditions, spores that adhere to produce may still be viable when crops are processed for juice. Chapter 4 describes the results of this study, showing that pressures of 345 MPa or more render spores of *E. cuniculi* non-infective in cell culture. Examination of pressure-treated spores by TEM microscopy did not reveal any morphological changes. This is not unexpected, since HPP acts primarily by disrupting hydrogen bonds and three-dimensional configuration of protein molecules. High pressure processing is a viable alternative for producers who do not wish to pasteurize juices. Pathogenic bacteria and protozoa can be eliminated, and the processing time required for HPP is shorter.

In Chapter 5, the effects of several commercially available disinfectants were examined to identify means of inactivating microsporidia spores in animal facilities and cages. All dilutions of bleach (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 5, 10%), HiTor™ and Roccal™ (0.1, 0.39%) and 70% ethanol were lethal to *E. cuniculi* spores after 10 minutes exposure at 4°C. Examination of the spore suspensions showed that *E. cuniculi* spores were completely destroyed by all concentrations of bleach. No spores were visible under the microscope, and only cellular debris was observed. Spores treated with HiTor, Roccal or ethanol appeared normal using light microscopy. All disinfectants examined should be effective for the killing of *E. cuniculi* spores in animal cages and facilities.

## **CHAPTER 7**

### **APPENDIX**

# **PREVALENCE OF AGGLUTINATING ANTIBODIES TO *TOXOPLASMA GONDII* AND *SARCOCYSTIS NEURONA* IN BEAVERS (*CASTOR CANADENSIS*) FROM MASSACHUSETTS.**

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## **ABSTRACT**

This study examined the seroprevalence of *Toxoplasma gondii* and *Sarcocystis neurona* in a population of beavers (*Castor canadensis*) from Massachusetts. Blood samples were taken on filter paper and shipped to Virginia Tech for testing. The samples were tested at various dilutions against each parasite antigen by modified agglutination tests to determine whether antibodies to either parasite were present in the blood. Six (9.7%) of 62 samples were positive for *T. gondii*, with 2 samples having titers of 1:25 and 4 having titers of 1:50. Four (6.5%) of 62 samples were positive for *S. neurona*, with 2 samples having titers of 1:25 and 2 having titers of 1:50.

## INTRODUCTION

*Toxoplasma gondii* is an important protozoal pathogen of humans and other warm-blooded vertebrates. Domestic and wild felids are the definitive host, and they excrete oocysts into the environment. Intermediate hosts can become infected by ingestion of sporulated oocysts in contaminated feed or water-or by ingesting tissue cysts in prey.

*Sarcocystis neurona* is an obligate heteroxenous parasite with the opossum as its definitive host. Intermediate hosts are infected by ingestion of sporocysts passed in opossum feces. *Sarcocystis neurona* is important because it is the causative agent of equine protozoal myeloencephalitis, a severe neurological disease that can occur when a horse accidentally ingests *S. neurona* sporocysts. Horses may become infected by drinking water contaminated with sporocysts.

This study examines the prevalence of *T. gondii* and *S. neurona* in beavers as an indication of contamination in local water sources. Little is known about the prevalence of apicomplexan parasites in beavers, *Castor canadensis*. The present study was done to determine the seroprevalence of *T. gondii* and *S. neurona* in beavers from Massachusetts.

## **MATERIALS AND METHODS**

Beavers sampled in this study originated in various locations in Massachusetts. Beavers were live-trapped as part of a larger study on rabies. Given the difficulty of venipuncture in beavers, an alternative method for collecting samples was employed. Drops of blood were blotted onto filter paper following a cutaneous tail stick using a hypodermic needle. This method of collecting blood has been used in toxoplasmosis testing of congenitally infected infants (Hsu et al., 1992; Patel and Holliman, 1994; Paul et al., 2001), sheep (Uggla and Nilsson, 1987) and cats (Nogami et al., 1992). The filter paper blots were kept on ice packs and mailed to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech for testing. One square centimeter sections containing the dried blood were cut from the filter paper blots and submerged in 200  $\mu$ l phosphate-buffered saline (PBS). The paper soaked overnight at 4°C to allow blood proteins to dissolve into the PBS. The eluate was removed and placed into clean tubes for use in agglutination testing.

The modified direct agglutination test (MAT) was used to examine beaver eluates for agglutinating IgG antibodies to *T. gondii* (Dubey and Desmonts, 1987) and *S. neurona* (Lindsay and Dubey, 2001). A dilution of 1:25 was used to screen sera. We examined serum samples from 31 beavers in 2002, 17 in 2003, and 14 beavers in 2004. Positive beaver sera were further examined at dilutions of 1:50 and 1:100.

## RESULTS

Positive *T. gondii* MAT results were found in 6 of 62 (9.7%) beavers from 2002. Four samples were positive at 1:50, and 2 samples were positive only at 1:25. Four of 62 (6.4%) beavers from 2002 were found positive for *S. neurona* by the MAT. Two were positive at 1:50, and 2 were positive only at 1:25. No samples from 2003 or 2004 were positive for *T. gondii* or *S. neurona*.

## DISCUSSION

*Toxoplasma gondii* has been isolated from a beaver in Kansas (Smith and Frenkel, 1995), and prevalence data is abundant for infection in wildlife. The prevalence of *T. gondii* in beavers reported here is low compared to data from other surveys of aquatic wildlife in the United States. One study in North Carolina found that 45% of river otters sampled were seropositive for *T. gondii* using a latex agglutination test (Tocidlowski et al., 1997).

The infected beavers in this study may be ingesting *T. gondii* oocysts excreted by wild or domestic cats from contaminated water or foliage. Bobcats are the only wild cats that live in Massachusetts, where they are most common in the central and western regions. Although no prevalence data exists for *T. gondii* in bobcats from Massachusetts, studies have been conducted in other parts of the United States. Serum from 52 bobcats from various states was tested for the presence of antibodies to *T. gondii*, and 50% of the animals were seropositive (Kikuchi et al., 2004).

We are unaware of any previous reports of *S. neurona* infection in beavers. However, a recent study examined other wildlife species from Connecticut for the presence of agglutinating antibodies to *S. neurona*. Eleven of 24 (46%) skunks and 12 of 12 (100%) raccoons had positive titers to *S. neurona* (Mitchell et al., 2002). Another study found that 24 of 25 (96%) raccoons from Massachusetts were seropositive for *S. neurona* (Lindsay et al., 2001). Compared to these and other studies, the prevalence of *S. neurona* infection in the beavers tested in this study is low.



Opossums are the definitive host for *S. neurona*, and they are abundant throughout Massachusetts. Prevalence data is not available for *S. neurona* infection in opossums from Massachusetts, but studies have been conducted in Maryland and Michigan. These studies found that 6 of 11 (54%) opossums from Maryland (Dubey, 2000), and 31 of 206 (15%) opossums from Michigan (Elsheikha et al., 2004) were infected with *S. neurona*.

The prevalence of *T. gondii* and *S. neurona* in beavers from Massachusetts is lower than what has been observed in other wildlife species. However, even a small number of infected animals indicates that the parasites are present in the area, and infective stages are likely to be found in water sources.

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## **CHAPTER 8**

### **VITA**

Carly Nicole Jordan was born October 12, 1981 in Houston, Texas to Janet and Monty Wetch. She moved to Pennsylvania in 1995 and graduated from Carlisle High School in 1999. In July 2003, she earned a Bachelors degree in biology from Virginia Tech. Following graduation she enrolled as a Masters student in the department of Biomedical Sciences and Pathobiology through the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech. There she studied parasitology with Dr. David Lindsay, focusing her work on microsporidia. During this time she was an active member of several professional societies, including the Southeastern Society of Parasitologists, American Society of Parasitologists, and American Society of Tropical Medicine and Hygiene. In June 2004, Carly was married to her high school love, Jason Jordan. Following completion of this degree, she will attend the University of Georgia where she will continue studying parasitology while pursuing a PhD in the department of Cellular Biology.