

# DIAGNOSIS AND EPIDEMIOLOGY OF MICROSPORIDIA INFECTIONS IN HUMANS

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**Abstract.** Microsporidia are single-celled, obligately intracellular parasites that are considered a cause of emerging and opportunistic infections in humans. The most common symptoms associated with microsporidiosis are persistent and self-limiting diarrhea in immune-deficient and immune-competent individuals, respectively. Species of microsporidia that infect humans also infect a wide range of animals which raises a concern for zoonotic transmission. Microsporidian spores are environmentally resistant and have been identified in various water sources, also raising a concern for water- and food-borne transmission. Microsporidia can be detected in patient specimens such as urine, feces, and tissue biopsies by staining with calcofluor white, concentrated trichrome, Gram, or immunofluorescent antibody but species identification usually requires detection by PCR-based techniques. Improved diagnostic methods are being used to address the epidemiology of microsporidiosis and recent studies indicate that individuals most at risk for infection include persons with AIDS, immune-suppressed organ transplant recipients, malnourished children, travelers, and the elderly. Risk factors have included eating undercooked meat and exposure to water through recreational or occupational exposure or through drinking water. Strategies, therefore, are being developed to identify and remove or disinfect species of microsporidia present in water sources that pose a risk for transmission to humans and animals.

## INTRODUCTION

Microsporidia were first recognized as the cause of pébrine (pepper) disease in silkworms in 1857 and since then have caused significant economic losses in the honeybee, fish, and mink industries (Wittner, 1999). Subclinical microsporidia infections in laboratory animals have interfered with biomedical research (Shadduck and Pakes, 1971; Canning and Lom, 1986; Shadduck and Greeley, 1989), and more recently, microsporidia have been identified as a cause of opportunistic infections associated with persistent diarrhea and weight loss in persons with AIDS (Desportes *et al.*, 1985; Modigliani *et al.*, 1985; Kotler and Orenstein, 1998; 1999). With the improvement of diagnostic methods and increased awareness, microsporidiosis has been recognized in organ transplant recipients, travelers, children, and the elderly (Bryan *et al.*, 1997; Bryan and Schwartz, 1999; Schwartz and Bryan, 1999; Deplazes *et al.*, 2000). Species of microsporidia that infect humans also have been identified in animals and water sources, raising public health concerns for zoonotic, food-borne, and

water-borne transmission of microsporidia (Franzen and Müller, 1999a; Deplazes *et al.*, 2000).

## BIOLOGY OF MICROSPORIDIA

### Structure / Morphology

Microsporidia are single-celled, obligately intracellular eukaryotic parasites that infect protozoa, invertebrates, and vertebrates (Canning and Lom, 1986). Spores of microsporidian species that infect mammals are small and oval in shape, measuring approximately 1.0 - 2.0  $\mu\text{m}$   $\times$  1.5 - 4.0  $\mu\text{m}$  (Canning and Lom, 1986; Vavra and Larrison, 1999). Microsporidian spores are surrounded by an outer electron-dense exospore composed of glycoprotein, an electron-lucent endospore composed of chitin, and an inner plasma membrane. The chitinous spore wall is believed to contribute to the persistence of microsporidia in the environment. The nucleus in microsporidia exists either as a monokaryotic single nucleus (*eg* *Encephalitozoon*, *Enterocytozoon*, *Pleistophora*, and *Trachipleistophora* species) or as a diplokaryon with two adjoined nuclei that function as a single unit (*eg* *Brachiola*, *Nosema*, and *Vittaforma* species). Mature microsporidian spores contain a distinct posterior vacuole which provides a useful diagnostic characteristic for identifying microsporidia in stained tissue sections or smears. An anchoring disc and Golgi-like membranous polaroplast are located in the anterior region of the spore. The polar filament, a

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unique structure found in microsporidia, is a hollow tube arising from the anterior region that coils in the mid-to-posterior region of the spore and is used during germination to infect the host cell (Undeen, 1990; Keohane and Weiss, 1999). Although microsporidia are true eukaryotes, microsporidia contain prokaryote-like ribosomes that are closely associated with endoplasmic reticulum scattered throughout the cytoplasm and they lack of true peroxisomes and mitochondria. Further descriptions on the ultrastructure and morphology of microsporidia may be found in the excellent reviews by Canning and Lom (1986), Cali and Takvorian (1999), Vavra and Larsson (1999), and Desportes-Livage (2000).

### Taxonomy and phylogeny

The first microsporidian was named *Nosema bombycis* by Nägeli in 1857 who grouped this organism with the Schizomycetes (Nägeli, 1857). Balbiani then created the order of Microsporidia in 1882, and in 1976, Sprague established the phylum Microspora which was then classified in the subkingdom Protozoa within the kingdom of Protista that was established in 1980 by Levine (Balbiani, 1882; Sprague, 1976; Levine *et al.*, 1980; Wittner, 1999). The phylum name was then changed to Microsporidia, Balbiani, 1882 (Sprague and Becnel, 1998).

The taxonomy and phylogeny of the microsporidia was questioned when Vossbrinck *et al.* (1987) reported that the small subunit rRNA gene sequences of *Variamorphia necatrix* were found to more closely resemble those of prokaryotes than eukaryotes, suggesting that microsporidia were ancient eukaryotes. The absence of typical eukaryote-like Golgi and mitochondria further supported this early lineage (Vavra and Larsson, 1999), but molecular phylogenetic analyses of microsporidian gene sequences for  $\alpha$ - and  $\beta$ -tubulin, the largest subunit of RNA polymerase II, TATA-box-binding protein, translation elongation factors EF-1 $\alpha$  and EF-2, and glutamyl tRNA synthase, supported a closer relationship between the microsporidia and the fungi (Edlind *et al.*, 1996; Germot *et al.*, 1997; Hirt *et al.*, 1997; 1999; Cavalier-Smith, 1998; Peyretailade *et al.*, 1998; Fast *et al.*, 1999; Keeling *et al.*, 2000; Van de Peer *et al.*, 2000; Katinka *et al.*, 2001; Keeling and Fast, 2002; Williams *et al.*, 2002). Based on studies of the *E. cuniculi* genome, few genes appeared to be related to energy production and tricarboxylic acid cycle-related genes were absent (Katinka *et al.*, 2001; Vivarès *et al.*, 2002). The presence of genes encoding for mitochondrion-like pyruvate dehydrogenase, superoxide dismutation, and Fe-S cluster assembly, however, suggested to Vivares *et al.* (2002) that microsporidia may contain a cryptic

organelle, the mitosome, that retains some mitochondrial functions, comparable to the residual mitochondrion-derived organelle described in *Entamoeba histolytica* and related to the mitochondrion of aerobic eukaryotes. Furthermore, the identification of mitochondrial-like HSP70 genes in several microsporidia suggested a secondary loss of mitochondria and that the endosymbiosis of mitochondria occurred prior to the emergence of the microsporidia (Germot *et al.*, 1997; Keeling *et al.*, 2000; Vivarès *et al.*, 2002; Williams *et al.*, 2002).

Of the nearly 1,200 species of microsporidia that have been identified, 14 species have been reported to infect humans (Weiss and Vossbrinck, 1999). The taxonomic classification of the microsporidia has been based primarily on life cycle and ultrastructural characteristics that include the size of developing and mature organisms, nuclear arrangement, number and alignment of polar filament coils, intracellular location of development, and modes of nuclear and cellular division (Sprague *et al.*, 1992; Cali and Takvorian, 1999; Canning and Vavra, 2000; Desportes-Livage, 2000; Weiss, 2000).

Molecular biology, biochemistry, and immunology approaches have become useful for higher taxonomic classification among the microsporidia including the identification of intermediate hosts of aquatic microsporidian species and the recognized variation within species of microsporidia that infect humans (Weiss and Vossbrinck, 1998; Weiss, 2000). Intraspecific variation of *E. cuniculi*, for example, was first raised by Weiser (1964, 1965) who observed that infections of dogs and foxes resulted in clinically-significant renal disease whereas rabbits and mice chronically infected with *E. cuniculi* rarely developed clinical signs of disease. This was further corroborated after eight isolates of *E. cuniculi* from dogs, rabbits, and mice were compared by SDS-PAGE, Western immunoblot assay, and rDNA nucleotide sequencing. By all three assays, the isolates segregated into three genotypes with the most notable marker being the number of 5'-GTTT'3' repeats identified in the ITS region of the rDNA sequences (Didier *et al.*, 1994). Strain (genotype) I, originally isolated from a rabbit, was found to contain three repeats. Strain II was originally isolated from mice and was found to contain two repeats, and strain III, originally isolated from dogs, contained four repeats. Currently, at least six genotypes of *E. cuniculi* have been identified by random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) analysis, analysis of repetitive sequences in genes encoding the spore wall protein and polar tube protein, and karyotype analysis by pulsed-field gel

electrophoresis (PFGE) (Biderre *et al.*, 1994, 1995, 1999; Mathis *et al.*, 1996; 1997; Xiao *et al.*, 2001a). *E. hellem* diversity was first noted by Hollister *et al.* (1995) based on protein profile analyses and four genotypes have been found by PFGE, rDNA ITS nucleotide sequencing, and single-stranded conformation polymorphism (SSCP) analysis (Mathis *et al.*, 1999; Sobottka *et al.*, 1999; Peuvel *et al.*, 2000; Delarbre *et al.*, 2001; Fedorko *et al.*, 2001; Xiao *et al.*, 2001b; Haro *et al.*, 2003). To date, no diversity has been identified among *E. intestinalis* isolates (Sobottka *et al.*, 1999; Liguory *et al.*, 2000).

Genotype diversity within *E. bienersi* was first described by Rinder *et al.* (1997) who detected nine polymorphic sites within the rDNA ITS region of 12 human fecal specimens which segregated into three genotypes. After *E. bienersi* was identified in pigs, dogs, cats, and non-human primates, five genotypes emerged with the genotype of the human *E. bienersi* isolates differing from the genotypes of *E. bienersi* identified in the other hosts (Breitenmoser *et al.*, 1999; Mathis *et al.*, 1999; Liguory *et al.*, 2000). As more isolates from a broader range of hosts were characterized, at least 31 genotypes of *E. bienersi* now have been identified including some that were shared between isolates from human and non-human hosts (Dengjel *et al.*, 2001; Sadler *et al.*, 2002; Sulaiman *et al.*, 2003; Rinder *et al.*, 2000; 2004).

### Genome

The microsporidian genome is relatively small among the eukaryotes, although estimates of genome sizes of different microsporidia suggest a broad range among these species. The haploid genome of *E. cuniculi* is only 2.9 megabase (Mb) pairs on 11 chromosomes with an estimated 1,997 protein-coding genes (Biderre *et al.*, 1995, 1999; Katinka *et al.*, 2001). The genome of *Encephalitozoon intestinalis* is even smaller at 2.3 Mb (Biderre *et al.*, 1999). *Glugea atherinae*, a microsporidian that infects fish, has a genome size of ~ 20 Mb, arrayed on 16 chromosomes (Biderre *et al.*, 1994), while the genomes of several *Nosema* species range from 5-7 Mb (Malone and McIvor, 1995). The compact genome of *E. cuniculi* is characterized by reduced intergenic spacers and by the shortness of most putative proteins relative to their eukaryote orthologues. A lack of genes for several biosynthetic pathways and the tricarboxylic acid cycle suggested to Katinka and colleagues (2001) that *E. cuniculi* exhibits a strong dependence on host metabolites. Few *E. cuniculi* genes have introns, and pseudogenes do not seem to be present. The genomes of several other microsporidian species are being studied, including *Spraguea lophii* (Hinkle *et al.*, 1997) and *Vittaforma corneae* (Mittleider *et al.*, 2002), both

of which include retrotransposable elements unlike *E. cuniculi*.

### Life cycle

The life cycle of microsporidia species that infect humans is direct and relatively simple (Canning and Lom, 1986; Canning and Hollister, 1992; Didier *et al.*, 1998). Most infections seem to occur through ingestion or inhalation of microsporidian spores because typical primary sites of infection include epithelium of the small intestinal and respiratory tracts, respectively. Vertical transmission has occurred in carnivores, horses, rabbits, rodents, and non-human primates, but has not been reported in humans (Canning and Lom, 1986; Shadduck and Orenstein, 1993; Snowden *et al.*, 1998; Didier *et al.*, 1998; 2000). Transmission through trauma was reported to occur rarely in humans (Canning and Lom, 1986), and experimentally, microsporidiosis has been transmitted to animals through intraperitoneal, intravenous, intrarectal, intratracheal, and intracerebral inoculation routes (Shadduck and Orenstein, 1993; Weber *et al.*, 1994; Snowden *et al.*, 1998).

Microsporidia infect host cells by injecting their spore contents into the host cell during germination. Germination is initiated through a change in pH or osmotic pressure, causing the posterior vacuole and polaroplast to swell due to water uptake which in turn exerts pressure within the spore and results in the propulsion of the spore contents through the everting polar filament into the host cell (Undeen, 1990; Keohane and Weiss, 1999). The organisms then multiply through the process of merogony and differentiate into spores through the process of sporogony (Canning and Lom, 1986; Canning and Hollister, 1992).

*E. bienersi*, the only species assigned to the genus *Enterocytozoon*, is the most common species reported to infect humans and infects a wide range of animals. Multinucleated merogonial plasmodia contain electron-lucent clefts and develop in direct contact with the host cell cytoplasm (Desportes *et al.*, 1985; Cali and Owen, 1990; Cali and Takvorian, 1999; Vavra and Larrison, 1999; Desportes-Livage, 2000). During sporogony, organelles develop further and electron-dense disks can be observed to join and form polar filaments around each nucleus within the sporogonial plasmodium. Cytokinesis then occurs resulting in individual mature spores separating from the plasmodium. *E. bienersi* infections usually remain localized to the small intestine and biliary tract with mature spores being shed with the feces (Weber *et al.*, 1994; Kotler and Orenstein, 1998, 1999). *E. bienersi*

spores measure approximately 1.0 x 1.5 µm and are among the smallest of the microsporidia. These spores are surrounded by a relatively thin chitinous endospore, possess a single nucleus, and contain a polar filament that usually coils six times in a double-row alignment.

*E. cuniculi* is the type species of the genus *Encephalitozoon* and also has a wide host range among mammals, including humans. *E. hellem* and *E. intestinalis* were identified later in patients with AIDS (Canning and Lom, 1986; Weber *et al*, 1994; Didier 1998, 2000; Cali and Takvorian, 1999), and a fourth species, *E. lacertae*, was recently described in skinks but has not been reported to infect mammals (Koudela *et al*, 1998). *Encephalitozoon* organisms replicate by binary division within a membrane-bound parasitophorous vacuole (PV). Meronts, the larger less mature stages, are primarily found closely associated with the PV membrane. During sporogony, the plasma membranes thicken and organelles such as the polar filament and endoplasmic reticulum can be observed. Sporonts may continue to divide by binary division, become more electron dense, and are more centrally located within the PV (Canning and Lom, 1986; Cali and Takvorian, 1999). *E. intestinalis* also secretes an extracellular matrix substance which surrounds the developing organisms in the PV (Orenstein *et al*, 1992; Cali *et al*, 1993). As organisms continue to replicate, the PV and host cell plasma membranes eventually rupture to release spores. During the early stages of infection while *Encephalitozoon* is still found in the intestinal tract, spores are shed in the feces. *Encephalitozoon* species typically disseminate to various sites, including the kidney, after which spores are typically shed with urine (Weber *et al*, 1994; Kotler and Orenstein, 1998, 1999). *Encephalitozoon* spores measure approximately 2.0 x 4.0 µm and contain a polar filament that coils 4 - 8 times, usually in single row alignment.

#### CLINICAL SYNDROMES

The competence of the immune system is a major factor that influences the clinical course of microsporidiosis in mammals. Immunologically competent hosts that were either naturally or experimentally infected with *E. cuniculi* usually developed clinically silent chronic infections, although clinical signs were sometimes evident early after infection (Shadduck and Orenstein, 1993; Weber *et al*, 1994; Kotler and Orenstein, 1998; 1999; Snowden *et al*, 1998; Snowden and Shadduck, 1999). Mice experimentally infected with *E. cuniculi*, for example, sometimes developed ascites which resolved within

two weeks of inoculation, and infected rabbits occasionally developed motor paralysis, convulsions, and torticollis (Snowden *et al*, 1998; Snowden and Shadduck, 1999). In otherwise healthy humans such as travelers, self-limiting diarrhea of about two-to-three weeks' duration has been reported (Sandfort *et al*, 1994; Albrecht and Sobottka, 1997; Raynaud *et al*, 1998; Thielman and Guerrant, 1998; Lopez-Velez *et al*, 1999; Okhuysen, 2001). *E. cuniculi*-infected rabbits and rodents typically remain persistently infected unless treated, but it is unclear if immunocompetent humans infected with microsporidia clear their infections or remain infected.

Immune-compromized hosts infected with microsporidiosis often developed disease that contributed to death (Shadduck and Orenstein, 1993; Weber *et al*, 1994; Snowden *et al*, 1998; 1999; Didier *et al*, 1998; 2000). Immunodeficient athymic, SCID, and gamma-interferon receptor knock-out mice experimentally infected with *Encephalitozoon* spp, *T. hominis*, or *V. corneae*, for example, developed lethal hepatitis and ascites, and rabbits chronically infected with *E. cuniculi* and then immunosuppressed with cyclophosphamide developed incontinence, ataxia, tremor, paresis, and paralysis of the hind limbs prior to death (Schmidt and Shadduck, 1983; Koudela *et al*, 1993; Silveira *et al*, 1993; Didier *et al*, 1994; Silveira and Canning, 1995; Hollister *et al*, 1996; Horvath *et al*, 1999; Khan and Moretto, 1999; Salat *et al*, 2001). Animals with immature immune systems also are at risk. Examples include *E. cuniculi* infections that were associated with spontaneous abortions in squirrel monkeys, renal failure in puppies, and placentitis and abortion in horses (Shadduck *et al*, 1978; Zeman and Baskin, 1985; van Rensburg *et al*, 1991; Snowden *et al*, 1999; Patterson-Kane *et al*, 2003).

Among immunocompromized humans, AIDS patients with  $\leq 100$  CD4<sup>+</sup> T cells/µl blood and infected with microsporidia species such as *E. bienewisi* and *E. intestinalis*, often developed persistent diarrhea accompanied by fever, loss of appetite, weight loss, and wasting disease (Weber *et al*, 1994; Kotler and Orenstein 1998, 1999; Dascomb *et al*, 1999). *Encephalitozoon* infections in persons with AIDS also disseminated to cause encephalitis, sinusitis, hepatitis, myositis, keratitis, pneumonia, or peritonitis (Weber *et al*, 1994; Kotler and Orenstein, 1998, 1999). Immunosuppressed organ transplant recipients with *E. bienewisi* and *Encephalitozoon* infections likewise developed fatigue, fever, nausea and diarrhea (Kotler and Orenstein, 1998; Gumbo *et al*, 1999). Children living in the tropics who were infected with microsporidia, primarily *E. bienewisi*, developed

persistent diarrhea, most likely exacerbated by immune compromise resulting from malnutrition (Hautvast *et al.*, 1997; Desportes-Livage *et al.*, 1998; Valperga *et al.*, 1999; Tumwine *et al.*, 2002; Wanachiwanawin *et al.*, 2002). The elderly in Spain also appeared to be more susceptible to microsporidiosis due to decreasing immune competency associated with aging (Lores *et al.*, 2002). *E. bienersi* infections in immune-deficient individuals usually remained localized to the small intestine, but biliary tract involvement leading to cholangitis and cholecystitis also has occurred. Malabsorption, decreased mucosal surface area, and immaturity of villus epithelial cells are believed to contribute to the diarrhea caused by *E. bienersi* infection (Kotler and Orenstein, 1998, 1999). *Encephalitozoon*, *Trachipleistophora* and *Pleistophora* species usually disseminate to cause sinusitis, keratoconjunctivitis, hepatitis, myositis, peritonitis, nephritis, encephalitis, or pneumonia in immune-deficient individuals.

In contrast to immunodeficient hosts, *E. cuniculi*-infected carnivores such as dogs and blue foxes developed hypergammaglobulinemia that led to immune-complex formation and often fatal renal disease (Nordstoga, 1976; Canning and Lom, 1986; Shadduck and Orenstein, 1993; Didier *et al.*, 1998; Snowden *et al.*, 1998). These findings support the importance of a well-regulated immune response for controlling the pathogenesis of microsporidia infections.

## DIAGNOSIS

### Serology

Several serological methods have been applied toward detection microsporidia-specific antibodies in mammals, including immunofluorescent antibody staining, complement fixation, enzyme-linked immunosorbent assay (ELISA), and Western immunoblot assays (Weber *et al.*, 1999, 2000; Garcia, 2002). These tests were limited to species of microsporidia that could be grown in culture for generating the antigens required to perform these assays. Earlier serological tests, therefore, were used to identify antibodies to *E. cuniculi* which was the first mammalian microsporidian that could be grown in culture (Shadduck, 1969) and were applied for culling infected animals to establish *E. cuniculi*-free colonies (Bywater and Kellett, 1978; Shadduck and Geroulo, 1979; Pakes *et al.*, 1984; Shadduck and Baskin, 1989; Boot *et al.*, 2000). *E. bienersi* has not yet been grown in long-term culture and HIV-infected immune-deficient individuals fail to produce antibodies, so serology for identifying humans with microsporidia

infections has been limited to non-HIV-infected persons infected with species that could be cultured *in vitro* (van Gool *et al.*, 1997; Kucerova-Pospisilova and Ditrich, 1998; del Aguila *et al.*, 2001; Kucerova-Pospisilova *et al.*, 2001). Serological applications for identifying humans with microsporidiosis are improving, but interpretation of serological results remain complicated because it is not yet possible to discern between current infections, resolved infections, or exposures to microsporidia that do not establish infection but induce cross-reacting antibodies. As such, more definitive diagnostic procedures in humans generally rely upon detection of microsporidia by microscopy and molecular-based methods.

### Microscopy

Transmission electron microscopy (TEM) long has been the standard for specifically identifying microsporidia based on observing the polar filament (Orenstein *et al.*, 1990, 1992). TEM is important for observing and describing the ultrastructural features of developing and mature organisms and often can be used to identify organisms to the genus and species level. TEM requires a high degree of technical expertise and is costly, time-consuming, and insensitive for routine diagnosis in large-scale studies (Franzen and Müller, 1999; Weber *et al.*, 1999, 2000).

Histochemical methods are commonly used in diagnostic laboratories to identify microsporidia in urine, stool, aspirates, and tissue biopsies, and excellent descriptions of these techniques can be found in reviews by Weber *et al.* (1994, 1999, 2000), Weber and Canning (1999), and Garcia (2001, 2002). Optical fluorescent brighteners such as Uvetix 2B, Calcofluor White, and Fungifluor are relatively sensitive and time-efficient for screening most types of specimens to detect microsporidia. These optical brighteners bind to chitin in the spore wall and stained microsporidia appear white-to-turquoise under fluorescence microscopy with an excitation filter in the range of 350-440 nm. Small yeasts also contain chitin and stain with optical fluorescent brighteners resulting in some false positive results. The modified trichrome (concentrated chromotrope 2R) and Gram-chromotrope stains, though perhaps less sensitive than the fluorescent brighteners, appear to be more specific for identifying microsporidia in fluids and stool specimens. Microsporidia appear pink against a blue or green counterstained background and contain a central pink band and posterior vacuole which are useful indicators for discerning these organisms from yeasts which also stain pink. Immunofluorescent antibody staining methods are useful for detecting microsporidia in fluids, stool specimens and tissue

biopsies but are limited by availability of specific antisera. Gram stains (Brown-Brenn and Brown-Hopps) are useful for detecting microsporidia in tissue biopsies where organisms appear dark blue-to-purple against a yellow background, and silver stains (*eg* Warthin-Starry) also have been applied for detecting microsporidia in tissue sections where organisms appear black against a yellow background. Gram and silver stains are not typically used for identifying microsporidia in fecal smears. Giemsa and modified trichrome stains are less commonly used for identifying microsporidia in tissue sections and microsporidia stain poorly with H&E stain.

### Polymerase chain reaction (PCR)-based methods

PCR-based methods are increasingly applied in diagnostic and research laboratories for species-specific identification of microsporidia (Fedorko and Hijazi, 1996; Weiss and Vossbrinck, 1998; Franzen and Müller, 1999b; Weiss, 2000; Garcia, 2002). DNA extraction from microsporidia requires the disruption of the spore wall, either by glass-bead beating, boiling, and digestion with proteinase K, lyticase, and chitinase or can be accomplished by use of commercially-available kits. Primers generally employed for diagnostics target, the rRNA genes of the microsporidia, and the most thorough compilation of these primer sequences can be found in the review by Franzen and Müller (1999b). Species identification can be accomplished by use of species-specific primers. Alternatively, if pan-microsporidian primers are used (*ie*, primers that amplify more than one microsporidian species), species identification can be determined by subjecting the amplicons to nested PCR with a second set of specific primers, restriction enzyme digestion to generate a restriction fragment-length polymorphism (RFLP) pattern, nucleotide sequencing for BLAST analysis, Southern analysis using species-specific probes, or heteroduplex mobility shift analysis (Franzen and Müller, 1999b).

## EPIDEMIOLOGY

Microsporidia species that infect humans also infect a wide range of animals that shed organisms into the environment, increasing the probability for human exposure to microsporidia. Although diagnostic methods are improving, microsporidiosis still is probably widely underdiagnosed in humans. Species of microsporidia that infect humans are small and easily overlooked and reagents for specific diagnostic methods such as species-specific antibodies are not always commercially available. Microsporidia often cause non-specific symptoms and often are not

included on differential diagnoses, and many parasitology diagnostics laboratories do not stain or test for microsporidia unless specifically requested.

### Prevalence and natural history

Microsporidiosis in humans occurs world-wide, but prevalence data vary widely because of concerns about the reliability of detection methods (Bryan and Schwartz, 1999). Prior to the AIDS pandemic, microsporidiosis was only rarely identified in humans (Canning and Lom, 1986; Kotler and Orenstein, 1999), and prevalence data for microsporidiosis in human populations before the era of AIDS relied upon serology for detecting antibodies to *E. cuniculi* which was the only mammalian-derived microsporidian that could be grown in culture to provide antigens needed for the assays. These early seroprevalence results ranged from 0 - 42% with the highest rates found in homosexual men in Sweden and in persons with other parasitic infections (Singh *et al.*, 1982; Canning and Lom, 1986; Hollister *et al.*, 1987; 1991). Additional species of microsporidia now have been identified in humans, and persons with other parasitic infections often undergo polyclonal activation of lymphocytes leading to the expression of cross-reacting antibodies and false-positive results for microsporidia. Serological techniques have been applied recently with additional species of microsporidia for detecting microsporidia-specific antibodies in HIV-seronegative individuals such as blood donors, slaughterhouse workers, dog breeders, forestry workers, and pregnant women. These seroprevalence rates ranged from 1.3 - 8.0 % suggesting that microsporidiosis exists in non-HIV-infected populations (van Gool *et al.*, 1997; del Aguila *et al.*, 2001; Kucerova-Pospisilova *et al.*, 2001).

Serological tests are unreliable in persons with HIV/AIDS due to immune deficiency so prevalence studies in these individuals have been based on microscopy or PCR-based detection of microsporidia in clinical specimens. In these studies, prevalence rates ranged from approximately 5 - 50%, varying by geographic location and diagnostic techniques (Weber *et al.*, 1994; Van Gool *et al.*, 1995; Kyaw *et al.*, 1997; Enriquez *et al.*, 1998; Sobottka *et al.*, 1998; Brasil *et al.*, 2000; Deplazes *et al.*, 2000; Ferreira, 2000). Bryan and Schwartz (1999) estimated an overall prevalence of microsporidiosis at 15% of AIDS patients.

Some debate existed about whether microsporidiosis in HIV-infected individuals was associated with clinical disease because some individuals who were found to shed microsporidia in their feces were not exhibiting gastrointestinal symptoms (Rabeneck *et al.*, 1993, 1995). A retrospective review of HIV-

infected patients with enteric microsporidia infections was conducted through the National Institutes of Health-sponsored AIDS Clinical Trials Groups (ACTG) to better understand the natural history of microsporidiosis (Dascomb *et al*, 1999). The results of this study found that most HIV-infected individuals with microsporidiosis remained symptomatic after six months of initial diagnosis with persistent diarrhea and weight loss greater than 10% of baseline body weight. In other studies, microsporidia were the only enteric pathogens detected in HIV-infected individuals with diarrhea, further supporting an association between microsporidia infection and ensuing disease in immunocompromised individuals (Kotler and Orenstein, 1998, 1999).

Microsporidiosis also has been identified in a wide range of non-HIV-infected individuals but prevalence data based on parasite detection per se are lacking (Bryan *et al*, 1997; Bryan and Schwartz, 1999). Ocular infections due to microsporidia in non-HIV-infected individuals have been reported prior to and since the AIDS pandemic (Ashton and Wirasinha, 1973; Pinnolis *et al*, 1981; Cali *et al*, 1991; Davis *et al*, 1991; Rastrelli *et al*, 1994; Silverstein *et al*, 1997; Theng *et al*, 2001; Mietz *et al*, 2002). Other groups of non-HIV-infected individuals with microsporidiosis included travelers (Sandfort *et al*, 1994; Fournier *et al*, 1998; Raynaud *et al*, 1998; Thielman and Guerrant, 1998; Lopez-Velez *et al*, 1999; Okhuysen, 2001; Müller *et al*, 2001), malnourished children (Hautvast *et al*, 1997; Desportes-Livage *et al*, 1998; Valperga *et al*, 1999; Mungthin *et al*, 2001), organ transplant recipients (Sax *et al*, 1995; Rabodonirina *et al*, 1996; Kelkar *et al*, 1997; Gumbo *et al*, 1999; Guerard *et al*, 1999; Goetz *et al*, 2001; Sing *et al*, 2001; Mohindra *et al*, 2002; Mahmood *et al*, 2003), and the elderly (Lores *et al*, 2002).

#### **Risk factors associated with infection**

Microsporidia have been found throughout the environment and in a wide range of hosts, but relatively little is known about the risk factors most commonly associated with transmission of microsporidia infections to humans. Among the first reports to address this was a serological study in which 33% of homosexual men in Sweden expressed antibodies to *E. cuniculi* suggesting that homosexual practices may contribute to horizontal transmission of microsporidiosis (Bergquist *et al*, 1984) and subsequent epidemiological studies further supported that homosexual preference was a risk factor for intestinal microsporidiosis among HIV-infected men (Birthistle *et al*, 1996; Hutin *et al*, 1998). Additional risk factors associated with microsporidiosis in HIV-infected individuals included immune deficiency, eating

undercooked beef at least once a month, exposure to water by swimming in a pool, use of a hot tub or spa, occupational contact with water, and animal exposure through contact with horses or being stung by a bee, hornet, or wasp (Hutin *et al*, 1998; Bryan and Schwartz, 1999; Schwartz and Bryan, 1999; Dascomb *et al*, 2000; Deplazes *et al*, 2000; Mota *et al*, 2000).

#### **Sources**

Likely reservoirs of microsporidia species that can be transmitted to humans include other infected humans, animals, and water. Microsporidia are shed into the environment with the feces and urine from infected hosts, and the observation that microsporidia infections commonly are found in the respiratory and intestinal tracts of infected individuals supports the likelihood that fecal-oral, oral-oral, inhalation of contaminated aerosols, and ingestion of contaminated food and water are probable modes of transmission (Kotler and Orenstein, 1998; Bryan and Schwartz, 1999; Schwartz and Bryan, 1999; Deplazes *et al*, 2000; Mota *et al*, 2000; Slifko *et al*, 2000; Weber *et al*, 2000). Species of microsporidia that infect humans also infect a wide range of animals with which humans have contact, and among the risk factors associated with microsporidiosis in HIV-infected individuals were association with animals and eating undercooked meat (Hutin *et al*, 1999; Dascomb *et al*, 2000). The only direct evidence for zoonotic transmission of microsporidiosis, however, was reported in a child who seroconverted after being exposed to a litter of puppies infected with *E. cuniculi* (McInnes and Stewart, 1991).

Indirect evidence also supports the possibility that insects serve as a source for microsporidia infections of humans. *Brachiola* (syn. *Nosema*) *algerae* is a natural pathogen of mosquitos and while this species failed to cause systemic infections in rats or athymic mice after iv or oral inoculations, localized infections developed after subcutaneous inoculations in the tail, feet, or ears of these hosts suggesting that the growth of *B. algerae* was limited to environments at temperatures below 37° C (Vavra and Undeen, 1970; Undeen and Alger, 1976; Trammer *et al*, 1997). *B. algerae*, however, sub-sequently was identified and isolated from the cornea of an immune-competent individual and adapted for culture at 37° C (Visvesvara *et al*, 1999; Moura *et al*, 1999; Lowman *et al*, 2000; Visvesvara, 2002). Subsequently, Koudela and colleagues (2001) found that application of *B. algerae* spores onto the eyes of SCID mice failed to cause clinical signs of infection at the site of inoculation but infections were observed 60 days later in the spleen and liver. Of particular interest was the report by Cali *et al* (2003; and this proceeding) who described a case

Table 1  
Microsporidia species of humans and putative sources.

Microsporidia species <sup>a</sup>	Other animal hosts	Environmental sources
<i>Brachiola</i> (syn. <i>Nosema</i> ) <i>algerae</i>	Mosquito	None reported
<i>Brachiola</i> (syn. <i>Nosema</i> ) <i>connori</i>	None reported	None reported
<i>Brachiola vesicularum</i>	None reported	None reported
<i>Encephalitozoon cuniculi</i>	Wide range of mammals including rodents, carnivores, non-human primates	None reported
Strain I	Rabbit	
Strain II	Mouse, blue fox, wild rat	
Strain III	Dog, prosimian	
<i>Encephalitozoon hellem</i>	Birds	None reported
<i>Encephalitozoon intestinalis</i>	Dog, donkey, pig, cow, goat, gorilla	Ground water, surface water sewage effluent, irrigation crop water
<i>Enterocytozoon bieneusi</i>	Pig, cat, dog, goat, chicken, cow, rabbit, muskrat, fox, racoon, beaver, and non-human primates	Surface water, swimming pool, river water
<i>Nosema ocularum</i>	None reported	None reported
<i>Nosema</i> spp	Insects	Ditch water
<i>Pleistophora</i> spp	Fish	Ditch water, river water, crop irrigation water
<i>Pleistophora ronneafiei</i>	None reported	None reported
<i>Trachipleistophora anthropophthera</i>	None reported	None reported
<i>Trachipleistophora hominis</i>	None reported	None reported
<i>Vittaforma corneae</i>	None reported	River water, tertiary effluent

<sup>a</sup> Microsporidia that infect humans but are unclassified include *Microsporidium africanum* and *Microsporidium ceylonensis*.

of myositis due to *B. algerae* in a diabetic woman with rheumatoid arthritis, now demonstrating that this organism of mosquitos can cause deep tissue infection in humans.

Mounting evidence supports the probability that water sources serve as reservoirs of microsporidia infections of humans. Contact with water was one of the risk factors associated with transmission of microsporidiosis in epidemiological studies (Hutin *et al*, 1998; Dascomb *et al*, 2000) and species of microsporidia that infect humans including *E. intestinalis*, *E. bieneusi*, *Nosema* species, *Pleistophora* species, and *V. corneae*, have been identified in ground,

surface, ditch, and crop-irrigation water sources (Avery and Undeen, 1987; Sparfel *et al*, 1997; Dowd *et al*, 1998; Fournier *et al*, 2000, 2002; Thurston-Enriquez *et al*, 2002). *E. bieneusi* spores were identified in the feces of fur-bearing animals that closely associate with surface water (Sulaiman *et al*, 2003), and an increased rate of microsporidiosis was associated with people living near water distribution subsystems in France (Cotte *et al*, 1999). In addition, several characteristics of microsporidia favor the probability for water-borne transmission. Microsporidia species infecting humans appear to lack host specificity and could easily be excreted with urine and feces of infected animals to



Table 2  
Diagnostics paradigm for microsporidiosis<sup>a</sup>.

	Feces / urine and other fluids	Tissue section (biopsy)
Screening	Fluorescent optical brighteners Calcofluor White Fungifluor Uvitex B	Fluorescent optical brighteners Calcofluor White Fungifluor Uvitex B
Corroboration	Modified trichrome stain (Chromotrope 2R)	Gram stain Brown-Brenn Brown-Hopps
Species identification	PCR-based methods (rDNA)	Immunofluorescent antibody stain PCR-based methods (rDNA)

<sup>a</sup> Protocols for (immuno)histochemistry methods can be found in Garcia and Bruckner (2001, 2002) and PCR-based methods can be found in Franzen and Müller (1999b).

contaminate water supplies, microsporidian spores are environmentally resistant and survive for extended periods of time in water, the spores are relatively small and not easily trapped by filtration, and the infectious dose is probably reasonably low (Franzen and Mueller, 1999a). These factors plus the identification of microsporidia in water prompted the United States Environmental Protection Agency to include *E. bienewisi* and *E. intestinalis*, the two most commonly-identified microsporidians that infect humans, on the microbial candidate contaminant list in response to the Safe Drinking Water Act (<http://www.epa.gov/safewater/ccl/ccdfs.html>).

## ENVIRONMENTAL STUDIES

### Methods to assess viability and infectivity

Questions exist about whether the microsporidia identified in water sources are viable and infectious, and thus pose a risk for transmission to humans and animals. Viability staining and infectivity assays are being applied to address these issues. Ethidium bromide and acridine orange have been used to identify dead and live *E. cuniculi* spores, respectively, under fluorescence microscopy and propidium iodide was found to be excluded by live spores (Peterson *et al*, 1988). A dual staining procedure using Calcofluor White and Sytox Green was developed to simultaneously discern between live and dead spores (Green *et al*, 2000). Viable spores excluded Sytox Green, stained with Calcofluor White, and appeared turquoise when viewed by fluorescent microscopy at an excitation wavelength of 395-415 nm while dead

spores stained with both Calcofluor White and Sytox Green, and appeared yellowish-green. Recently, a fluorescent *in situ* hybridization (FISH) assay has been applied in which viable spores with intact rRNA stained brightly while dead spores that lost their rRNA stained weakly (Graczyk *et al*, 2002).

A limiting-dilution focus-forming assay to assess infectivity of *E. cuniculi in vitro* was first described by Pye and Cox (1979). This assay was performed by adding serial dilutions of microsporidian spores to culture wells of confluent host cells and either counting the number of infected host cells or identifying the highest dilution at which host cells were infected approximately one week later. Modifications of this procedure have been applied for assessing immune responses and drug effects on microsporidia survival *in vitro* (Schmidt and Shadduck, 1984; Beauvais *et al*, 1994; Didier *et al*, 1994; Franssen *et al*, 1995). Infectivity of microsporidia also has been assessed in athymic and SCID mice which develop lethal disease after inoculation with as few as 10 *E. cuniculi* spores (Schmidt and Shadduck, 1983), and Koudela *et al* (1999) used this system to measure survival of *E. cuniculi* after treatment in water at various temperatures over time.

### Environmental persistence

The chitinous spore wall of the microsporidia appears to afford these organisms some protection from various environmental conditions. In laboratory experiments, at least some *E. cuniculi* spores remained infectious in tissue culture after incubation in medium for 16 days at 22°C and after 98 days at 4°C, and spores

that were dried and then incubated at 22°C at 0 - 2% humidity for 28 days also remained infectious (Waller, 1979). Freezing and thawing or incubation in distilled water failed to kill all *E. cuniculi* spores, and organisms survived at least 24 hours after incubation at pH 4 and pH 9 (Shaddock and Polley, 1978). Koudela *et al* (1999) reported similar results by demonstrating that sufficient numbers of *E. cuniculi* spores remained capable of causing lethal infections in SCID mice after incubation in distilled water for two years at 4°C or after freezing at -12°C and -24°C for 24 hours. Recently, Fayer and colleagues determined that *E. intestinalis* and *E. hellem*, like *E. cuniculi*, were still able to infect host cells in culture after incubation in water at temperatures ranging from 10° - 30°C for weeks to months (Li *et al*, 2003). Interestingly, *E. intestinalis* was found to be hardier than *E. hellem* and *E. cuniculi*. These findings then, indicated that microsporidia have the potential to persist in fresh water, salt water (*ie* after incubation in tissue culture medium), or after dehydration for extended periods of time at ambient temperatures and thus likely pose a health risk for transmission to susceptible hosts.

#### Capture and identification strategies

The United States Environmental Protection Agency published methods 1622 and 1623 for identifying and determining the concentration of the parasites, *Cryptosporidium parvum* and *Giardia lamblia* from water sources, and these approaches now are being modified and applied for detecting and identifying microsporidia (<http://www.epa.gov/nerlcwww/>). These procedures utilize pre-enrichment filtration followed by immunomagnetic bead separation (IMS) assay and detection by immunofluorescence antibody staining (FA). Variations on this approach for detecting microsporidia in water samples include application of the IMS followed by PCR (Dowd *et al*, 1999), water filtration followed by PCR (Sparfel *et al*, 1997; Sorel *et al*, 2003), and concentration of microsporidia by continuous separation channel centrifugation (Borchardt and Spencer, 1997). To simplify the enrichment phase of these protocols, Orlandi and Lampel (2000) applied an extraction-free, FTA filter-based template for PCR detection of microsporidia in fecal specimens and *Cyclospora cayetanensis* from raspberries under experimental spiking conditions. The authors suggested that this approach could be applied to detecting pathogens in a wide range of clinical, environmental, and food samples. Standardized methods to capture and identify microsporidia in food and environmental sources beyond the use of microscopy, however, are still being developed and evaluated (Orlandi *et al*, 2002).

#### PREVENTION AND TREATMENT

Common sense approaches have been recommended to individuals at risk for developing life-threatening microsporidiosis such as persons with AIDS or organ transplant recipients, who have been urged to drink bottled or boiled water and to wash hands appropriately (Bryan and Schwartz, 1999; Schwartz and Bryan, 1999). Other preventive strategies include thorough cooking of meat, fish, and seafood, as well as washing fruits and vegetables prior to ingestion. Since animals are known to be infected with microsporidian species that infect humans, limited exposure to animals suspected of carrying microsporidiosis may be warranted under some circumstances.

Disinfection strategies have been evaluated and applied to reduce the viability and potential infectivity of microsporidia present in the environment. Microsporidia could be killed by boiling for at least five minutes and by application of disinfectants including quarternary ammonium, 70% ethanol, formaldehyde (0.3% or 1%), phenolic derivatives, 1% hydrogen peroxide, chloramine, sodium hydroxide, or amphoteric surfactants for 30 minutes at 22°C (Shaddock and Polley, 1978; Waller, 1979; Santillana-Hayat *et al*, 2002). Microsporidia survival and infectivity were reduced in water by coagulation, sedimentation, and mixed media filtration (Gerba *et al*, 2003). Ozone treatment, ultraviolet exposure, gamma irradiation, and chlorination at pH 7 were effective at reducing viability and infectivity of *Encephalitozoon* species, as well (Wolk *et al*, 2000; Khalifa *et al*, 2001; Huffman *et al*, 2002; Li *et al*, 2002; John *et al*, 2003; Johnson *et al*, 2003; Marshall *et al*, 2003).

Currently, two drugs are primarily being used clinically to treat microsporidiosis in animals and humans. Albendazole, a benzimidazole with anthelmintic and antifungal activity, inhibits the polymerization of tubulin, and has been found to be effective against *Encephalitozoon* species of microsporidia. Albendazole, however, is only variably effective against *E. bieneusi* (Blanshard *et al*, 1992; Dieterich *et al*, 1994; Kotler and Orenstein, 1999; Conteas *et al*, 2000). Fumagillin, an antibiotic produced by the fungus *Aspergillus fumigatus*, is highly effective when used topically to treat keratoconjunctivitis due to infections with *Encephalitozoon* species (Diesenhouse *et al*, 1993; Rosberger *et al*, 1993; Garvey *et al*, 1995; Conteas *et al*, 2000; Chan *et al*, 2003). When administered systemically to humans at a dose of 20 mg three times per day, fumagillin was highly effective against *E. bieneusi*, but caused

neutropenia and thrombocytopenia in some patients (Molina *et al*, 2002). TNP-470 (also named AGM-1470) was less toxic in laboratory animals and was as effective as fumagillin against several species of microsporidia in tissue culture and in infected athymic mice, but has a shorter half-life than fumagillin (Didier, 1997; Coyle *et al*, 1998). Additional drugs with variable results that have been reported for treating microsporidiosis in humans, include metronidazole, furazolidone, sinefungin, atovaquone, azithromycin, itraconazole, octreotide, and sulfa drugs (Canning and Lom, 1986; Weber *et al*, 1994; Kotler and Orenstein, 1998, 1999; Contreas *et al*, 2000). The highly active antiretroviral therapy (HAART) presently used to treat AIDS patients has resulted in HIV reduction and concomitant improvement in CD4<sup>+</sup> T cell levels with a subsequent reduction in the prevalence of many opportunistic infections including microsporidiosis (Goguel *et al*, 1997; Carr *et al*, 1998; Contreas *et al*, 2000).

#### CONCLUSION

The improvement in diagnostic methods and greater awareness has resulted in microsporidia infections being increasingly recognized in humans. The presence of microsporidia in water sources and in pets and food-producing animals, along with epidemiological risk factors that have associated exposure to water and eating undercooked meat with microsporidiosis in HIV-infected individuals have further raised the concern that microsporidia infections may be food- and water-borne parasitic zoonoses. Additional epidemiological studies focusing on risk factors associated with microsporidiosis will define more clearly the sources of microsporidia in the environment that pose a risk for transmission so that better preventive strategies can be implemented. Continued studies also are needed to identify with better accuracy the presence of viable and infectious microsporidia that may pose a risk for transmission from various environmental sources. Methods to remove or inactivate microsporidia in water sources still need to be developed, and more effective, less toxic drugs are needed for effectively treating microsporidiosis in humans and animals.

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