Microsporidia

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1. Infectious Agent and Morphology
Microsporidia

1. Infectious Agent and Morphology

Microsporidia are obligate intracellular parasites with a unique mode of entering host cells via a polar tube. They are true eukaryotes with a nucleus, chromosome separation and an intracytoplasmic membrane system. However, they lack a classical stacked Golgi apparatus, centrioles, peroxisomes and the mitochondria have been substituted by a mitochondria-like organelle called mitosome (Franzen and Muller 1999; Keeling 2011).

Microsporidia have a unique structure in nature, the polar tube or polar filament that is involved in host cell invasion (Bigliardi and Sacchi 2001). Their infective stage is the spore (Figure 1.1), ranging 1-20 µm long, but species infecting mammals are smaller (1-3µm),

- *Encephalitozoon* sp spores measure 2.0 to 2.5 by 1.0 to 1.5 µm, and
- *Enterocytozoon bieneusi* spores measure 0.7 to 1 by 1-1.6 µm.

**Figure 1.1:** Morphology and diagram of a microsporidian spore. (Parasitology Laboratory, Universidad San Pablo-CEU).
Microsporidia

1. Infectious Agent and Morphology

1.2 Morphology

They are gram-positive and environmentally resistant, with a thick wall composed of three layers:

(i) an electron-dense proteinaceous outer layer, called **exospore**,  
(ii) an electron-lucent inner chitinous layer called **endospore**, and  
(iii) a **plasma membrane** enclosing an infective sporoplasm (Franzen and Muller 1999, Vavra 1999).

The content of the spores is composed of two functionally different parts: the **sporoplasm** and the **extrusion apparatus**. The sporoplasm may be considered as the infectious material of microsporidia. It may contain a single nucleus, as it is the case of *Encephalitozoon* and *Enterocytozoon*, or two nucleus in *Nosema, Brachiola* and *Vittaforma*, which consist of two closely apposed nuclei functioning as a single unit (Bigliardi and Sacchi 2001), with ribosomes and endoplasmic reticulum membranes (Delbac and Polonais 2008).
The extrusion apparatus is composed of a polar tube, coiled around the posterior region of the spore, an anchoring disk, an anterior membrane-bounded organelle, termed polaroplast, and a vacuole at the posterior end (Figure 1.2).

The number and disposition of polar filament coils vary among microsporidia, showing 5 to 7 coils in a single row in *Encephalitozoon* species and *Enterocytozoon* with double row.

Under certain conditions such as alkaline pH, or increased concentrations of Na\(^+\), K\(^+\), Cl\(^-\), Ca\(^{2+}\) ions, the spore germinates and an inflow of water dramatically increases the pressure inside the spore.

Figure 1.2: Life cycle of microsporidia. Infection phase (A), Sporogony phase (B). (Parasitology Laboratory, USP-CEU).
2. Life Cycle
2.1 Life Cycle Overview

This eventually ruptures the wall at the anterior end, where the exospore is less thick, forcing the polar filament to eject turning inside out to form a tube (Delbac and Polonais 2008; Keeling 2009). Germination is a very quick process: the polar tube acts as a projectile if penetrates the host cell membrane, it delivers the sporoplasm inside the host cell cytoplasm (Figure 2.1A).

Once inside the host cell cytoplasm, microsporidian proliferation (merogony) will occur within a parasitophorous vacuole (as in the case of *Encephalitozoon* species), or in direct contact with the host cell cytoplasm (e.g. *Enterocytozoon*). At the end of the proliferative phase (Figure 2.1B), the cytoplasm of the host cell is completely filled with spores and the cell membrane will disrupt releasing the mature spores that can infect new cells to continue the life cycle (Franzen and Muller 1999; Bigliardi and Sacchi 2001; Delbac and Polonais 2008).

Figure 2.1: Life cycle of microsporidia. Infection phase (A), Sporogony phase (B). (Parasitology Laboratory, USP-CEU).
3. Host Parasite Relationship and Immune Response
To date, the knowledge of the real human *Encephalizoon* and *Enterocytozoon* infections is not easy to discern, as they are often misdiagnosed or overlooked. *E. intestinalis* has been described with variable presence in all continents depending on the country. However, in general, in HIV+ patients, varies from 0.9% in Switzerland (Coyle et al. 1996) to 12.8% recently detected in Russian HIV+ patients. In this case, the prevalence was unexpectedly higher than that observed for *E. bieneusi* (Sokolova et al. 2011). However, the data on immunocompetent individuals are still scarce. It has been associated mainly to travellers' diarrhoea although serologic studies have shown high seroprevalence up to 8% (Raynaud et al. 1998; Wichro et al. 2005; Mathis, Weber, and Deplazes 2005).

The zoonotic potential of these species has been recognized, since it has been occasionally described in a great variety of mammals, such as donkeys, dogs, pigs, cows, goats and recently in cats and avian hosts so, the most likely source of this infection may be other infected humans and animals (Didier 2005; Mathis, Weber, and Deplazes 2005; Velasquez et al. 2012).

Figure 3.1: *Encephalitozoon intestinalis* spores from cell culture. Spores detected by IFAT with polyclonal antibodies. The arrows mark the extruded polar tubes. (Parasitology Laboratory, USP-CEU).
3. Host-Parasite Relationship and Immune Response

3.1 Epidemiology

Water-borne transmission must be considered, since the spores are environmentally resistant and can survive in water. For example, in spite of their small size, recent publications have detected the presence of *E. intestinalis* in water (Dowd, Gerba, and Pepper 1998; Izquierdo et al. 2011; Galvan et al. 2013). These spores have been concentrated using the IDEXX Filta-Max®, recommended by the USEPA, to detect *Cryptosporidium* and *Giardia* in water (U.S.E.P.A 2005; Izquierdo et al. 2011).

In the case of *E. intestinalis* infections, an oral-faecal transmission route is indicated, given the multiple sites of infection and that spores can be released via stools. Other modes of infection could be inhalation and contaminated fingers, although waterborne route must be taken in consideration, since spores of *E. intestinalis* have been detected in recreational water (Dowd, Gerba, and Pepper 1998; Didier and Weiss 2006; Izquierdo et al. 2011).

Human infections by *E. cuniculi* are traditionally described as less frequent than *E. intestinalis* infections in studies carried out with HIV+ patients and in patients with severe immunosuppression, in Europe and the USA, revised in Talabani et al. (2010) and recently, in Africa (Ojuromi et al. 2012). However, studies on immunocompetent individuals have shown a prevalence higher than expected indicating that human exposure may be more common than previously suspected.

![Figure 3.2: Natural bath (Madrid, Spain) (Parasitology Laboratory, USP-CEU).](image-url)
In the case of *E. hellem* infections, reports in humans are rare. Around 50 HIV+ patients infected with *E. hellem* have been described in Europe, United States and Africa (Mathis, Weber, and Deplazes 2005). However, recent studies in immunocompetent individuals have shown higher prevalence (Sak, Brady et al. 2011). This species has been described mainly in avian hosts, such as parrots, ostriches, peach-face lovebirds and pigeons, some of them closed to humans and is considered of zoonotic origin (Black et al. 1997; Gray, Puette, and Latimer 1998; Snowden, Logan, and Phalen 2000; Haro et al. 2005). The parrots or pigeons excrete spores in faeces which, although not yet demonstrated, suggest that birds may be a source for the zoonotic origin of this microsporidium.

Finally in the case of *E. bieneusi*, the immunocompromised and immunocompetent populations are affected with this infection, with data on microsporidia presence not only in HIV/AIDS patients but also in HIV-negative patients including travellers, the elderly, organ transplant recipients, Crohn’s disease patients and the immunocompetent population. Similar to data described worldwide, *E. bieneusi* is the species most frequently identified. Animal hosts including dogs, goats and rabbits, pigeons and soil and faecal samples (presumably from cats and dogs) from urban parks have also been described for microsporidia in several regions of Spain, with *E. bieneusi* and *Encephalitozoon hellem* as the species identified. All these data suggest that human pathogenic microsporidia circulate in the environment in Spain and support the idea that the most frequent microsporidia associated with human infection are of zoonotic origin (Galván-Díaz, 2014).
4. Clinical and Pathological Features
4.1 Clinical Features

Clinical features of microsporidiosis due to *Encephalitozoon* sp and *Enterocytozoon* are broad because to the possibility to disseminate and produce systemic diseases. Included among the variety of manifestations are intestinal, ocular, renal and pulmonary disorders, although the immune status of the host is important in the clinical course of the disease (Didier 2005).

Clinically silent chronic infections are usually developed in immunologically competent host, even though clinical signs could be developed after early infections (Snowden et al. 1998; Didier 2005; Sak, Kvac et al. 2011). In immunocompromised hosts, microsporidial infections develop serious diseases that may produce death. The most common clinical symptoms are chronic diarrhoea and malabsorption although, systemic diseases can be developed. Due to their dissemination capacity these parasites have been detected in numerous varieties of locations producing a great variety of manifestations.
4.2 Microsporidial Diarrhoea and Biliary Pathology

*E. intestinalis* and *Enterocytozoon* are the species more frequently implicated in intestinal and biliary pathology. Chronic diarrhoea and extreme slimming are frequent in immunocompromised patients. The diarrhoea is not bloody, there are no fever, anorexia is joined to abdominal pain, sickness and vomiting. Intestinal malabsorption is frequent but unspecific. A case with a small bowel perforation has been described (Soule et al. 1997).

*E. cuniculi* and *E. hellem* have only occasionally been found in the intestine of immunocompromised patients (del Aguila, Moura et al. 2001; Garcia 2007; Chabchoub et al. 2009). However, whether the presence of spores in faecal samples in these patients could be related with an actual infection, or on the contrary, be the result of spores passing through the intestine, inhaled or ingested, is under discussion (Haro et al. 2005; Graczyk et al. 2007). It is important to note that spores are shed primarily in urine rather than faeces (Didier and Weiss 2011).

4.3 Microsporidial Hepatitis and Peritonitis

The cases described are scarce and isolates and have been attributed to *E. intestinalis* and *E. hellem*. To date, only two cases have been reported with hepatic involvement, one case the hepatitis was described at autopsy produced by *E. cuniculi* (Terada et al. 1987) and in the second case, a fulminant hepatic failure was referenced in a HIV+ patient with previous diarrhoea lasting 2 months (Sheth et al. 1997). Peritonitis is an unusual presentation of microsporidia infections.
4.4 Disseminated Infections

The three *Encephalitozoon* species and *Enterocytozoon bieneusi* have been described in immunocompromised patients, and in transplant recipients with multiple–organ failure, although they can also produce disseminated infections in the immunocompetent (Weber et al. 1994; Cali, Kotler, and Orenstein 1993; Galvan et al. 2011; Ditrich et al. 2011; Galván-Díaz et al. 2011). The clinical manifestations are variable. Beyond gastrointestinal manifestations, keratoconjunctivitis, bronquiolitis, sinusitis, nephritis, urethritis, cystitis, prostatitis, hepatitis and peritonitis manifestations are frequent (Figures 4.1a and 4.1b). Other organs can be involved such as brain and genital tract (Weber et al. 1994; Mohindra et al. 2002; Didier 2005; Torres et al. 2012).

*E. intestinalis* has been associated to enteric disease, although it can infect kidneys, gallbladder, eyes, nasal mucosa, and skin. This microsporidium has been detected in saliva, urine and bronchoalveolar lavage fluid. *E. hellem* has been described as cause of pulmonary disease, keratitis/ketratoconjunctivitis, kidney disease and nasal polyps. Finally, *E. cuniculi* can infect intestine, liver, peritoneum, kidneys, brain and eyes (Weber et al. 1994; Kotler and Orenstein 1998; Anane and Attouchi 2010; Ditrich et al. 2011; Robinson et al. 2011).
4.5 Ocular Microsporidiosis

Ocular infections due to microsporidia have been described in both immunocompetent and immunocompromised patients and have recently received considerable attention (Loh et al. 2009). This infection is mainly associated to *E. hellem* where a superficial punctate keratoconjunctivitis was described (Didier et al. 1991). Although, these manifestations have been described in immunocompromised individuals or in contact lens wearers, recent studies indicated that this manifestation can occur in immunocompetent individuals (Chan et al. 2003; Sridhar and Sharma 2003).

4.6 Pathology and Immunology 1

Studies on the pathology of *Encephalitozoon* and *Enterocytozoon* have been carried out in immunocompromised patients, especially in AIDS patients. In the case of *E. intestinalis* infection, although the parasite infects primarily small-intestinal enterocytes, it may affect the perinuclear zone of intestinal lamina propria of macrophages, fibroblasts and endothelial cells (Weber et al. 1994; Conteas et al. 2000). It seems that via infected macrophages, the parasite can disseminate, to liver, pancreas, colon, lungs sinuses, kidneys, and conjunctiva (Orenstein, Dieterich, and Kotler 1992; Weber et al. 1994). In the intestine, a severe ulceration of the small bowel with mucosal atrophy, along with acute and chronic inflammation, has been described. Bile ducts infections are related to papillary stenosis, bile duct dilatation, alithiasic cholecystitis, and sclerosing cholangitis (Weber et al. 1994).

Alterations produced by dissemination of the *E. intestinalis*, *E. hellem* and *E. cuniculi* out of intestine are related with the tissue affected. An inflammatory reaction has been observed in the liver, pancreas, lung, kidneys and eyes. In the case of respiratory tissues infection parasites have been detected in lining epithelium of almost the entire length of the tracheobronchial tree (Schwartz et al. 1992).
The immunity against these parasites depends mainly on cell-mediate immunity. This fact was suspected from the beginning, when clinicians observed that patients with severe AIDS and with low CD4$^+$ T cells showed a more severe manifestation of infection. Recent studies in animal models have shown that protective immunity is mainly dependent on CD8$^+$ T cells. In contrast, the role of CD4$^+$ cells does not seem necessary to control the infection, and the lack of CD4$^+$ T cells does not affect induction CD8$^+$ T cells (Moretto et al. 2000; Ghosh and Weiss 2009).

In the case of humoral immunity it is known that the infection activates antibody production and their presence is related with a latent infection. However, their presence alone does not look to have a protective role, although a study carried out in mice experimentally infected with *E. cuniculi* has shown that antibodies can contribute to resistance (Weber et al. 1994; Sak et al. 2006).
5. Diagnosis
5. Diagnosis

5.1 Diagnosis and Detection

The diagnosis in the case of human infection and the detection in environmental samples, is based on the observation of the characteristics spores. However, due to the small size of spores, multiple and specific diagnostic methods are required.

Originally microsporidia identification was based on electron microscopic examination to determine the genus species, although not always reaching the species level. Nowadays, that method has been substituted by new staining methods for light microscopy (more available in diagnostic laboratories), immunofluorescence procedures and molecular test.

![ELISA](image1)
![IFAT](image2)
![TEM](image3)
![PCR](image4)

**Figure 5.1**: Pictures from Parasitology Laboratory, USP-CEU.
5.2 Electron Microscopy

Electron microscopy was considered the gold standard to confirm the diagnosis of microsporidiosis and specie identification, based in the characteristic of the polar tube (Figures 5.2a and 5.2b). However compared to others, this method is less sensitive and is not available in most laboratories for clinical diagnosis. Nowadays, it is limited to taxonomic studies.

**Figure 5.2a:** Immunogold electron micrographs of *Encephalitozoon intestinalis* spores. (Arrows mark the polar tube.) (Parasitology Laboratory, USP-CEU).

**Figure 5.2b:** Immunogold electron micrographs of *Encephalitozoon intestinalis* spores. (Arrows mark the polar tube.) Parasitology Laboratory, USP-CEU.)
5.3 Staining Methods

Staining methods for microsporidia detection include Modified Trichrome stains (Weber et al. 1992). The spore wall should stain pinkish to red, with the interior of the spore being clear or showing a horizontal or diagonal stripe, which represents the polar tube (Figure 5.3a). This method can be used mainly for stools and body fluids. In the case of tissue sections a Gram-chromotrope staining is recommended (Visvesvara and Garcia 2002) and could be used in stools samples too (Figure 5.3b). It does not allow the species identification.

Figure 5.3a: Microsporidia spores stained in faecal smear. *Enterocytozoon bieneusi* (Modified Trichrome). (Gram-Chromotrope). (Parasitology Laboratory, USP-CEU).

Figure 5.3b: Microsporidia spores stained in faecal smear. *Encephalitozoon intestinalis* (Gram-Chromotrope). (Parasitology Laboratory, USP-CEU).
5.4 Fluorescent Staining Techniques

Fluorescent staining techniques using optical fluorochrome is another staining method used, in addition to Modified Trichrome stain to confirm microsporidia spores. This method is based in the capacity of some chemo-fluorescent agents to stain chitin, the main component of the wall spores (Calcofluor White, or Uvitex 2B) (Garcia 2002) and allows the detection of spores that could be missed by the Trichrome-stain (Figure 5.4).

Figure 5.4: Encephalitozoon intestinalis spores in fecal smear stained by the Calcofluor technique. (Parasitology Laboratory, USP-CEU).
5.5 Immunofluorescent Test

Species determination can be carried out with the use of indirect immunofluorescence test (Figures 5.5A and 5.5B). This method has been improved since the development of monoclonal antibodies against the different species of *Encephalitozoon* sp (Hoffman et al. 2007; Li et al. 2011; Peer et al. 2012). It has been described as having high sensibility and specificity and comparable to PCR methods, the most used routinely in spite of their high cost and complexity (Polley et al. 2011).

Figure 5.5: *Encephalitozoon cuniculi* spores from cell culture. A and B: Spores detected by IFAT with polyclonal antibodies. The arrows mark the extruded polar tubes. (Parasitology Laboratory, USP-CEU)
5.6 PCR Methods

The genetic diagnosis has been increasingly employed because it allows the detection of small numbers of spores or other stages of the parasite that may be undetected under the microscope. Molecular techniques are methods that provide the species identification, although routinely used in clinical diagnosis, to date there are no commercial kits available, and their use is limited to specialized laboratories. During the last year, PCR-based methods have been increasingly used in order to improve sensitivity and specificity (Figure 5.6).

The application of PCR in clinical and environmental samples is subject to limitations such as the viable concentration of spores and the presence of inhibitors of the PCR. The latter is of special interest in the case of stool samples and water proceeding from wastewater sources. Furthermore, the sensibility of this technique is much higher than optical microscopy (Menotti et al. 2003).

Figure 5.6: PCR amplification of rDNA coding region of the ITS of *Encephalitozoon intestinalis*. 
5.7 Cell Culture

All the species of *Encephalitozoon* can be cultured *in vitro* but cell culture is not a routine method in diagnostic laboratories. Nevertheless, in vitro culture can provide information: the culture mass can be used to immunological, biochemical, physiological and molecular characterization studies, and even for the development of immunological or molecular reagents as well as different studies on the efficacy of antimicrobial agents. A great variety of cell lines has been used proceeding from monkey (*Figure 5.7*), rabbit and human (Garcia 2002). Unfortunately, one of the most common human microsporidial pathogens, *E. bieneusi*, has been cultured only in short-term cultures lasted anywhere from 6 weeks to 6 months (Visvesvara, 2002).

*Figure 5.7: Encephalitozoon cuniculi* cell culture (*Vero*-E6). The arrows mark several parasitophorous vacuoles. (Parasitology Laboratory, USP-CEU).
6. Treatment and Prevention
The treatment of microsporidiosis is an unsolved problem, as it is an intracellular parasite, and also, to the resistance of spores. Since the discovery of microsporidia as infectious agents, a number of drugs has been used with variable effectiveness. In the case of intestinal and disseminated (non-ocular) *Encephalitozoon* infections, Albendazole, a Benzimidazole that inhibits tubulin polymerization and has antihelminthic and antifungal activity, is the drug of choice, as demonstrated by the complete elimination of the parasite after 7 days of treatment (Didier 2005; Anane and Attouchi 2010; Robinson et al. 2011). However, Albendazole is less-than optimal therapy in HIV patients with *E. bieneusi* and not result in clearance of the organisms from the stools. Fumagillin, an antibiotic and antiangiogenic is more broadly effective against *Encephalitozoon* spp. and *E. bieneusi*, however, is toxic when administered systemically to mammals (Didier, 2005).

Since the use of antiretroviral therapy (HAAT), available in developed countries, a reduction in the prevalence of opportunistic infections, including microsporidiosis, has been observed without use of a specific treatment, simply by induction of a progressive reconstitution of the immune system. However, we remember that the different situation in developing countries, where the rapid expansion of AIDS, together with limited access to HAART, has contributed to an increased incidence of this disease (Ojuromi et al. 2012).
6.2 Prevention

Since the sources of human infection are uncertain, specific measures of prevention are difficult to establish. Primary infection can occur by inhalation or ingestion of spores from environmental sources or by zoonotic transmission, suggesting the need of adequate hygiene rules, especially in the case of high-risk patients (HIV-positive patients, transplant recipients, elderly and children) (Garcia 2007). The measures included all those developed to prevent any infection of fecal-oral transmission related to hands, water and food ingestion (Didier 2005; Garcia 2007). Moreover, since clinical encephalitozoonosis is related with the immune-status of the host, in the case of HIV+ patients, reconstitution of immune-system by the use of HAART is an important measure of prevention (Anane and Attouchi 2010).

Figure 6.1: Natural bath (Madrid, Spain) (Parasitology Laboratory, USP-CEU).
7. Public Health Risks
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7.1 Overview

Measures related to animal contact should be developed, since one of the sources of human infection is animal-to-human (Didier 2005). Of interest is the description of the presence of *E. hellem* in park pigeons in their relation with humans, mainly children and the elderly people, or other immunosuppressed people. (Haro et al. 2005).

The presence of microsporidia related to waterborne transmission, has been documented. Studies in different types of water, including ditch and raw water, water treatment effluents, surface and ground water, irrigation and river water, recreational lakes, and wastewater, have shown the presence of *Encephalitozoon* sp and *E. bieneusi* (Graczyk et al. 2007; Galvan et al. 2013). Their viability after treatment of drinking water and wastewater has been demonstrated (Graczyk et al. 2004). Wastewater requires special attention, since this type of water can be discharged to a river or can be used for urban, agricultural, industrial, recreative and environmental practices, and may contribute to the contamination of the environment.

These observations suggest that source of infection and modes of transmission require more definitive information in order to take measures to avoid health risks from microsporidia.
References
References

Coy-Did


Did-Gar


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Izq-Mat

Men-Pee

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Referenc  es

Pol-Sak


Sch-Sri

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References