human philophthalmosis was reported from the United States (Gutierrez et al., 1987). The patient was a 66-year-old man, and the parasite obtained from his left eye was identified as *P. gralli*, although the position of the corrus sac in the specimen was not the same as that described for this species. A case was also reported from Israel (Lang et al., 1993). The patient was a 13-year-old girl, and the worm obtained from her right eye was apparently mature, but the species was not identified. Our case is the first in Mexico, the first human case of *P. lacrimalis* infection in the world, and the 24th case of human philophthalmosis overall.

The authors are grateful to Dr. A. Villegas-Alvarez, who extracted the worm; QFB Javier Sedano Millan for his collaboration; and Dr. Scott Monks, Universidad Autonoma del Estando d Hidalgo, Dra. Virginia Leon Regagon and M. en C. Luis Garcia Prieto, Instituto de Biologia, UNAM, and Biol. Maria Antonieta Arizmendi, Facultad de Ciencias, UNAM, for their valuable help and advice.

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Infectivity of Microsporidia Spores Stored in Water at Environmental Temperatures

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**ABSTRACT:** To determine how long waterborne spores of *Encephali-

*tozoon cuniculi*, *E. hellem*, and *E. intestinalis* could survive at environmental temperatures, culture-derived spores were stored in water at 10, 15, 20, 25, and 30 C and tested for infectivity in monolayer cultures of Madin Darby bovine kidney (MDBK) cells. At 10 C, spores of *E. intestinalis* were still infective after 12 mo, whereas those of *E. hellem* and *E. cuniculi* were infective for 9 and 3 mo, respectively. At 15 C, spores of the same species remained infective for 10, 6, and 2 mo, and at 20 C, for 7, 5, and 1 mo, respectively. At 25 C, spores of *E. intesti-

*naulis* and *E. hellem* were infective for 3 mo, but those of *E. cuniculi* were infective for only 3 wk. At 30 C, the former 2 species were infective for 3 wk and 1 mo, respectively, and the latter species for only 1 wk. These findings indicate that spores of different species of *En-

*cephalitozoon* differ in their longevity and temperature tolerance, but at temperatures from 10 to 30 C, all 3 have the potential to remain infective in the environment long enough to become widely dispersed.

Of the 13 species of microsporidia that infect humans, *Encephalito-

*zoon cuniculi*, *E. hellem*, *E. intestinalis*, and *E. bieneusi* have been re-

ported in a variety of domesticated animals (Deplazes et al., 1996; Mansfield et al., 1997; Mathis et al., 1999; Rinder et al., 2000) and wildlife (Hersteinsson et al., 1993; Mathis et al., 1996; Thomas et al., 1999; Doek et al., 2000). *Encephalitozoon hellem* and *E. hellem*—like microsporidia have been found in psittacine birds in the United States and Australia as well as in budgerigar chicks and wild, yellow-

streaked iory (reviewed by Bryan and Schwartz, 1999; Doek et al., 2000). *Encephalitozoon intestinalis* spores were identified in feces of farm animals in Mexico (dog, pig, cow, and goat) (Bornay-Linares et al., 1998). Although the actual routes of transmission are not well known, it is possible that the infective spore stage in urine or feces can contaminate the environment and enter surface water used for recreation or drinking water (Sparfel et al., 1997; Dowd et al., 1998; Cotte et al., 1999, Fournier et al., 2000). Isolation of spores and molecular detection of microsporidia in ditch water and river water along with circumstantial evidence of waterborne transmission is accumulating (reviewed by Bry-

ian and Schwartz, 1999). The longevity of insect-infecting microsporidia stored under different conditions has been studied (Oshima, 1964; Hen-

ry and Oma, 1974; Fuxa and Brooks, 1979; Teeter-Barsch and Kramer, 1979), but data on the effect of environmental temperatures on longevity of spores of species that infect animals and humans are sparse. Such information can be extremely helpful in understanding the epidemiology and evaluating the risk of transmission. The present study was designed to investigate the effect of temperature and storage in water on infect-

*ivity of spores of *E. cuniculi*, *E. hellem*, and *E. intestinalis* by testing in cultured mammalian cells.

Spores of *E. cuniculi* and *E. hellem* were purchased from the Amer-

ican Type Culture Collection, Manassas, Virginia (ATCC Nos. 50502 and 50545, respectively). Spores of *E. intestinalis* originally isolated from an acquired immune deficiency syndrome (AIDS) patient and grown in culture (Didier et al., 1996) were provided by Elizabeth Didier, Tulane Regional Primate Research Center, Covington, Louisiana. *En-

*cephalitozoon cuniculi* and *E. intestinalis* were propagated in monolayer cultures of MDBK cells, and *E. hellem* was propagated in human lung fibroblasts (WI-38) in T-75 flasks. The MDBK cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 1% nonessential amino acids (NEAA), 2% N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES), 5% fetal calf serum (FCS), and 1% penicillin or streptomycin in a 5% CO2 atmosphere incubator at 35 C. WI-38 cells were similarly cultured in minimum essential medium
was removed from each water bath, and an equivalent quantity of 5
ly, except during weekends. At weekly intervals for 4 wk, and then at
20, 25, and 30 C, respectively. Temperatures were monitored twice dai-
were in the spore stage. Spore concentrations were adjusted with de-
ppeared to be uniform, suggesting that virtually all the forms examined
3 lighter-staining bodies per 100 organisms, staining intensity also ap-
to be of fairly uniform size and shape, and with the exception of 2 or
Portsmith, New Hampshire), and counted with the aid of an epi¯uoresc-
(MEM) supplemented with 1% NEAA, 2%HEPES, 10% FCS, 1% pen-
cillin or streptomycin, 1% l-glutamine, and 1% sodium pyruvate. Mi-
crosporidia were harvested from culture supernatant by centrifuging at
1,500 g for 15 min and were resuspended in deionized water. An aliquot
of organisms was stained with calco¯uor white (Becton Dickinson Mi-
crobiology Systems, Sparks, Maryland), pipetted into a well of heavy,
teflon-coated 3-well glass microscope slide (Cel-Line, Erie Scientific,
Portsmouth, New Hampshire), and counted with the aid of an epifluores-
cence microscope. Within each species examined, the bodies appeared
be of fairly uniform size and shape, and with the exception of 2 or
3 lighter-staining bodies per 100 organisms, staining intensity also ap-
ppeared to be uniform, suggesting that virtually all the forms examined
were in the spore stage. Spore concentrations were adjusted with de-
ionized water, and spores were pipetted into microcentrifuge tubes that
were capped and held in each of 5 circulating water baths at 10, 15,
20, 25, and 30 C, respectively. Temperatures were monitored twice dai-
ly, except during weekends. At weekly intervals for 4 wk, and then at
monthly intervals (28–30 days), 1 tube of each species of microsporidia
was removed from each water bath, and an equivalent quantity of 5 ×
10^5 spores was aspirated from that tube, and 2.5 × 10^4 spores were
inoculated into each of the 2 wells of an 8-well Lab-Tek chamber slide
(Nalge Nunc International, Naperville, Illinois), with each well contain-
ing a monolayer of MDBK cells. After 6 days incubation at 35 C in a
5% CO₂ atmosphere incubator, culture medium was removed, and cul-
tures in all 8 wells were fixed in situ with 100% methanol for 30 min
at room temperature. The plastic frame and silicon gasket that formed
the wells were removed, and each slide was air dried and stained by
the quick-hot Gram-chromotome method and examined by brightfield microscopy. Enceph-
alitozoon cuniculi, E. hellem, and E. intestinalis had been stored for 2,
8, and 10 mo, respectively, at the time that they were examined for
filament extrusion.
Spore infectivity for all 3 species decreased with increasing temper-
aturer or increased time of storage (Table I). Spores of E. intestinalis
stored at 10 C were still infective after storage for 12 mo, but at higher
temperatures infectivity was lost more quickly until at 30 C, spores were
not infective after 1 mo of storage. Similar patterns were observed for
E hellem and E. cuniculi. However, E. cuniculi lost infectivity more
rapidly than did E. hellem, which lost infectivity more rapidly than did
E. intestinalis (Table I). Intracellular clusters of developing microspor-
idia were found in all positive control wells, but none was found in negative
control wells.

<table>
<thead>
<tr>
<th>Time</th>
<th>10 C</th>
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<th>20 C</th>
<th>25 C</th>
<th>30 C</th>
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<tr>
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<td>12 mo</td>
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</table>

* ND, not done.

Results based on observation of 1 culture inoculated with spores.
at 37.5°C lost viability after 2–4 days, whereas those held at room temperature or under refrigeration survived for 6 days to 3 mo, respectively (White, 1919).

Compared with N. apis, only 1 report provides data on the effect of temperature and long-term storage on infectivity of E. hellem or E. intestinalis (Kucerova-Pospisilova et al., 1999). Data on E. cuniculi is sparse. After 2 yr of storage at 4°C, E. cuniculi spores were still infective for SCID mice (Koudela et al., 1999). Encephalitozoon cuniculi spores in Medium 199 survived for 98 days at 4°C, 6 days at 22°C, and 2 days at 37°C, as determined by infectivity for canine kidney cells in vitro (Waller, 1979). Another study found that E. cuniculi spores stored in cul- ture medium remained infective for at least 24 days at 4 and 20°C, but lost infectivity after 12 days at 37°C (Shadduck and Polley, 1978). In the present study E. cuniculi spores in deionized water appeared less vigorous than those reported by Waller (1979) and Koudela et al. (1999), with a few remaining infective for only 3 mo at 10°C. However, direct comparisons are not possible because the spores used in the pre- sent study were not held at 4 or 5°C.

Infection with microsporidia begins with germination, when the polar filament is triggered, everts from the spore, and forms a tube through which the spore content (sporoplasm) is expelled (Vavra and Larsson, 1999). It is not clear if all spores that undergo germination have the potential to be infective. Artificially induced germination is technique dependent and may either fail to initiate extrusion of polar filament from spores that are potentially infective or stimulate extrusion of filaments from spores that lack infectivity. One study found that after artificial stimulation the germination rate of the fish parasite Loma salmonae decreased from 51 to 0% after 100 days of storage in seawater at 4°C (Shaw et al., 2000). Microscopic appearances differentiating in- tact spores from recently germinated spores, and from spores that had lost extruded polar filaments, have been reported (Kucerova-Pospisilova et al., 1999). Germination was induced in spores of E. intestinalis, E. hellem, and E. cuniculi stored in culture medium at 4°C for 48 mo (Kucerova-Pospisilova et al., 1999). Spores that retained infectivity for cultured mammalian cells were found in only 1 of 6 pools of spores for each species in 24-mo-old samples (Kucerova-Pospisilova et al., 1999).

In the present study spores were examined to determine if those stored at 30°C had extruded polar filaments, which would render them non- infective when inoculated into cell cultures. Although aqueous suspensions of spores of E. cuniculi, E. hellem, and E. intestinalis were examined by DIC microscopy, and chromotrope-stained spores were examined by brightfield microscopy, no extruded filament was detected, suggesting that factors other than extrusion of the filament were involved in the loss of infectivity. Different methods applied in the present study might account for differences in observations, i.e., the lack of visible extruded filaments.

The present study demonstrated that time and temperature of storage affect infectivity of microsporidian spores and that longevity varies among species. As demonstrated with the protozoan parasite Cryptosporidium parvum, the period of time which organisms remain infective decreased as environmental temperatures increased (Fayer et al., 1998). As temperatures were increased or decreased beyond those normally found in nature, C. parvum lost infectivity rapidly, so that organisms were rendered noninfective within 5 sec at 71.7°C and within 24 hr at ~10°C (Fayer, 1994; Fayer and Nerad, 1996; Harp et al., 1996). Those extreme values have yet to be examined for infectivity of E. cuniculi, E. hellem, and E. intestinalis.

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STAT-6 is an Absolute Requirement for Murine Rejection of *Hymenolepis diminuta*

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**ABSTRACT:** Infection with helminth parasites typically evokes a Th helper 2-cell response in the mammalian host. Interleukin (IL)-4 and IL-13 are important in the rapid expulsion of parasitic nematodes, with the absence of either cytokine being compensated for by the other. However, the IL-4 and IL-13 common signaling molecule, signal transducer and activator of transcription 6 (STAT-6) appears to be mandatory for the spontaneous expulsion of enteric helminths. Mice genetically deficient in IL-4, IL-13, or STAT-6 were infected with the cestode *Hymenolepis diminuta* and worm infectivity and jejunal goblet cell responses assessed 12–18 days postinfection (PI). Only the STAT-6 knockout (KO) animals harbored adult worms; neither gravid adult nor stunted *H. diminuta* was obtained from the infected IL-4 KO or IL-13 KO mice ≥12 days PI. Also, the establishment of worms in the intestine of STAT-6 KO animals was associated with a reduced goblet cell response. These findings support the hypothesis that increased mucin production is an important part of the host response to tapeworm infection and that functional STAT-6 signaling may be an absolute requirement for the rejection of intestinal cestodes and thus, helminth parasites in general.

Many parasitic helminth–rodent infections have been used to define the immunological and (physiological) events that lead to the rejection of the worm from a nonpermissive host. A variety of effector mechanisms are now known to contribute to helminth expulsion, including increased mucus production, mast activation, eosinophil attack, antibody-dependent cytotoxicity, and altered peristalsis and water secretion (Andreassen et al., 1999); the relative importance of these responses reflects the complexity of the host–parasite relationship. However, it is clear that the rapid spontaneous rejection of helminth parasites, for example *Hymenolepis diminuta* from mice, is a T cell–dependent phenomenon (McKay et al., 1990). Moreover, the majority of parasitic helminth infections in mammalian hosts is accompanied by a T helper 2-cell (Th2)–dominated response (Bancroft et al., 2000) and appropriately so because Th2 cells orchestrate immune events aimed at combating extracellular parasites, i.e., drive humoral immunity and activate or promote mast cell and eosinophil activities.

Interleukin (IL)-4 and IL-13 are Th2-type cytokines that share a common peptide chain in their receptors, i.e., that designated the IL-4 receptor α chain, and mobilize common, e.g., signal transducer and activator of transcription (STAT-6), and unique intracellular signaling molecules in their target cells. These cytokines display closely related and overlapping biological activity, as well as nonredundant functions (McKenzie, 2000). Moreover, IL-4 and IL-13 have been implicated as key players in the expulsion of parasitic nematodes (Urban et al., 1998, 2000), but considerably less data have been presented with respect to their importance in the immunologically mediated loss of flatworm parasites.

Using gene knockout (KO) animals, this study examined the ability of mice lacking IL-4, IL-13, or STAT-6 to expel *H. diminuta*. Male mice deficient in IL-4 (BALB/c background), IL-13 (BALB/c background), or STAT-6 (C57/B6 background) and the appropriate parental strains were infected by oral gavage with 5 *H. diminuta* cysticercoids in 100 μl of sterile saline (n = 4 to 8) (McKay et al., 1991). BALB/c and C57/B6 expel *H. diminuta* with similar kinetics. Noninfected, age-matched BALB/c and C57/B6 mice were also included in this study. Mice were killed 12–18 days postinfection (PI), a time at which immunocompetent mice have expelled their worms (McKay et al., 1990; Palmas et al., 1997). The entire small intestine was removed, flushed with cold saline, and a portion of midjejunum fixed in formalin, processed to wax, and sections stained with periodic acid–Schiff (PAS) reagent for the enumeration of goblet cells. Goblet cell hyperplasia is characteristic of the murine gut response to this parasite (McKay et al., 1990). The intestinal contents were examined for the presence of *H. diminuta*.

On opening the abdomen of STAT-6 KO *H. diminuta*–infected mice...