Infectivity of Microsporidia Spores Stored in Seawater at Environmental Temperatures

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ABSTRACT: To determine how long spores of Encephalitozoon cuniculi, E. hellem, and E. intestinalis remain viable in seawater at environmental temperatures, culture-derived spores were stored in 10, 20, and 30 ppt seawater at 10 and 20 C. At intervals of 1, 2, 4, 8, and 12 wk, spores were tested for infectivity in monolayer cultures of Madin Darby bovine kidney cells. Spores of E. hellem appeared the most robust, some remaining infectious in 30 ppt seawater at 10 C for 12 wk and in 30 ppt seawater at 20 C for 2 wk. Those of E. intestinalis were slightly less robust, remaining infectious in 30 ppt seawater at 10 and 20 C for 1 and 2 wk, respectively. Spores of E. cuniculi remained infectious in 10 ppt seawater at 10 and 20 C for 2 wk but not at higher salinities. These findings indicate that the spores of the 3 species of Encephalitozoon vary in their ability to remain viable when exposed to a conservative range of salinities and temperatures found in nature but, based strictly on salinity and temperature, can potentially remain infectious long enough to become widely dispersed in estuarine and coastal waters.

Fourteen species of microsporidia have been reported to infect humans (Kotler and Orenstein, 1999; Cali and Takvorian, 2003). Of these, Encephalitozoon cuniculi, E. hellem, E. intestinalis, and Enterocytozoon bieneusi are zoonotic, infecting domesticated animals (Deplazes et al., 1996; Mansfield et al., 1997; Breitenmoser et al., 1999; Mathis et al., 1999; Rinder et al., 2000; Buckholt et al., 2002; Fayer, Santin, and Trout, 2003) and wildlife (Hersteinsson et al., 1993; Mathis et al., 1996; Thomas et al., 1997; Sulaiman et al., 2003). Encephalitozoon cuniculi has been identified in wild and pet rabbits, wild rats and mice, dogs, cats, foxes, mink, and a variety of monkeys (Bryan and Schwartz, 1999; Deplazes et al., 2000). Encephalitozoon hellem and E. hellem-like microsporidia have been found in psittacine birds, budgerigar chicks, a wild yellow-streaked lory (Bryan and Schwartz, 1999; Deplazes et al., 2000), and have experimentally infected domesticated chickens (Fayer, Santin, Palmer et al., 2003). Encephalitozoon intestinalis spores have been reported from feces of farm animals in Mexico (dog, pig, cow, goat) (Bornay-Linares et al., 1998). Although the actual routes of transmission are not known, it is possible that the infectious spore stage in urine or feces can contaminate surface waters used for recreation or drinking water (Sparfel et al., 1997; Dowd et al., 1998; Cotte et al., 1999; Fournier et al., 2000). Microscopic and molecular detection of spores in surface waters and circumstantial evidence of waterborne transmission has been reviewed by Bryan and Schwartz (1999). Under experimental conditions, spores of E. cuniculi, E. hellem, and E. intestinalis stored in water at environmental temperatures ranging from 10 to 30 C remained infectious long enough to become widely dispersed if exposed to similar conditions in the environment (Li et al., 2003). For example, at 10 C, spores of E. intestinalis were still infectious after 12 mo, whereas those of E. hellem and E. cuniculi were infectious for 9 and 3 mo, respectively. At 30 C, the former 2 species were infectious for 3 wk and 1 mo, respectively, and the latter species for 1 wk. Little is known of how long microsporidians remain infectious in seawater. On the basis of artificially induced filament extrusion from spores of the microsporidian fish parasite Loma salmonae, a decrease was found after storage in seawater, suggesting that spores lost viability (Shaw et al., 2000). Using a similar experimental design as that of Li et al. (2003), the present study was conducted to determine the effect of salinity and temperature on longevity of spores of zoonotic species of Encephalitozoon that might be found in estuaries and coastal marine
Table I. Viability of *Encephalitozoon hellem* spores held in 0, 10, 20, and 30 ppt of seawater at 10 and 20 C for 1, 2, 4, 8, and 12 wk, as determined by observation of clusters of proliferating intracellular parasites in Madin Darby bovine kidney cells. (0, 0 clusters; 1, 1–10 clusters; 2, 11–100 clusters; 3, 101 + clusters.)

<table>
<thead>
<tr>
<th>TP*</th>
<th>Positive control</th>
<th>0 ppt, 10 C</th>
<th>10 ppt, 10 C</th>
<th>20 ppt, 10 C</th>
<th>30 ppt, 10 C</th>
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<th>10 ppt, 20 C</th>
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<tr>
<td>8</td>
<td></td>
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</tbody>
</table>

* TP: time period (number of weeks spores were held before in vitro viability testing).
† Number of clusters of proliferating parasites within each of 3 wells of MDBK cells.

Table II. Viability of *Encephalitozoon intestinalis* spores held in 0, 10, 20, and 30 ppt of seawater at 10 and 20 C for 1, 2, 4, 8, and 12 weeks, as determined by observation of clusters of proliferating intracellular parasites in Madin Darby bovine kidney cells. (0, 0 clusters; 1, 1–10 clusters; 2, 11–100 clusters; 3, 101 + clusters.)

<table>
<thead>
<tr>
<th>TP*</th>
<th>Positive control</th>
<th>0 ppt, 10 C</th>
<th>10 ppt, 10 C</th>
<th>20 ppt, 10 C</th>
<th>30 ppt, 10 C</th>
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</tbody>
</table>

* TP: time period (number of weeks spores were held before in vitro viability testing).
† Number of clusters of proliferating parasites within each of 3 wells of MDBK cells.

waters used for recreation and shellfish harvesting. Knowledge of the effect of salinity and temperature on infectivity of microsporidia in seawater is necessary for evaluating the risk of waterborne contamination.

Many isolates of *E. cuniculi*, *E. hellem*, and *E. intestinalis* have been propagated in vitro in many types of cells (Visvesvara, 2002). In the present study, spores were obtained and propagated as described previously (Li et al., 2003). Briefly, *E. cuniculi* and *E. intestinalis* were propagated in monolayer cultures of Madin Darby bovine kidney (MDBK) cells, and *E. hellem* was propagated in human lung fibroblasts (WI-38). MDBK cells were cultured in Dulbecco modified Eagle medium supplemented with 1% nonessential amino acids, 2% N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, 5% fetal calf serum (FCS), and 1% penicillin–streptomycin in a 5% CO<sub>2</sub> atmosphere at 35 C. WI-38 cells were similarly cultured, but in minimum essential medium with 10% FCS, as well as 1% l-glutamine, and 1% sodium pyruvate. Spores harvested from culture supernatant by centrifuging at 1,500 g for 15 min were resuspended in deionized water, stained with calcifluor white (Becton Dickinson Microbiology Systems, Sparks, Maryland), pipetted into a well of a Teflon-coated 3-well glass microscope slide (Cel-Line, Erie Scientific, Portsmouth, New Hampshire), and counted with the aid of an epifluorescence microscope. For each species, morphologic features and staining intensity appeared uniform, indicating that the forms examined were spores. Spores of each species were pipetted at 1.5 × 10<sup>4</sup> spores per tube into 40 microcentrifuge tubes, centrifuged, and the pellets resuspended in artificial seawater at concentrations of 10, 20, and 30 ppt salinity or in deionized water (Tables I–III). Seawater was constituted from Forty Fathoms Crystal Sea Marine Mix (Marine Enterprises International, Inc., Baltimore, Maryland) dissolved in deionized water. Spores suspended in deionized water served as controls. Tubes were capped and held in either of 2 circulating water baths at 10 and 20 C that were monitored for temperature twice daily, except on weekends. At 1, 2, 4, 8, and 12 wk, 1 tube for each species of microsporidia at each concentration of salinity was removed from each water bath, spores were aspirated from that tube, and 5 × 10<sup>4</sup> spores were inoculated into each of the 3 wells of an 8-well Lab-Tek chamber slide (Nalge Nunc Intl., Naperville, Illinois), each well containing a monolayer of MDBK cells. After 4 days incubation at 35 C in a 5% CO<sub>2</sub> atmosphere, the culture medium was decanted, wells were flooded with 100% methanol for 30 min, and slides were air dried. After removing the plastic frame and silicon gasket that formed the wells, each slide was stained by the quick-hot gram-chromotrope method (Moura et al., 1997), a coverslip was affixed, and the entire area of cells within each well was examined by brightfield microscopy. Spores were considered viable and infectious on finding intracellular clusters of pro-
lifering microsporidia. The number of clusters in each well was counted, and the numbers 0, 1, 2, and 3 were assigned to represent counts of 0, 1–10, 11–100, and 101 or more clusters per well, respectively (Tables I–III). At the onset (time 0) and termination (12 wk) of the study, 5 × 10³ spores of each species held as positive controls in deionized water at 5 C were also added to each of 3 wells containing MDBK cell monolayers, then processed and examined in the same manner as wells that received spores stored in artificial seawater.

For spores of all 3 species held in deionized water at 10 and 20 C compared with those held in deionized water at 5 C for 12 wk, infectivity decreased both with elevated temperatures and length of storage time (Tables I–III). For all time periods for all species at both 10 and 20 C (with 2 exceptions), spores held in seawater were less infectious than those held in deionized water (Tables I–III), indicating a negative effect on infectivity from elevated salinity alone. The degree to which spores were affected was species dependent. Some spores of E. hellem remained infectious at 30 ppt at 10 and 20 C for 12 and 2 wk, respectively, fewer spores of E. intestinalis remained infectious at 30 ppt at 10 and 20 C for 1 and 2 wk, and spores of E. cuniculi remained infectious at only 10 ppt at 10 and 20 C for 2 wk.

Spores of microsporidia have been detected in a variety of surface waters (Avery and Undeen, 1987; Dowd et al., 1998), and water as a source of human infections has been implied from epidemiological data (Cotte et al., 1999), but information is lacking on the presence of and survival in seawater of microsporidia infectious to humans and other mammals. General interest in survival of microsporidian spores dates back nearly 90 yr, with most efforts to determine the effects of time versus temperature on the viability of spores of Nosema apis, a microsporidian parasite of bees, held in water at various temperatures (White, 1919; Revell, 1969; Kramer, 1970; Bailey, 1972; Vavra and Maddox, 1976; Malone et al., 2001) or the mammalian microsporidia E. hellem and E. intestinalis (Kucerova-Pospisilova et al., 1999; Li et al., 2003) and E. cuniculi (Shadduck and Polley, 1978; Waller, 1979; Koudela et al., 1999; Kucerova-Pospisilova et al., 1999; Li et al., 2003) held in water or culture medium. The only study to determine the effect of seawater on spores of microsporidia was that of Shaw et al. (2000), who examined the microsporidian fish parasite L. salmonae.

Infection with all microsporidia begins when the polar filament extruded from the spore forms a tube through which the sporoplasm passes into a host cell (Vavra and Larsson, 1999). This process of germination can be artificially induced. However, it is technique dependent, and polar filaments can fail to extrude from spores that are potentially infectious or can extrude from spores that lack infectivity. Shaw et al. (2000) examined the germination rate of L. salmonae and found that it decreased from 51 to 0% after 100 days storage at 4 C, suggesting that spores lost viability, although after 95 days, infectivity for fish appeared not to be diminished. Germination was induced in spores of E. intestinalis, E. hellem, and E. cuniculi stored in culture medium at 4 C for 48 mo, and the microscopic appearance of intact versus recently germinated spores versus those that had lost extruded polar filaments was reported (Kucerova-Pospisilova et al., 1999). When spores of E. cuniculi, E. hellem, and E. intestinalis stored in deionized water at elevated temperatures for 2, 8, and 10 mo were examined by DIC microscopy and chromatoprobe-stained spores were examined by brightfield microscopy, no extruded filament was detected despite the fact that other spores stored under the same conditions were infectious to cultured mammalian cells (Li et al., 2003). These findings suggested that factors other than extrusion of the filament were involved in the loss of infectivity (Li et al., 2003). On the basis of those findings, spores in the present study were not examined for polar filament extrusion but were considered infectious based solely on their ability to actually invade and multiply within cultured mammalian cells.

The present study demonstrated that the temperature of storage in deionized water increased from 5 to 20 C, infectivity of microsporidian spores decreased and as salinity increased from 0 to 30 ppt, infectivity of microsporidian spores decreased. At the highest level of salinity (30 ppt) at both 10 and 20 C, spores of E. hellem were more robust, i.e., remained infectious longer or more were infectious for longer periods, than those of E. intestinalis, which were more robust than those of E. cuniculi, indicating species-based responses to the effects of salinity. These findings suggest that spores of E. hellem and E. intestinalis could potentially remain infectious in estuarine and ocean waters for weeks and those of E. cuniculi could remain infectious in low-salinity estuarine waters for weeks, which is sufficient to infect humans and marine mammals or to contaminate shellfish.

The technical assistance of Robert Palmer is gratefully acknowledged.

LITERATURE CITED


ABSTRACT: Ixodid ticks were collected and identified from 8 wild carnivore species in Phu Khieo Wildlife Sanctuary, northeastern Thailand. Six tick species belonging to 4 genera were recovered and identified from 132 individuals. These included Amblyomma testudinarium (n = 36), Haemaphysalis asiatica (n = 58), H. hystricis (n = 31), H. sememis (n = 3), Rhizophus haemaphysaloides (n = 3), and Ixodes granulatus (n = 1). Leopard cats (Prionailurus bengalensis) (n = 19) were infested with 4 tick species, whereas yellow-throated marten (Martes flavigula) (n = 4), clouded leopard (Neofelis nebulosa) (n = 2), and dhole (Cuon alpinus) (n = 1) were infested with 3 tick species. Asiatic golden cat (Catopuma temminckii) (n = 2) with 2 species, and marbled cat (Pardofelis marmorata) (n = 1) with 1 species. This information contributes to the knowledge available on the ectoparasites of wild carnivores in Southeast Asia.

The collection and identification of ectoparasites from wild carnivores in North America and Europe is well documented in the literature, including host species such as raccoon (Procyon lotor) (Sonenshine and Stout, 1971; Rhodes and Norment, 1979; Whitaker and Goff, 1979; Brillhart et al., 1994; Pung et al., 1994), striped skunk (Mephitis mephitis) (Durden and Richardson, 2003), coyote (Canis latrans) (Eads, 1948; Pence et al., 1981), red fox (Vulpes vulpes) (Aubert, 1975; Toutoungi et al., 1991), and river otter (Lutra canadensis) (Eley, 1977). Tick research in Southeast Asia has mainly covered tick identification, distribution, and disease transmission (Toomanoff, 1944; Petney and Keirans, 1994, 1995, 1996; Voltzit and Keirans, 2002), a checklist of Thai ticks (Tanskul et al., 1983), and a tick survey of Malaysian carnivores and other mammals (Hooogstraal and Wassef, 1984). Except for research on the endoparasites of Thai wild cats (Patton and Rabinowitz, 1994), parasitological research on wild carnivores in Thailand remains largely unstudied. Our objective was to add information about the tick-host relationships of carnivores from Southeast Asia.

Ticks were collected as part of an ecological study of carnivores in northeastern Thailand (Grassman, 2004). Situated in Chaiyaphum Province (16°S–16°35′N, 101°20′–101°55′E), Phu Khieo Wildlife Sanctuary (PKWS) is a large, 1,560-km² evergreen forest dominated by an approximately 1,000-m elevation plateau (Anonymous, 2000). Carnivores were livetrapped from October 1998 to October 2002. Captured carnivores were anesthetized for physical examination and to attach a radio