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Effect of low and high temperatures on infectivity of *Encephalitozoon cuniculi* spores suspended in water

Břetislav Koudela[1,2], **Štěpánka Kučerová**[1,2] and **Tomáš Hudcovic**[3]

[1]Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic;

[2]Department of Parasitology, Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic;

[3]Laboratory of Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, 549 22 Nový Hrádek, Czech Republic

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Abstract. The survival of *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 spores suspended in distilled water and exposed at defined temperatures was investigated. Infectivity of *E. cuniculi* spores was tested by inoculation of SCID mice. There was no marked loss of infectivity of spores stored at 4°C for two years or frozen at -12°C and -24°C for 1, 8, and 24 h. Although there was a remarkable loss of infectivity, spores remained infective after freezing at -70°C for 1 and 8 h. Heating at 60°C and 70°C for 5 min and 1 min, respectively, rendered the microsporidia non-infective. These findings demonstrate that *E. cuniculi* spores suspended in water can survive freezing temperatures but lost infectivity in water that reached a temperature of 60°C at 5 min.

Microsporidia are obligate intracellular parasites which commonly infect arthropods and may infect members of all classes of vertebrates. Recently, microsporidia have been recognised as a cause of a variety of opportunistic infections in immunodeficient patients (Weber et al. 1994, Didier et al. 1998). The most common microsporidian associated with AIDS, *Enterocytozoon bieneusi,* primarily infects enterocytes of the small intestine and causes chronic diarrhoea. The genus *Encephalitozoon* presently contains three distinct species: *E. cuniculi, E. intestinalis* and *E. hellem. E. cuniculi* infects a wide range of mammalian hosts, including rodents, rabbits, dogs, blue foxes, non-human primates, and man. Infections with *E. cuniculi* have been detected in persons with AIDS who had hepatitis, pneumonia, peritonitis, keratoconjuctivitis, cystitis, sinusitis, bronchiolitis, or encephalitis (Weber et al. 1994, 1997, Mertens et al. 1997, Didier et al. 1998). Other microsporidial species, *Vittaforma corneae,* formerly named *Nosema corneum* (Silveira and Canning 1995), *Nosema ocularum, N. connori, Pleistophora* sp., *Brachiola vesicularum, Trachipleistophora hominis* and *T. anthropophtera* have been demonstrated less frequently in AIDS patients (Weber et al. 1994, Hollister et al. 1996, Didier et al. 1998, Cali et al. 1998, Vávra et al. 1998).

The assumed modes for transmitting infections with *Encephalitozoon* spp. are ingestion and inhalation (Didier et al. 1998). Recent surveys of the occurrence of human microsporidia *E. bieneusi*, *V. corneae* and *E. intestinalis* indicate that microsporidia are frequently present in an aquatic environment and may be water-borne pathogens (Dowd et al. 1998). Therefore, information about viability and infectivity of microsporidia in water is of considerable importance for controlling the risk of infection.

We previously described the usefulness of the severe combined immunodeficient (SCID) mice for studies of *E. cuniculi* microsporidiosis (Koudela et al. 1993). This study was undertaken to determine survival of *E. cuniculi* spores suspended in water at defined temperatures. The infectivity of the treated microsporidia was assayed in SCID mice.

MATERIALS AND METHODS

Parasite. A murine isolate of *Encephalitozoon cuniculi* Levaditi, Nicolau et Schoen, 1923 was isolated by the authors from a dexamethazone-treated laboratory mouse (Koudela et al. 1993). Parasites were grown in E6 cells (Vero green monkey kidney cells) in modified RPMI 1640 medium supplemented with 5% fetal calf serum. The organisms used for inoculation were freshly collected from the culture supernatant, counted with a hemocytometer, and adjusted to the appropriate concentration.

For assays performed in this study, microsporidia spores were first passaged through SCID mice inoculated with 10^7 *E. cuniculi* spores. On 17-21 days post infection (DPI), the SCID mice were euthanised, necropsied and liver was homogenised in sterile distilled water. Microsporidia were then washed three times by centrifugation for 15 min at 400 × g with sterile

Address for correspondence: B. Koudela, Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic. Phone: ++420 38 777 5408; Fax: ++420 38 47743; E-mail: koudela@paru.cas.cz

distilled water. To remove host cell debris, the pellets were resuspended with sterile distilled water and mixed with a equal volume of 100% Percoll (Pharmacia, Sweden), and centrifuged at 400 × g for 30 min (Jouvenaz 1981). The pellets containing the purified microsporidia were washed, counted with a hemocytometer, and adjusted to the appropriate concentrations.

Low temperature treatment. Microcentrifuge tubes containing 5×10^6 freshly purified spores of *E. cuniculi* in 500 µl of sterile distilled water were placed in glycerol bath stored in freezers at -12°C and -24°C, for 1, 8, and 24 h, and at -70°C for 1 and 8 h (two tubes for each interval). Freezing tests were performed in the following freezers: DENLEY Instruments UK, model FR 130 and Electrolux UK, model F 210.

High temperature treatment. Microcentrifuge tubes containing 5×10^6 freshly purified spores of *E. cuniculi* suspended in 500 µl of sterile distilled water were inserted into wells in the heated metal block of a thermal DNA cycler (Techne UK, model FGENECO). These wells had a few drops of mineral oil placed in them to facilitate the transfer of heat from the block to the vial. At each temperature setting four tubes were used. Six temperatures were programmed into thermal cycler: 50, 60, 70, 80, 90, and 95°C. After 15 seconds was allowed for the initial heat transfer, for each temperature setting, two tubes were removed after 1 min. The two remaining tubes were removed after 5 min. Immediately after removal from heating block, the tubes were held in crushed ice until SCID mice were inoculated.

SCID mice. Severe combined immunodeficient (SCID) mice were housed in flexible isolators (BEM, Znojmo, Czech Republic) with high-efficiency particulate air (HEPA) filters. All cages, food, water and bedding were sterilised before use. A total 46 eight- to ten-week-old mice were used in the present study.

Bioassay in SCID mice. Two SCID mice were given spores treated at each temperature-time interval by intra-peritoneal injection (i. p.). Four additional SCID mice were inoculated i. p. with 5×10^6 freshly purified spores of *E. cuniculi* suspended in distilled water and held at room temperature (21°C). Two other SCID mice were inoculated i. p. with 5×10^6 *E. cuniculi* spores stored in distilled water with 0.1% gentamicin for 24 months at 4°C. The effect of temperature treatment on infectivity of *E. cuniculi* spores was evaluated by daily observation for clinical signs or death due to microsporidiosis. The *E. cuniculi* infection in the moribund and dead SCID mice were verified by detection of spores in peritoneal exudate cell (PEC) smears by staining with the optical brightening agent Calcofluor White 2MR (Sigma) (Weber et al. 1994).The remaining SCID mice were euthanised and necropsied at DPI 49.

RESULTS

The results of experimental infections of SCID mice with *E. cuniculi* spores exposed at defined temperatures are shown in Tables 1 and 2. Of SCID mice inoculated with spores frozen at -12°C and -24°C for 1, 8, and 24 h, all mice were infected and died of acute micro-sporidiosis within three weeks post infection. SCID mice inoculated with spores frozen at -70°C for 1 and 8 h survived without clinical signs five weeks post infection, and first developed clinical signs of microsporidiosis at 38 DPI. These mice died 42 DPI and 43 DPI, respectively. The majority of PEC cells from SCID mice with clinical microsporidiosis, contained *E. cuniculi* spores. Many of PEC were destroyed and free spores were seen.

All SCID mice inoculated i. p. with *E. cuniculi* spores heated at 50°C for 1min and 5min, and at 60°C for 1 min, were infected and died of acute micro-sporidiosis within three weeks post infection. In contrast, SCID mice inoculated with *E. cuniculi* spores exposed to 60°C for 5 min were completely resistant and survived through 42 DPI without any clinical symptoms. As expected, SCID mice that received spores heated to 70°C or higher remained alive up to end of experiment. Moreover, no microsporidia were found in the PEC smears of these mice when examined by fluorescent microscopy.

SCID mice infected with spores stored at 4°C became ill after 15-17 DPI and also died within three weeks post infection. The inoculation of fresh purified *E. cuniculi* spores into four SCID mice resulted in development of acute microsporidiosis with clinical signs of wasting and lethargy at 14 DPI and death within three weeks post infection.

DISCUSSION

It is well know that *E. cuniculi* spores are resistant to environmental influences. The methods which have been used for *E. cuniculi* viability and/or infectivity determination include only *in vitro* techniques. Shadduck and Polley (1978) studied some factors, including high and low temperature, and disinfectants, influencing the infectivity and replication of *E. cuniculi* seeded on rabbit choroid plexus cells. Waller (1979) exposed spores of *E. cuniculi* to various temperatures or disinfectants and then tested their infectivity on canine kidney cells. Peterson et al. (1988) have used the flow cytometric technique for quantitation of viable versus dead *E. cuniculi* spores. However, survival of *E. cuniculi* spores at different temperatures has not been critically investigated in an animal model.

The fact that all SCID mice inoculated with frozen *E. cuniculi* spores developed lethal microsporidiosis suggested that spores can survive freezing temperatures up to -70°C for 8 h without presence of cryoprotectants. However, SCID mice that were given spores exposed to -70°C for 1 and 8 h survived longer than SCID mice that received spores frozen at -12°C and -24°C for 1, 8 and 24 h or unfrozen fresh purified spores. These findings suggest that freezing at -70°C tested for 1 and 8 h rendered a portion of the spores noninfective. Previous studies on the effect of low temperature on the viability of *E. cuniculi* spores have been performed with

Table 1. Number of SCID mice surviving infection by *Encephalitozoon cuniculi* spores after low temperature exposure.

Temperature/ exposure time (°C /hours)	-12/1	-12/8	-12/24	-24/1	-24/8	-24/24	-70/1	-70/8
Number of SCID mice inoculated/survived	2/0	2/0	2/0	2/0	2/0	2/0	2/0	2/0

Table 2. Number of SCID mice surviving infection by *Encephalitozoon cuniculi* spores after high temperature exposure.

Temperature/ exposure time (°C /min)	50/1	50/5	60/1	60/5	80/1	80/5	85/1	85/5	90/1	90/5	95/1	95/5
Number of SCID mice inoculated/survived	2/0	2/0	2/0	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2

E. cuniculi spores suspended in 10% DMSO or 10% glycerol as cryoprotectants (Shadduck and Poley 1978), or in medium 199 (Waller 1979), which makes it difficult to compare those data with our results.

In the present study, in which pure aqueous suspensions of *E. cuniculi* spores free of extraneous debris were used, infectivity remained unchanged when spores were exposed to 60°C for 1 min. Infectivity was eliminated when spores were held in water that reached 60° C for 5 min. Moreover, when spores were exposed to temperatures of 70°C or higher with heat-up times of 1 min, infectivity could not be detected in any of inoculated SCID mice. These results are in general agreement with previous published data. Shadduck and Polley (1978) reported that *E. cuniculi* suspended in Hank's balanced salt solution remained infective when exposed to 56°C for 10 min. The infectivity declined continuously over the next 120 min, although a few spores remained infective after 120 min. Similarly, Waller (1979) reported that only 2.5% of *E. cuniculi* spores suspended in medium 199 remained infective when temperature reached 56°C for 30 min.

This investigation also confirmed previous data concerning long-time survival of *E. cuniculi* spores at 4°C in water without any preservatives (Canning and Lom 1986). Although spores were stored in distilled water with 0.1% gentamicin for 24 months at 4°C, there was no loss of infectivity due to the effects of gentamicin. Shadduck and Poley (1978) also reported that *E. cuniculi* spores were not affected by gentamicin in their studies.

The robust nature of microsporidian spores has long been recognised. They are well known to be resistant to many environmental factors that would prove lethal to other infectious agents (Canning and Lom 1986). The fact that tertiary sewage effluent, surface water and groundwater may be contaminated by microsporidian spores (Dowd et al. 1998) stresses the importance of knowledge on the infectivity of microsporidian spores in water under various factors including temperatures.

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REFERENCES

CALI A., TAKVORIAN P.M., LEWIN S., RENDEL M., SIAN C.S., WITTNER M., TANOWITZ H.B., KEOHANE E., WEISS L.M. 1998: *Brachiola vesicularum*, n. g., n. sp., a new microsporidium associated with AIDS and myositis. J. Euk. Microbiol. 45: 240-251.

CANNING E.U., LOM J. 1986: The Microsporidia of the Vertebrates. Academic Press, Inc., New York City, NY., 289 pp.

DIDIER E.S., SNOWDEN K.F., SHADDUCK J.A. 1998: Biology of microsporidian species infecting mammals. Adv. Parasitol. 40: 283-320.

DOWD S.E., GERBA C.P, PEPPER I.L. 1998: Confirmation of the human pathogenic microsporidia *Enterocytozoon bieneusi, Encephalitozoon intestinalis*, and *Vittaforma corneae* in water. Appl. Environ. Microbiol. 64: 3332-3335.

HOLLISTER W.S., CANNING E.U., WEIDNER E., FIELD A.S., KENCH J., MARRIOT D.J. 1996: Development and ultrastructure of *Trachipleistophora hominis* n. g., n. s., after *in vitro* isolation from an AIDS patient and inoculation into athymic mice. Parasitology 112: 143-154.

JOUVENAZ D.P. 1981: Percoll: an effective medium for cleaning microsporidian spores. J. Invertebr. Pathol. 37: 319.

KOUDELA B., VÍTOVEC J., KUČEROVÁ Z., DITRICH O., TRÁVNÍČEK J. 1993: The severe combined immunodeficient mouse as a model for *Encephalitozoon cuniculi* microsporidiosis. Folia Parasitol. 40: 279-286.

MERTENS R.B., DIDIER E.S., FISHSTEIN M.C., BERTUCCI D.C., ROGERS L.B., ORENSTEIN J.M. 1997: *Encephalitozoon cuniculi* microsporidiosis: infection of the brain, heart, kidneys, trachea, adrenal glands,

and urinary bladder in a patient with AIDS. Mod. Pathol. 10: 68-77.

PETERSON N., LIU J.A., SHADDUCK J. 1988: *Encephalitozoon cuniculi:* quantitation of parasites and evaluation of viability. J. Protozool. 35: 430-434.

SHADDUCK J.A., POLLEY M.B. 1978: Some factors influencing the *in vitro* infectivity and replication of *Encephalitozoon cuniculi*. J. Protozool. 25: 491-496.

SILVEIRA H., CANNING E.U. 1995: *Vittaforma corneae* n. comb. for the human microsporidium *Nosema corneum* Shadduck, Meccoli, Davis & Font, 1990, based on its ultrastructure in the liver of experimentally infected athymic mice. J. Euk. Microbiol. 42: 158-162.

VÁVRA J., YACHNIS A.T., SHADDUCK J.A., ORENSTEIN J.A. 1998: Microsporidia of the genus *Trachipleistophora* – causative agents of human microsporidiosis: description of *Trachipleistophora anthropopthera* n. sp. (Protozoa: Microsporidia). J. Euk. Microbiol. 45: 273-283.

WALLER T. 1979: Sensitivity of *Encephalitozoon cuniculi* to various temperatures, disinfectants and drugs. Lab. Anim. 13: 227-230.

WEBER R., BRYAN R.T., SCHWARTZ D.A., OWEN R.L. 1994: Human microsporidial infections. Clin. Microbiol. Rev. 7: 426-461.

WEBER R., DEPLAZES P., FLEPP M., MATHIS A., BAUMAN R., SAUER B., KUSTER H., LÜTHY R. 1997. Cerebral microsporidiosis due to *Encephalitozoon cuniculi* in a patient with human immunodeficiency infection. N. Engl. J. Med. 336: 474-478.

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