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***Encephalitozoon*: Tissue Culture, Cryopreservation and Murine Infection**

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Abstract

Microsporidia are eukaryotic unicellular parasites that have been studied for more than 150 years. They are found throughout the world and are capable of infecting various invertebrate and vertebrate hosts. They can cause disease in both immune compromised and immune competent humans. In immune compromised individuals, infections can be severe leading to the death of the host. Microsporidia possess a unique, highly specialized invasion mechanism that involves the polar tube and spore wall. During germination of spores, the polar tube will rapidly discharge from the spore and deliver the sporoplasm into the host cell. Spores are the only stage of microsporidia that can survive outside of host cells. Since the first attempt to culture microsporidia *in vitro* in 1930s, the cultivation of microsporidia has served a critical role in studying and diagnosis of these parasites. In this chapter, we include methods on the cultivation, isolation and cryopreservation of *Encephalitozoon cuniculi*, which can infect humans, and provides a useful model for other microsporidia. These methods can also be utilized for the culture of *E. hellem* or *E. intestinalis*.

Keywords

Microsporidia; Encephalitozoon; Animal Model; RK13 cells; PCR; cell passage and maintenance; cryopreservation

INTRODUCTION

Microsporidia are parasites of both invertebrate and vertebrate animals (Louis M Weiss & Becnel, 2014). Once considered as protozoa, recent genomic characterization has supported their reclassification as being related to Fungi (Corradi, 2015), with genome-scale phylogenies placing microsporidia together with Cryptomycota at the basal branch of the fungal kingdom (or alternatively as a sister phylum) (Jones et al., 2011). Microsporidian infections in immunocompetent hosts are often chronic and asymptomatic, but they can cause lethal disease in immunocompromised individuals. Since the discovery of microsporidia by the identification of *Nosema bombycis* in the European silkworm in the 19th century, more than 1500 species and over 200 genera of microsporidia that have been

identified (Vávra & Lukeš, 2013);(Louis M Weiss & Becnel, 2014). Shadduck reported the propagation of *Encephalitozoon cuniculi* in rabbit choroid plexus cells in 1969 and was able to continuously maintain the culture by sub-passage for four months (Shadduck, 1969). Since then, *E. cuniculi* has been used *in vitro* by many research groups for studies on microsporidia, utilizing a wide variety of cell lines. Several species of microsporidia infecting humans and other mammals have now been cultivated *in vitro* including *E. hellem*, *E. intestinalis*, *Vittaforma corneae*, *Trachipleistophora hominis*, *T. anthropopthera*, and *A. algerae* (reviewed in (Louis M Weiss & Becnel, 2014). These cultures have been established from a variety of clinical specimens, including conjunctival scrapings, corneal biopsies, urine, sputum, bronchoalveolar lavage, feces, duodenal aspirates, cerebrospinal fluid, muscle biopsies, and brain tissue. The most common cell lines used for culturing these pathogens are derived from epithelium or fibroblast tissues from rabbits, mice, monkeys, or humans. Researchers have not yet been successful in their attempts to establish continuous *in vitro* cultures of *Enterocytozoon bieneusi*, which is the most common microsporidium seen in patients with diarrhea and AIDS (Louis M Weiss & Becnel, 2014). Cultures of the majority of mammalian infective microsporidia are available from ATCC (<https://www.atcc.org/Products/Cells%20and%20Microorganisms/Fungi%20and%20Yeast/Microsporidia.aspx>)

Microsporidia possess a unique cell structure and infection apparatus that includes the spore wall, sporoplasm, and polar tube (Weidner, 1976). Instead of typical mitochondria, they possess mitosomes, which are thought to be mitochondrial remnants (Katinka et al., 2001; Williams, Hirt, Lucocq, & Embley, 2002). The spore wall is made up of various spore wall proteins and chitin. It contains three layers, an electron dense outer layer, the exospore layer and an electron-lucent inner endospore layer. The spore wall plays a crucial role in both microsporidiosis and in the extracellular survival of spores (Bhat, Bashir, & Kamili, 2009; Southern, Jolly, Lester, & Hayman, 2007). The polar tube is a highly specialized infection organelle of microsporidia. In the spore, the polar tube is connected at the anterior end and is coiled around the sporoplasm (Thelohan, 1892, 1894). Upon appropriate environmental stimulation, the polar tube rapidly discharges out of the spore and serves as a conduit for sporoplasm passage into the new host cell (Frixione et al., 1992; Weidner, 1972).

Microsporidia are common parasites of vertebrate animals (Louis M Weiss & Becnel, 2014). At least 17 different microsporidian species have been shown to infect humans including *Encephalitozoon cuniculi*. Microsporidia are listed as priority pathogens by the National Institutes of Health and as waterborne contaminants of concern by the Environmental Protection Agency (E. Didier & Weiss, 2006). Immune-deficient hosts, including AIDS patients, athymic mice, or severe combined immune deficiency (SCID) mice, can develop a lethal infection due to these pathogens (Khan & Didier, 2004).

Microsporidiosis induces a cascade of immunological events that involve both components of innate and adaptive immunity in vertebrate hosts (Sak & Ditrich, 2005). Cell-mediated immunity is currently believed to be the main defense mechanism that protects the host against microsporidiosis (Khan, Moretto, & Weiss, 2001), suggesting that immune T cells are critical in the control of *E. cuniculi* infection in the normal host. CD8⁺ T cells are activated as early as day 3 post-infection by *E. cuniculi*, and the major killing mechanism exhibited by CD8⁺ T cells during microsporidia infection is via the perforin pathway

(Denkers et al., 1997; I. Khan, J. Schwartzman, L. Kasper, & M. Moretto, 1999). No significant increase in CD4⁺ T cells was observed during the infection of *E. cuniculi* (I. Khan et al., 1999). Many microsporidian infections occur through the gastrointestinal tract and important barriers preventing microsporidiosis are the intraepithelial lymphocytes and humoral antibodies produced in the intestine (M. Moretto, L. Weiss, & I. Khan, 2004). The immune response involves not only microsporidia specific antibodies, but also mucosal antimicrobial molecules such as defensins that may play a role in preventing microsporidia infection by reducing spore germination and enterocyte infection (Texier, Vidau, Viguès, El Alaoui, & Delbac, 2010). Macrophages are a critical link between the innate and adaptive immune response and are therefore critical for mounting a protective immune response against microsporidia (Mathews, Hotard, & Hale-Donze, 2009; Texier et al., 2010). These local macrophages can quickly recognize microsporidia through a Toll-like receptor (TLR2), resulting in a slew of host defense mediators including Th1 cytokines such as IFN- γ and IL-12, which have been implicated in protective immunity for many intracellular viral, bacterial and parasitic infections, including microsporidia (Mathews et al., 2009).

Safety Concerns:

Encephalitozoonidae (*E. cuniculi*, *E. hellem*, and *E. intestinalis*) are human pathogens and are considered to be Biohazard Level II agents. To this end, these organisms should be handled using standard BSL2 practices as described in the latest edition of Biosafety in Microbial and Biomedical Laboratories; which can be obtained from the Centers for Disease Control (<https://www.cdc.gov/biosafety/publications/bmbl5/>). This includes: the wearing of a laboratory coat, use of gloves, use of a biosafety cabinet (level II) for cell culture, minimize the use of sharps, and disposal of waste as a biohazard.

NOTE: All solutions and equipment that come into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: Tissue culture should be performed in a humidified incubator, set at 37°C for mammalian cells, and with 5% CO₂ unless otherwise indicated. For microsporidia from insects or fish, lower temperature incubators are utilized.

Basic Protocol 1: IN VITRO CULTURE OF HUMAN FORESKIN FIBROBLASTS (HFF) AND RABBIT KIDNEY (RK13) AS HOST CELLS FOR ENCEPHALITOZOON INFECTION

Encephalitozoon cuniculi was the first microsporidium of mammalian origin that was successfully cultured *in vitro* (J. A. Morris, J. M. McCown, & R. E. Blount, 1956). *E. cuniculi* has been demonstrated to be able to be maintained in a number of cell lines. Among these, human foreskin fibroblast (HFF) cells and rabbit kidney (RK13) cells are widely used for culturing all of the Encephalitozoonidae (e.g., *E. cuniculi*, *E. hellem*, and *E. intestinalis*). Either of these cell lines can be used to culture, harvest spores and perform experiments such as an invasion assay (Han et al., 2017). Cultivation of microsporidia in these cell lines has allowed the study of their life cycles, metabolism, pathogenesis and the development of diagnostic tests (Molestina, Becnel, & Weiss, 2014).

Materials

For HFF cell culture—Human foreskin fibroblast cells, HFF (ATCC #CRL-2522)

Dulbecco's Modified Eagle Medium (DMEM; Gibco, #11965-092)

For RK13 cell culture—Rabbit Kidney cells, RK13 (ATCC #CCL-37)

Minimum Essential Medium Eagle (MEM; Corning, #10-010-CV)

For either type of cell culture—Fetal bovine serum heat inactivated (FBS) (Gibco or HyClone)

NOTE: It is critical to use heat-inactivated FBS (e.g. FBS incubated for 45 min at 56° C), as complement in the serum can interfere with parasite growth in vitro.

Penicillin-Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin; Gibco #154140–122)

NOTE: 6ml aliquots should be stored in 10ml screw top tubes at –20°C and then thawed at 37°C prior to use.

Dulbecco's phosphate-buffered saline without calcium and magnesium (1× DPBS; Corning #21–031-Cv),

0.05% Trypsin-EDTA (Gibco #25300–054)

NOTE: 5ml aliquots should be stored in 10ml screw top tubes at –20°C and then thawed at 37°C prior to use.

25-cm² T25 flasks (Corning, Falcon, or similar plastic flasks)

NOTE: 175- cm² T175 flasks can be used if larger cell cultures are needed (in this case 20ml of medium rather than 5ml is used in each flask).

Cell culture CO₂ incubator maintained at 37°C; 5% CO₂

Water bath set at 37°C

1.5-ml sterile microcentrifuge tubes

5ml sterile pipets

70% ethanol (diluted in distilled water)

Laminar flow cell culture hood (for microsporidia this should be a Biohazard Level II hood)

NOTE: If a hood blower system is turned off, after turning on the blower a hood should not be used until 30 minutes have passed. The internal work area of the hood should be sprayed with 70% ethanol prior to starting cell culture, to limit contamination.

Microcentrifuge maintained at 4°C

Thawing HFF and RK13 cells:

1. Work in a hood while wearing a laboratory coat, disposable latex or nitrile exam gloves, and if using infected cells, protective eye goggles.
2. Remove a frozen cryovial of cells from liquid nitrogen storage tank and place in a 37°C water bath and shake gently by hand for 1 minute until completely thawed.

NOTE: Liquid nitrogen within the cryovial can expand during thawing and can lead to pressure build up in the tube. Appropriate safety measures, such as a covered bucket or box should be used during transport and warming.

3. Place the thawed cryovial in the laminar hood after spraying the tube with 70% ethanol in order to avoid contamination from organisms in the water bath.
4. Material in the cryovial can be added to pre-warmed media (as described in the section on cell culture).
5. The empty cryovial and all pipets should be discarded as biohazard waste.

Establishing an HFF or RK13 cell culture:

- 6a. HFF cells are maintained in Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin-streptomycin supplemented with 10% heat inactivated FBS.
- 6b. RK13 cells are maintained in Minimum Essential Medium Eagle (MEM) with 1% penicillin-streptomycin supplemented with 10% heat inactivated FBS.

NOTE. Media should be stored at 4°C and warmed prior to use.

NOTE: 1% Amphotericin B (250ug/ml; Gibco catalogue #15290018) can also be added to the medium to decrease fungal growth as it does not affect microsporidia growth.

7. Warm medium by placing a bottle in a 37°C water bath for 10 min, and after removal dry the bottle and then spray with 70% ethanol in order to avoid contamination from organisms in the water bath.
8. Add 5 ml of medium into T25 flask and then add the cells from a thawed cryovial.
9. Incubate the flask in 37°C, 5% CO₂ incubator.
10. The next day, replace the medium in the flask with fresh medium to remove DMSO which is present in freezing medium.

NOTE: DMSO is toxic to cell when the concentration in medium is higher than 1%. Above that concentration DMSO can significantly decreased cell survival (Chen & Thibeault, 2013; Timm, Saaby, Moesby, & Hansen, 2013).

11. Change medium twice a week by aspirating the medium and replacing with fresh medium using a 5 ml sterile pipet.

12. All pipets should be discarded as biohazard waste.
13. Examine the growth of cells using an inverted microscope every day until the cells achieve a confluent monolayer.

Changing Medium

NOTE: Medium replacement should be done twice a week.

14. Warm medium (supplemented DMEM for HFF; supplemented MEM for RK13) by placing bottles in a 37°C water bath for 10 min, and after removal, dry the bottles and then spray them with 70% ethanol in order to avoid contamination from organisms in the water bath.
15. Take the T-25 flask from the incubator and place them in the laminar flow hood after spraying them with 70% ethanol.
16. Aspirate the medium from the flasks and replace with 5 mL of fresh medium.
17. Examine the growth of cells using an inverted microscope every day until the cells achieve a confluent monolayer.

Sub-culturing an established HFF or RK13 cell culture:

NOTE: Infected or uninfected cell cultures can be expanded using this sub-culturing technique.

18. Warm medium (supplemented DMEM for HFF; supplemented MEM for RK13) and DPBS by placing bottles in a 37°C water bath for 10 min, and after removal, dry the bottles and then spray them with 70% ethanol in order to avoid contamination from organisms in the water bath.
19. Thaw a tube of 0.05% Trypsin-EDTA (1×) in a 37°C water bath for 10 min, after removal, dry the tube and then spray it with 70% ethanol in order to avoid contamination from organisms in the water bath.
20. Place supplemented medium (DMEM for HFF; MEM for RK13), 0.05% Trypsin-EDTA, and DPBS into the laminar flow.
21. Take the cell flask out of the 5% CO₂ incubator, spray with 70% ethanol, and place in the laminar flow hood.
22. Aspirate the medium from the flask and then add 3ml of DPBS using a 5ml sterile pipet. Incubate the flask for 5 minutes.

NOTE: The cell medium contains calcium and magnesium which inhibits trypsin activity. FBS also contains proteins that are trypsin inhibitors.

Before using trypsin to detach adherent cells, it is highly recommended to use DPBS without Ca²⁺/Mg²⁺, as described, to remove these inhibitors from the cell cultures.

23. Aspirate the DPBS from T25 flask and replace with 1ml of 0.05% Trypsin-EDTA. Make sure that this solution covers the entire bottom of flask. Incubate

the flask until all the cells are detached from the bottom of the flask (at 37°C this occurs in about 1 minute).

24. Pipette 10 ml of fresh medium into T25 flask, mix well and dispense 5ml of suspended cells into each of two new T25 flasks.

NOTE: 175- cm² T175 flasks can be used if larger volume cell cultures are needed. To establish T175 cultures, 5ml of the suspended cells and then 15ml of fresh medium are added to a flask.

25. Swirl the flasks gently to make sure the entire bottom of each flask is covered and return these flasks to the incubator (37°C, 5% CO₂).
26. Examine the growth of cells using an inverted microscope every day until the cells achieve a confluent monolayer.

Basic Protocol 2: GROWTH OF *ENCEPHALITOZOON SPP.* IN VITRO

The methods described below for the cell culture of *E. cuniculi* can also be utilized for the culture *E. hellem* or *E. intestinalis*.

Materials

For HFF cell culture—Human foreskin fibroblast cells, HFF (ATCC #CRL-2522)

Dulbecco's Modified Eagle Medium (DMEM; Gibco, #11965–092)

For RK13 cell culture—Rabbit Kidney cells, RK13 (ATCC #CCL-37)

Minimum Essential Medium Eagle (MEM; Corning, #10–010-CV)

Encephalitozoon cuniculi (or alternatively *E. hellem* or *E. intestinalis* if these microsporidia are to be cultivated). Frozen spores of these organisms are available from ATCC (<https://www.atcc.org/Products/Cells%20and%20Microorganisms/Fungi%20and%20Yeast/Microsporidia.aspx>).

For either type of cell culture

Fetal bovine serum heat inactivated (FBS) (Gibco or HyClone)

NOTE: It is critical to use heat-inactivated FBS (e.g. FBS incubated for 45 min at 56° C), as complement in the serum can interfere with parasite growth in vitro.

Penicillin-Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin; Gibco #154140–122)

NOTE: 6ml aliquots should be stored in 10ml screw top tubes at –20°C and then thawed at 37°C prior to use.

Dulbecco's phosphate-buffered saline without calcium and magnesium (1× DPBS; Corning, #21–031-Cv),

0.05% Trypsin-EDTA (Gibco #25300–054)

NOTE: 5ml aliquots should be stored in 10ml screw top tubes at -20°C and then thawed at 37°C prior to use.

25-cm² T25 flasks (Corning, Falcon, or similar plastic flasks)

NOTE: 175-cm² T175 flasks can be used if larger cell cultures are needed (in this case 20ml of medium rather than 5ml is used in each flask).

Cell culture CO₂ incubator maintained at 37°C ; 5% CO₂

Water bath set at 37°C

1.5-ml sterile microcentrifuge tubes

5ml, 10ml, 25ml sterile pipets

70% ethanol (diluted in distilled water)

Laminar flow cell culture hood (for microsporidia this should be a Biohazard Level II hood)

NOTE: If a hood blower system is turned off, after turning on the blower a hood should not be used until 30 minutes have passed. The internal work area of the hood should be sprayed with 70% ethanol prior to starting cell culture, to limit contamination.

Microcentrifuge maintained at 4°C

Cell scraper (Corning, #CLS3010)

Diluted bleach solution (1 part bleach:4 parts dH₂O)

Additional material for purification of spores

5- μm pore-size durapore (PVDF) filters (Millipore sigma, #SLSV025LS)

250ml conical screw top tube

Thawing Encephalitozoon spores or infected cells

1. Work in a hood while wearing a laboratory coat, disposable latex exam gloves, and if using infected cells protective eye goggles.
2. Remove a frozen cryovial of spores or cells from a liquid nitrogen storage tank and place in a 37°C water bath and shake for 1 minute until completely thawed.

NOTE: Liquid nitrogen within the cryovial can expand during thawing and can lead to pressure build up in the tube. Appropriate safety measures, such as a covered bucket or box should be used during transport and warming.

3. Place the thawed cryovial in the laminar hood after spraying the tube with 70% ethanol in order to avoid contamination from organisms in the water bath.

Establishing an infected host cell culture:

4. Warm supplemented medium (DMEM for HFF or MEM for RK13 containing FBS and Penicillin/Streptomycin) by placing bottles in a 37°C water bath for 10

min, and after removal, dry the bottles and then spray them with 70% ethanol in order to avoid contamination from organisms in the water bath.

5. Take the confluent host cell (HFF or RK13) flask from the 37°C CO₂ incubator, spray the flask with 70% ethanol in order to avoid contamination, and place it into a laminar flow hood.
6. Place the thawed cryovial (of either spores or infected host cells) in the laminar hood after spraying the tube with 70% ethanol in order to avoid contamination from organisms in the water bath.
- 7a. Thawed spores can be added to a tissue culture flask containing a monolayer of a suitable cell line capable of sustaining microsporidian growth (HFF or RK13 cells).
- 7b. Thawed infected host cells can be added directly to a tissue culture flask for culture. Additional uninfected host cells can be added to the culture at the same time, but this is usually not required as an infected host cell culture will contain both infected and uninfected cells.
8. Incubate the flask at 37°C in a 5% CO₂ incubator.
9. The next day, replace the medium in the flask with fresh medium to remove DMSO which can inhibit cell growth.
10. Change medium twice a week by aspirating medium and replacing with fresh medium using a sterile pipet (5ml of medium for a T25 flask and 25ml of medium for a T175 flask).
11. Infection can be observed using an inverted microscope (Figure 1A). Free spores can be seen in the supernatant. If frozen spores are used to infect uninfected host cells, intracellular infection can usually be visualized after 7 days. If frozen infected cells are used to establish a culture then infection can usually be visualized within 3 days.
12. Cultures can be maintained by changing media twice weekly for several weeks. Cultures can be expanded using the sub-culturing technique described in the previous section on host cells.

NOTE: 175- cm² T175 flasks can be used if larger volume cell cultures are needed.

Purification of microsporidia spores from collected cell culture supernatant

1. Warm complete medium (DMEM for HFF or MEM for RK13 containing FBS and Penicillin/Streptomycin) and DPBS by placing bottles in a 37°C water bath for 10 min, and after removal, dry the bottles and then spray them with 70% ethanol in order to avoid contamination from organisms in the water bath.
2. Take out of the infected cell flask from 5% CO₂ incubator and leave it in laminar flow hood. Spray the flask with 70% ethanol.

3. Aspirate the medium using a pipette and place this medium into a sterile 250 ml conical bottle.
4. Replace the medium in the cell culture flask with fresh medium and return the flask to a 37°C 5% CO₂ incubator.
5. The collected medium which contains released spores and detached host cells should be kept at 4 °C. Spores remain viable for over a year at 4 °C.

NOTE: The bottle containing the spores can be centrifuged and the medium replaced with DPBS. This limits the growth of contaminating organisms, which is more likely to occur in medium stored for long periods of time.

6. Centrifuge the 250 ml conical bottle containing the medium with spores at 1,500 × g for 20 min at 4 °C.
7. Resuspend the pelleted spores and debris in 10 ml DPBS and transfer to a 15-ml polystyrene sterile conical centrifuge tube.
8. Pass the resuspended material three times through a 27-G needle attached to a 10 ml syringe to ensure that the majority of the host cells are lysed.
9. Filter the syringe-lysed cell suspension through a 5-µm Nucleopore filter to remove debris. The concentration of spores can be determined by counting the spores in the filtered suspension using a hemocytometer (Figure 1B).
10. A spore pellet can be obtained by centrifugation of the filtered material at 1,500 × g for 20 min at 4°C.

Purification of microsporidia spores directly from infected host cells

1. Scrape the monolayer of infected RK13 cells with a cell scraper and transfer into a 15-ml polystyrene sterile conical centrifuge tube.
2. Pass the cell suspension three times through a 27-G needle to ensure that most of the host cells are lysed and the parasites are released from the host cells.
3. Filter the syringe-lysed cell suspension by passing through a 5-µm Nucleopore filter, and then centrifuge at 1,500 × g for 20 min, 4°C in a benchtop centrifuge.
4. After centrifugation, discard the supernatant, resuspend the pellet with 1 ml of MEM medium.
5. Count the number of spores present in cell suspension by transferring 10 µl of the 1:100 diluted cell suspension into a standard hemocytometer and by examining under an inverted microscope.

NOTE: Among the species of microsporidia currently recognized as pathogens in humans, four species within two genera (Enterocytozoon and Encephalitozoon) predominate. The Encephalitozoon species infecting humans consist of Encephalitozoon cuniculi, Encephalitozoon hellem and Encephalitozoon intestinalis, all these three of these species can be grown in tissue culture and can cause infection in mice. Because of this, they have

been studied more than other microsporidia. *Enterocytozoon bienersi* is the most prevalent microsporidium detected in humans but it cannot be grown in a long-term culture and no useful small animal model exists. *E. cuniculi*, *E. hellem*, and *E. intestinalis* cannot be distinguished by light microscopy; however, PCR can be used to identify these species. DNA extracted from spores or tissue culture can be amplified using the following primers (see table 1) using a thermal cycler programmed as follows: initial denaturation for 5 min at 95°C, followed by 29 cycles, 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and the final termination lasted 10 min at 72°C ((Louis M Weiss & Becnel, 2014; L. M. Weiss & Vossbrinck, 1998) to verify the species:

NOTE: There are 3 major lineages of *E. cuniculi* that are distinguished by the number of GTT repeats in the ITS region (mouse strain 2 repeats; rabbit strain 3 repeats; and dog strain 4 repeats) (E. S. Didier et al., 1995). The ITS region can be amplified using the primers TGCAGTTAAAATGTCCGTAGT and TTCTACTCGCCGCTACTCAG; annealing temperature 55° C, amplicon 1000bp. Sequencing of the amplified region can then be used to distinguish and validate these strain isolates.

Basic Protocol 3: CRYOPRESERVATION OF *ENCEPHALITOZOON* IN INFECTED CELLS

Materials

For freezing infected cells: Freezing medium: 20% (v/v) dimethylsulfoxide (DMSO; Sigma, #D2438) and 80% fetal bovine serum

For freezing spores 20% DMSO (v/v) and 80% DPBS

hemocytometer

15ml screw top plastic tubes

Sterile 5 ml and 10 ml syringes

18-gauge needles

27-gauge needles

15 ml tubes

Hemostat

Sterile cryovials

1. Warm DPBS by placing bottles in a 37°C water bath for 10 min, and after removal, dry the bottles and then spray them with 70% ethanol in order to avoid contamination from organisms in the water bath.

2. Thaw a tube of 0.05% Trypsin-EDTA (1×) in a 37°C water bath for 10 min, after removal, dry the tube and then spray it with 70% ethanol in order to avoid contamination from organisms in the water bath.
3. Remove an infected (at least 10% of cells should be infected) T25 flask from the incubator, place it into the laminar flow hood, and spray the flask with 70% ethanol in order to limit contamination.
4. Aspirate the medium from the flask and dispose of as a biohazard.
5. Add 3ml DPBS to the flask using a 5ml sterile pipet and incubate for 5 minutes.
6. Aspirate the DPBS from the flask and replace with 1ml 0.05% Trypsin-EDTA. Make sure that the solution covers the entire bottom of flask. Incubate until all of the cells are detached from the bottom of the flask (takes about 1 minute at 37°C).
7. Aspirate the detached cells using a pipette and place them into a sterile cryovial.
8. Centrifuge the cryovial at $1,300 \times g$ for 10 min.
9. Aspirate the supernatant from the cryovial and dispose of the aspirated material into a biohazard waste container containing 1:4 bleach solution.
10. Resuspend the pelleted cells in the cryovial by adding 1ml of freezing medium and aspirating the cell pellet several times until resuspended.
11. Label the cryovial with the date, host cell, and parasite information.
12. The cryovials should be placed in a freezing unit that cools at $-1^{\circ}\text{C}/\text{min}$ to reach -40°C , and then transferred to liquid nitrogen. If a controlled-rate freezing unit is not available, then place the vials in a Nalgene 1°C freezing chamber and put the chamber at -80°C for a minimum of 2 hours. The vials can then be transferred to liquid nitrogen.

Cryopreservation of *Encephalitozoon* spores

13. Take a confluent, heavily infected (at least 50% of cells) host cell (HFF or RK13) flask from the 37°C CO₂ incubator, spray the flask with 70% ethanol in order to avoid contamination, and place it into a laminar flow hood.
14. Harvest the infected T175 culture flask by transferring all but approximately 3 ml of the 20 ml culture medium into two 15 ml plastic centrifuge tubes.
15. Detach the remaining tissue culture cells by scraping the surface of the flask.
16. Transfer the remaining ~3ml of cell suspension into a 5ml syringe fitted with an 18-gauge needle and aspirate several times.
17. Change the needle on the syringe to a 27-gauge needle and pass the previously aspirated culture fluid through the syringe. Combine this aspirate material with the cell culture medium from step 14

NOTE: This procedure involves needles and has a risk for sharps injury. A hemostat should be used to grab the needle when it is being removed from the syringe to avoid injury. All needles should be disposed of as biohazard sharp waste. To minimize the use of sharps blunt ended gavage needles can be used for these procedures.

18. Centrifuge the 15 ml tubes at $1300 \times g$ for 10 min.

NOTE: To avoid aerosolization, tubes should be capped and, if possible, buckets with lids should be used to minimize biohazard risk.

19. Add 1ml DPBS to each tube and pool the cell pellets.
20. Perform a spore count under the microscope using a hemocytometer and adjust the concentration of spores in the suspension to $2.0 - 4.0 \times 10^7$ spores per ml using DPBS as a diluent.
21. Mix the spore preparation with an equal volume of 20% DMSO in DPBS and incubate in this cryoprotectant solution for 30 minutes on ice.
22. Dispense 0.5 ml aliquots into sterile plastic screw-capped cryovials.
23. The cryovials should be placed in a freezing unit that cools at $-1^\circ\text{C}/\text{min}$ to reach -40°C , and then transferred to liquid nitrogen. If a controlled-rate freezing unit is not available, then place the vials in a Nalgene 1°C freezing chamber and put the chamber at -80°C for a minimum of 2 h. The vials can then be transferred to liquid nitrogen.

Basic Protocol 4: *ENCEPHALITOZOON CUNICULI* MURINE INFECTION MODEL

Encephalitozoon cuniculi can infect mice which have been used to investigate the host immune response (Snowden, Didier, Orenstein, & Shadduck, 1998) and also to test compounds for *in vivo* efficacy against microsporidiosis (Bacchi et al., 2002). Experimental animals can be infected either via oral gavage, the natural route of infection, or via intraperitoneal injection. Both routes of infection induce a potent immune response dependent on CD8 T cell cytotoxicity which has been shown to be critical for protection (I. A. Khan, J. D. Schwartzman, L. H. Kasper, & M. Moretto, 1999; MM Moretto, Weiss, Combe, & Khan, 2007). However, the role of CD4 T cells is more complex. During intraperitoneal infection, the CD4 T cell response is dispensable for induction of the protective CD8 T cell response, whereas these cells play a synergistic role during oral infection (M. Moretto, Casciotti, Durell, & Khan, 2000; M. Moretto, L. M. Weiss, & I. A. Khan, 2004). Therefore, special care should be taken when choosing the route of infection to address questions of the immune response to *E. cuniculi*.

NOTE: Protocols using live animals must be reviewed and approved by the Institutional Care and Use Committee (IACUC) and must adhere to governmental regulation regarding the use and care of animals.

NOTE: The same procedures described below can be used to infect mice with *E. hellem* or with *E. intestinalis*.

Materials

E. cuniculi spores propagated *in vitro*

6 to 8 week old mice (BALB/c, C57BL/6)

NOTE: Immune deficient mice (CD8^{-/-}, Interferon γ ^{-/-}, Balb/c^{nude/nude}) have been used for studies of drug efficacy as infection with *E. cuniculi* is lethal in these animals.

1× Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium

50 ml sterile polystyrene conical centrifuge tubes

Refrigerated centrifuge with adaptors for buckets with aerosol-tight lids capable of holding 50 ml tubes

Hemocytometer

1 ml sterile syringes

18 gauge feeding needle for oral infection

27 gauge ½ inch needle for intraperitoneal infection

NOTE: Additional information is available for intraperitoneal injection of mice (Donovan & Brown, 2006), oral infection of mice (Pawlowic, Vinayak, Sateriale, Brooks, & Striepen, 2017) and counting cells using a hemocytometer (Strober, 2001).

***Encephalitozoon cuniculi* Infection of Mice**

1. Harvest *E. cuniculi* spores from *in vitro* cell culture flask(s) in a laminar flow hood as described above (personnel protective equipment should include a protective laboratory coat, protective eye goggles, and nitrile gloves).
2. Spin the parasite suspension in a 50 ml conical tube(s) for 15 min at 1,500 × g, 4°C (use buckets with aerosol-tight lids).
3. Remove the lid covered buckets from the centrifuge and transfer to a laminar flow hood prior to retrieving the conical 50 ml tube(s).
4. Aspirate the supernatant without disturbing the pellet.
5. Resuspend the pellet in 40 ml of ice cold PBS.
6. Repeat the wash steps 2 to 4 twice (2 washes) in order to remove remaining cell culture media and supplements.
7. Resuspend *E. cuniculi* spores in 1 ml cold DPBS.
8. Perform a 1:1000 dilution with PBS before counting spores with an inverted microscope under 20× magnification.

9. Inoculate 200 µl of the spore suspension (2×10^7 *E. cuniculi* spores/mouse) either orally using an 18 gauge feeding needle (Pawlowic et al., 2017) or intraperitoneally using a 27gauge 1/2-inch needle (Donovan & Brown, 2006).
10. In immune competent animals infection will occur and become chronic over the course of 3 to 4 weeks with minimal mortality during acute infection. In immune deficient animals mortality typically occurs at 3 to 6 weeks post infection; depending on the degree and type of immune deficiency, dose of organisms, and route of infection.

BASIC PROTOCOL 5: DETERMINING MICROSPORIDIA BURDEN IN MURINE TISSUES USING PCR

Materials

70% ethanol spray bottle

200 proof molecular grade ethanol

Molecular grade water

Sterile scissors and forceps

Disposable benchtop protector

Sterile cryovials

Qiagen DNeasy Blood and tissue kit (# 69506)

Forward and reverse primer set specific for the *E. cuniculi* small subunit ribosomal RNA gene TGTGAGACCCTTTGACGGTGTCT and ACATTCAAAGCAGCTTCGTCAGCC

NOTE: Other primer pairs for *E. cuniculi* as well as other microsporidia are described in (Louis M Weiss & Becnel, 2014) and (L. M. Weiss & Vossbrinck, 1998)

SsoAdvanced™ Universal SYBR® Green supermix (BIO-RAD t# 1725270)

60×15 mm polystyrene petri dish

Single edge razor blades #12

Microcentrifuge tubes

15 ml polystyrene tubes

PCR plate and PCR sealing film (BIO-RAD t# 9601 and #MSB1001)

Vortex mixer

Microcentrifuge

Heated block/water bath

Real time thermal cycler (Biorad CFX96)

PCR Assay for Microsporidia in Tissues

1. Euthanize infected mouse from Basic Protocol 3 using CO₂ asphyxiation (Donovan and Brown 2006).
2. Spray the mouse body with 70% ethanol.
3. Using scissors and forceps, incise the skin and harvest organs to be tested (spleen, liver, brain, and kidney).
4. Place the organs in labeled cryovials and freeze (−80°C).
5. Weigh each organ to determine the volume of ATL buffer and proteinase K needed for the digestion of the entire organ (180µl ATL buffer and 20µl proteinase K per 25 mg of tissue).
6. Add the calculated amount of ATL buffer and proteinase K to a 15 ml tube.
7. Extract and cut each frozen organ into small pieces (2 mm or smaller) in a petri dish using forceps and a clean razor blade for each tissue.

NOTE: Used razor blades should be disposed in a biohazard sharp waste container.
8. Transfer the tissue to the tube containing ATL buffer and proteinase K.
9. Vortex vigorously for 20-30 sec.
10. Place in a water bath at 56°C and vortex vigorously every 15 min until the tissue is completely lysed.
11. Transfer 1ml of the lysate to a new tube and add AL buffer (200µl AL buffer per 25 mg of tissue).
12. Vortex vigorously for 30 sec and place in a water bath at 70°C for 10 min.
13. Add 200µl 100% ethanol per 25mg tissue and mix thoroughly by pipetting up and down.
14. Pipet lysate into spin column(s) and follow the protocol for “purification of total DNA from Animal Tissues (Qiagen DNeasy Blood and tissue kit; cat# 69506; spin-column protocol)”.
15. Elute the column with 100 µl of molecular grade water.
16. Measure DNA concentration and assess DNA purity with a spectrophotometer.
17. Add the following to a PCR tube:
 - 1µl of the forward and reverse primers
(TGTGAGACCCTTTGACGGTGTCT and
ACATTCAAAGCAGCTTCGTCAGCC) stock concentration 6.25µM)
 - 10µl of the SsoAdvanced supermix (BIO-RAD, cat#1725270)
 - 100-200ng of DNA template from the organ being assayed
 - Molecular grade water to bring the total reaction mix volume to 25µl

18. Amplification is then conducted with the following conditions in a PCR machine:
 - 5 min initial incubation of 95°C, then
 - 40 cycles of 45 sec at 95°C and 50 sec at 58°C
 - Use a 1°C increment/cycle (melt curve) for ramping
19. Parasite DNA equivalents (purified from a set concentration of spores using the kit using the Qiagen DNeasy Blood and tissue kit and the protocol for purification of total DNA from cells) can be amplified concomitantly to generate a standard curve providing a quantitative assay of microsporidian DNA per µg of tissue DNA in this assay. The limit of detection of the assay is 5 spores.

COMMENTARY

Background Information

Microsporidia were initially described about 150 years ago with the identification of *Nosema bombycis* as the organism responsible for the disease pébrine in silkworms. They are ubiquitous in the environment and infect almost all invertebrates and vertebrates, as well as some protists. Microsporidia are obligate intracellular parasites related to Fungi and, are probably, members of the Cryptomycota (Han & Weiss, 2017; Louis M Weiss & Becnel, 2014). These organisms are eukaryotes that have a nucleus with a nuclear envelope, an intracytoplasmic membrane system, chromosome separation on mitotic spindles, vesicular Golgi, and a mitochondrial remnant organelle lacking a genome termed a mitosome. Most microsporidian infections are thought to result from fecal-oral transmission of spores from infected humans and animals through contaminated food or water (E. Didier & Weiss, 2011). However, transmission of various species of microsporidia has also been reported to occur from organ transplantation, direct inoculation through broken skin, contact with mucosa, inoculation into the eye, or sexual activity (E. Didier & Weiss, 2011). The spore is the only stage of microsporidia known to survive outside of host cells. Survival of microsporidia spores occurs in a wide range of salinities and temperatures facilitating environmental transmission. *E. cuniculi* spores were able to infect mice after being stored in water at 4 °C for 2 years (Koudela, Kucerova, & Hudcovic, 1999). When spores of *E. cuniculi*, *E. hellem*, and *E. intestinalis* are stored in water temperatures ranging from 10 °C to 30 °C, they have been shown to remain infectious for weeks to as long as a year (Li, Palmer, Trout, & Fayer, 2003). Due to the concern that water could be a source of microsporidian infection they have been included as pathogens on the Environmental Protection Agency Contaminant Candidate List. They are also classified as Biodefense Category B Priority Pathogens by the National Institutes of Health.

There are three phases to their life cycle: the infective or environmental phase; the proliferative phase (e.g. merogony); and the sporogony or spore forming phase (Keeling & Fast, 2002). During the infective phase cells release mature spores which are environmentally resistant. When a host ingests spores, they germinate and infect cells of the gastrointestinal tract. During spore germination the polar tube is extruded from the spore and acts as a conduit for injecting the sporoplasm into its host cell. Other than germinating

naturally during infection in their hosts, spores can also germinate when exposed to various specific conditions such as pH change (Undeen & Avery, 1984; Louis M Weiss & Becnel, 2014). Once in the host cell, the microsporidia enters the proliferative stage, replicating by merogony, which is followed by sporogony ending in the formation of infectious spores. The combination of multiplication during both merogony and sporogony results in a very large number of spores being produced from a single infection and is the basis of the enormous reproductive potential of these pathogens. These pathogenic organisms can have significant effects on their hosts and host cells, with infection resulting in juvenilization, feminization, or other changes to host physiology, as well as the formation of xenomas or other multinucleate cellular structures.

Microsporidia have a wide host range, with various species infecting invertebrate and vertebrate hosts. In the field of veterinary medicine, microsporidiosis has been recognized as causes of disease in laboratory and food-producing animals since the early 20th century (Louis M Weiss & Becnel, 2014). Microsporidia are also responsible for economic losses due to their adverse effects on insects and animals of economic importance such as silkworm, fish and honey bee (Pasteur, 1870; Louis M Weiss & Becnel, 2014). There are over 1500 species and 200 genera of microsporidia. Seventeen species of microsporidia have been reported to cause infection in humans, primarily in patients with immune suppression such as those with HIV/AIDS or recipients of organ transplants; however, infection also occurs in immune competent humans (Cali, Weiss, & Takvorian, 2005; Louis M Weiss & Becnel, 2014). The following genera have been demonstrated to cause infections in humans: *Nosema* (*Nosema corneum*, renamed *Vittaforma corneae*; *Nosema algerae*, reclassified initially as *Brachiola algerae* and now as *Anncaliia algerae*), *Pleistophora*, *Encephalitozoon*, *Enterocytozoon*, *Septata* (reclassified as *Encephalitozoon*), *Trachipleistophora*, *Brachiola*, *Anncaliia*, *Tubulonosema*, *Endoreticulatus*, and *Microsporidium*.

Critical Parameters and Troubleshooting

Encephalitozoonidae infect a wide range of mammals including mice, and there have been no reports indicating any mammalian host restriction for the Encephalitozoonidae that infect humans. Initial descriptions of *E. cuniculi* isolated from different hosts indicated that there might be host specificity at the genotype level; however, subsequent reports have indicated that all of the described *E. cuniculi* mammalian genotypes are able to infect humans (E. Didier et al., 1996; Kucerovala et al., 2011). Cultures of *Encephalitozoon* species appear to be easily established by inoculation of tissue or fluid containing spores onto a variety of cell lines from rabbit and monkey kidney, human fibroblast and rabbit cornea (Molestina et al., 2014; J. Morris, J. McCown, & R. Blount, 1956; Visvesvara, 2002). Many insect microsporidia have also been relatively easy to culture *in vivo* in a variety of insect cells, and insect microsporidia seen in humans have been cultured in a variety of mammalian cell lines (Molestina et al., 2014). In contrast, microsporidia that infect fish have been found to be quite host specific, at least at the genus level, which makes it difficult to culture these species *in vitro* compared to parasites of insect or mammalian origin (Molestina et al., 2014).

For most microsporidia species, temperature is a critical factor regarding success or failure of infection. These pathogens tend to grow best at temperatures typical of their hosts' temperature range. Growth *in vitro* of microsporidia of insect origin, for example, occurs at 25 to 30°C in insect cell lines; while mammalian microsporidia like *Encephalitozoon cuniculi* grow best at 37 °C (and *E. hellem* which is found in birds can grow well at temperatures up to 40 °C). *A. algerae*, which was initially described from insects grows at a wide range of temperatures (29 – 37 °C) and can infect both insects and humans (Lowman, Takvorian, & Cali, 2000; Undeen & Avery, 1984).

Mycoplasma contamination is one of the main problems in cell culture within laboratories. It has been estimated that 10 to 85% of cell lines may be contaminated by mycoplasma depending on the laboratory. Mycoplasma contamination will result in morphologic and growth rate changes in cell cultures. There are three major sources leading to mycoplasma contamination: infected cells sent from another laboratory; contaminated cell culture medium reagents such as serum and trypsin; and laboratory personnel infected with *M. orale* or *M. fermentans* (Nikfarjam & Farzaneh, 2012). Thus, any cell culture received from another laboratory should be screened for the presence of mycoplasma. Laboratory biohazard standard operating procedures should be strictly followed to prevent the contamination from laboratory persons when performing cell culture. It is recommended that all mycoplasma contaminated cells be discarded since they are a source of contamination for clean cell lines. For cell cultures which cannot be discarded, treatment of contaminated cell lines with a mycoplasma removing agent is necessary. However, both tetracyclines and fluoroquinolones are reported to inhibit the growth of *E. cuniculi* in cell culture which can limit therapy (Ridoux, Foucault, & Drancourt, 1998). Microsporidia can be passed through a mouse model to eliminate mycoplasma, with spores from tissue or ascites being used to establish infection in mycoplasma free host cells. The presence of mycoplasma can be evaluated using commercial testing reagents, such as the Invitrogen Plasmotest, Thermo-Fisher MycoSEQ, and ATCC Universal Mycoplasma Detection Kit.

Anticipated Results

Both HFF and RK13 cells have been widely used to cultivate microsporidia. It is anticipated that a single T175 flask can be split into ten T25 flasks and those flasks would be confluent within a week. *E. cuniculi* vacuoles will be observable within 3 to 5 days following cell culture infection with spores (see figure 1).

Between 2 and 4 weeks following infection immune competent mice will show some lethargy, but will then recover and go on to have a chronic infection. Assay of organs will demonstrate pathologic inflammatory changes consistent with infection, but organisms will be difficult to find. Using PCR the presence of microsporidian DNA can be confirmed and quantified in tissue. In contrast, when infection is done in immune deficient animals mortality typically occurs at 3 to 6 weeks post-infection; depending on the degree and type of immune deficiency, dose of organisms, and route of infection. Tissues from these animals usually contain easily found clusters of microsporidian spores. If immune deficient mice are inoculated intraperitoneally ascites is usually seen at 3 to 4 weeks and microsporidia can be recovered from the ascites and used to start cell cultures.

Time Considerations

Thawing RK13 or HFF cells takes about 30 minutes. Subculture of these cells can be accomplished in about 30 minutes. It takes about 3 days for a subculture to grow to confluence. Changing the medium of a single flask takes about 5 minutes. Freezing cells takes about 45 minutes. Thawing spores from a liquid nitrogen tank requires 30 minutes. Following infection of a cell monolayer (using spores), it takes 7 to 10 days for spores to be spontaneously released from infected host cells. Purification of spores from cell culture requires about 1 hour. Taking tissue from an infected mouse and the purification of microsporidia genomic DNA from infected mouse tissue takes from 2 to 3 hours, and the diagnostic and other PCR assays require about 2 hours to complete.

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FIGURE 1. *Encephalitozoon cuniculi*

(a) Infected host cells demonstrating parasitophorous vacuoles (white arrows) and free spores (black arrows) Bar, 50 μm

(b) Spores (black arrows) purified from infected cell culture medium on a hemocytometer. Bar, 50 μm .

Table 1:Diagnostic Primers for *Encephalitozoon spp.*

Species	Primers	Annealing Temperature	Amplicon Size (bp)
E. cuniculi	ATGAGAAGTGATGTGTGTGCG TGCCATGCACTCACAGGCATC	55° C	549
E. hellem	TGAGAAGTAAGATGTTTAGCA GTAAAAAGACTCTCACACTCA	55° C	547
E. intestinalis	TTTCGAGTGTAAGGGAGTCGA CCGTCCTCGTTCTCCTGCCCCG	55° C	520