Isolation and characterization of an avian isolate of *Encephalitozoon hellem*

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SUMMARY

Members of the phylum Microspora are a group of unusual, obligate intracellular eukaryotic parasites that infect a wide range of hosts. However, there are a limited number of microsporidial infections reported in avian hosts, and no parasite species has been defined as an avian pathogen. A microsporidian organism was recovered from the droppings of a clinically normal peach-faced lovebird (*Agapornis roseicollis*) and established in *in vitro* culture. Intermittent parasite spore shedding was documented over a 2-month period using calcofluor M2R staining of cloacal swabs. The organism was identified as *Encephalitozoon hellem* based on protein and antigenic profiles and molecular sequencing of the small subunit and internal transcribed spacer regions of the ribosomal RNA gene.

Key words: *Encephalitozoon*, Microspora, psittacine bird, zoonosis.

INTRODUCTION

Parasites of the phylum Microspora are obligate intracellular protozoans that infect a wide range of vertebrate and invertebrate hosts. A number of species from several genera have now been identified as human pathogens causing enteric, ocular, respiratory, neurological or systemic disease, particularly in immunocompromised patients (Kotler & Orenstein, 1998). The reservoirs for infection and routes of transmission for these pathogens have not been clearly defined for most of these human infections. However, a limited body of evidence suggests that animals may have some role as sources of human infection (Deplazes *et al*. 1996; Didier *et al*. 1996; Mathis *et al*. 1997; Bornay-Llinares *et al*. 1998).

The species, *Encephalitozoon hellem*, is the third most frequently identified microsporidial species causing clinical disease in humans. It was first identified and characterized using isolates from ocular lesions in 3 AIDS patients (Didier *et al*. 1991). Additional cases in immunocompromised humans have also been described in the eye, the respiratory tract, the urogenital tract, and as fatal disseminated systemic infections (Kotler & Orenstein, 1998).

Although microsporidia have been reported in a wide range of vertebrate hosts, only a few reports of microsporidial infections in birds are included in scientific literature as single case reports or flock outbreaks in psittacine birds. Peach-faced, masked and Fischer's lovebirds (*Agapornis roseicollis*, *A. personata*, *A. fischeri*) are the most frequently reported hosts (Kemp & Kluge, 1975; Novilla & Kwapien, 1978; Lowenstine & Petrak, 1980; Branstetter & Knipe, 1982; Randall *et al*. 1986; Powell *et al*. 1989; Norton & Prior, 1994). Microsporidiosis has also been reported in a flock of budgerigars (*Melopsittacus undulatus*) (Black *et al*. 1997), in a double yellow-headed Amazon parrot (*Amazona ochrocephala*) (Poonacha, William & Stamper, 1985), in 2 eclectus parrots (*Eclectus roratus*) (Pulparampil *et al*. 1998) and in a yellow-streaked lory (*Chalcopsitta scintillata*) (Suter *et al*. 1998). A single report describes microsporidiosis in a non-psittacine avian host, an ostrich (Gray, Puette & Latimer, 1998).

No microsporidian species has been categorized as a parasite that commonly uses birds as its natural host (Canning & Lom, 1986; Snowden & Shadduck, 1999). Until recently, the species identity of microsporidia in avian tissues had not been determined. The organisms were simply identified as members of the phylum Microspora or as members of the genus, *Encephalitozoon* based on morphological features. In recent reports, the molecular method of Southern blotting was used to identify *E. hellem* from tissues of budgerigars (Black *et al*. 1997). Partial sequencing of the small subunit ribosomal RNA gene (SSU rRNA) was used to identify the same parasite species in tissues of eclectus parrots (Pulparampil *et al*. 1998) and an ostrich (Snowden & Logan, 1999), and partial sequencing of the internal transcribed spacer region of the ribosomal RNA gene (ITS) was used to identify *E. hellem* in gut contents of a yellow-streaked lory (Suter *et al*. 1998).

This work supports and extends previous reports

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by detecting persistent shedding of spores in cloacal swabs and droppings from a live, clinically normal peach-faced lovebird. An avian microsporidian isolate was established in tissue culture from this bird. This parasite was identified as *E. hellem* using protein profiles, immunological comparisons and molecular sequence comparisons with human isolates of *E. hellem* and the closely related species, *E. cuniculi*.

MATERIALS AND METHODS

Parasite isolation

A mixed flock of 41 peach-faced, masked and Fischer's lovebirds were submitted by a commercial pet store wholesaler to the Texas A & M College of Veterinary Medicine for diagnostic purposes. All birds were clinically normal on physical examination. As part of a health evaluation, cloacal swabs, and sometimes droppings, were collected from each bird. Samples were examined for the presence of microsporidia using a calcofluor white M2R staining method, similar to that used for parasite detection in human body fluids and faeces (Didier *et al*. 1995*a*). A bird with a high number of spores in a cloacal swab was identified, and droppings were collected for further evaluation. Cloacal swabs were examined intermittently over a 2-month period to further document parasite shedding from an asymptomatic animal.

To isolate spores for *in vitro* culture, several grams of droppings from this bird were disbursed in phosphate-buffered saline (PBS, pH 7·3) with 3X antibiotic–antimycotic solution (Gibco, Gaithersburg, MD), passed through several layers of gauze to remove large clumps, and washed 3 times in PBS. The washed slurry of spores was inoculated into flasks of confluent monolayers of RK-13 cells (CCL-37, American Type Culture Collection, Rockville, MD) using RPMI-1640 tissue culture media supplemented with 5% fetal bovine serum and 3X antibiotic–antimycotic (Gibco, Gaithersburg, MD), as previously described (Didier *et al*. 1991). Medium was changed daily for the first 2 weeks, and twice weekly thereafter.

SDS–PAGE and Western blot analysis

Spores (1×10^7/well) from the avian isolate designated MU5, from a human isolate of *E. hellem* and from a rabbit isolate of *E. cuniculi* were electrophoresed on duplicate 10% polyacrylamide gels under reducing conditions. The human *E. hellem* isolate was first described by Didier *et al*. (1991) and molecular characterization was reported by Vossbrinck *et al*. (1993). The *E. cuniculi* was first isolated by Shadduck (1969) (both isolates kindly provided by E. S. Didier). Protein banding patterns were revealed by silver staining one of the gels (Silverstain Plus, BioRad, Hercules, CA). Proteins from the second gel were electrophoretically transferred onto a supported nitrocellulose membrane (0·45 μm, BioRad) for Western blot immunodetection. Electrophoresis and blotting were conducted in a minigel format following the manufacturer's instructions (BioRad). Western blotting was performed using a 1:100 dilution of polyclonal rabbit anti-*E. hellem* antiserum and horseradish peroxidase-labelled Protein G (BioRad) following manufacturer's instructions. The parasite-specific antiserum was produced by immunizing rabbits 3 times with 10^8 lysed spores of the human *E. hellem* isolate. Bound antibodies were detected using diaminobenzidine substrate (Sigma, St Louis, MO) following manufacturer's instructions. The silver-stained protein gel and the blot were photographed using a gel documentation system (Alpha-Innotech IS-500 systems, San-Leandro, CA).

DNA isolation

Parasite spores were purified from culture supernatants using Percoll™ density gradient centrifugation, and purified spores were processed for DNA extraction using a previously described method (Didier *et al*. 1995*b*). DNA was isolated by a standard phenol-chloroform extraction method (Sambrook, Fritsch & Maniatis, 1989) using a partitioning gel microfuge tube system (Light Phase Lock Gel [LPLG]™ system, 5 Prime → 3 Prime Manufacturer Inc., West Chester, PA) to optimize DNA recovery. The DNA was ethanol precipitated, the pellet resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7·6), and the DNA concentration was estimated using A260/A280 ratios by UV spectrophotometry (Pharmacia Ultraspec Plus®, Piscataway, NJ). DNA extracted from *E. cuniculi* was used as a positive control for all reactions.

Cloning of the small subunit and ITS regions of ribosomal RNA gene

The SSU rRNA gene from the avian isolate was amplified with the primer pair, 18f and 1537r using methods similar to those previously described (Weiss & Vossbrinck, 1998). A separate DNA fragment including a 3′ portion of the SSU and the ITS region of the ribosomal gene was amplified using the primer pair 1061f and int 580r (Didier *et al*. 1995*b*; Weiss & Vossbrinck, 1998). The PCR reactions were performed following manufacturer's instructions (GeneAmp PCR Core Reagents, N808-0009, Perkin Elmer Cetus, Roche Molecular Systems, NJ). The amplification was carried out in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA). After initial denaturation for 5 min at 95 °C, *Taq* polymerase (0·5 U) was added to each 25 μl reaction. The samples then underwent 35 cycles of denaturation

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(94 °C, 30 sec), annealing (58 °C, 30 sec) and extension (72 °C, 1 min) followed by 20 min at 72 °C for final extension. Unincorporated nucleotides were removed using Micro Bio-Spin 30 Chromatography Columns (BioRad).

The TOPO-TA cloning kit (Invitrogen, San Diego, CA) was used to insert the PCR product of approximately 1300 bases for the small subunit and 500 bases for the ITS region directly into a plasmid vector. Following the manufacturer's instructions, appropriately sized and purified amplicons were ligated within 24 h of PCR amplification into pCR 2.1 vectors and transformed into One Shot® competent *E. coli* cells. Plasmid DNA isolation was achieved using the Qiagen Plasmid MiniPrep Kit (Qiagen, Valencia, CA).

DNA automated sequencing

Multiple clones containing SSU sequence were amplified for automated sequencing using an ABI Prism Big Dye Terminator Cycle Sequencing Ready-Reaction Kit (Promega). Primers 530f, 1061f, 350f, PMP1, 1537r, 1047r, and 530r were used to completely sequence forward and reverse strands of DNA (Weiss & Vossbrinck, 1998). Two clones of the SSU rRNA gene were completely sequenced in both directions, and 7 additional clones were partially sequenced to confirm the identity of several bases. Two clones containing the ITS region were amplified for automated sequencing using the 1061f and int580r primers (Didier *et al*. 1995*b*; Weiss & Vossbrinck, 1998). All reactions were run in a MJ Research Minicycler thermocycler following the manufacturer's instructions. The samples underwent 25 cycles of denaturation (96 °C, 30 sec), annealing (50 °C, 15 sec) and extension (60 °C, 4 min). Extension products were purified using the Micro Bio-Spin 30 Chromatography Columns (Bio-Rad), dried in a vacuum centrifuge (Savant Instruments Inc., Holbrook, NY) and stored at −20 °C until sequenced in an automated sequencer (Perkin Elmer 377 ABI Applied Biosystems, Inc, Foster City, CA).

Sequences were aligned using the Sequencher software program (Gene Codes Corp., Ann Arbor, MI) to generate a consensus sequence. Sequence homology with other eukaryotes was determined using a BLASTN search from NCBI GenBank database. The consensus sequence for the SSU and ITS regions of the MU5 isolate were submitted to GenBank.

PBFDV testing

Since microsporidial infections in humans are often associated with immunosuppression, this lovebird was tested for the presence of the immunosuppressive avian circovirus, Psittacine Beak and Feather Disease virus (PBFDV). Heparinized blood was submitted to a commercial laboratory (Research Associates Laboratories, Milford, OH) for PCR detection of the virus.

RESULTS

A microsporidian parasite, designated MU5, was identified in calcofluor M2R stained cloacal swabs and droppings from a live, clinically normal peach-faced lovebird. Shedding of low numbers of parasites was observed during a 2-month sampling period. The MU5 avian isolate was established in tissue culture. Infected RK-13 cells were visible in cultures within 4 days, and free spores were identified in culture supernatants within 5 days after inoculation.

When purified spores of the MU5 isolate, and human isolates of *E. hellem* and *E. cuniculi* were compared using silver-stained polyacrylamide gel electrophoretic banding patterns, avian and human *E. hellem* isolates were essentially identical in the number and intensity of protein bands, while the closely related *E. cuniculi* showed multiple distinctly different bands of various molecular masses (Fig. 1 A).

The banding patterns of the 2 *E. hellem* isolates in the Western blot were immunologically very similar but not identical, both were distinctly different from the *E. cuniculi* isolate profile (Fig. 1 B).

DNA sequencing of the MU5 isolate generated a consensus sequence of 1484 bases that was assigned the GenBank accession no. AF177920. The small subunit region of the gene was 1314 bases long and showed greater than 99% homology with the SSU rRNA genes of several human isolates of *E. hellem*. Comparison of the first 1299 of 1314 bases in the SSU rRNA sequence, showed that the avian isolate was 100% homologous with one of the human isolates (L19070|EZOSRRNAX) (Visvesvara *et al*. 1994) and there were only 3 base differences from a second human isolate, L39108|EZORGOB (Baker *et al*. 1995). Comparisons between the MU5 avian isolate and partial SSU rRNA gene sequences from 2 eclectus parrots showed a single base difference (AF039229 and AF0390230) (Pulparampil *et al*. 1998).

In separate cloning and sequencing reactions, a 502 base fragment including the 3′ end of the small subunit, the ITS region and the 5′ end of the large subunit of the ribosomal gene was generated. Molecular sequencing of the ITS region showed 100% homology between the MU5 isolate and several human isolates (L29557|EZORGLSA, L13331) (Katiyar, Visvesvara & Edlind, 1995; Vossbrinck *et al*. 1993).

PBFDV testing was positive indicating that the bird was viraemic at the time of sampling.

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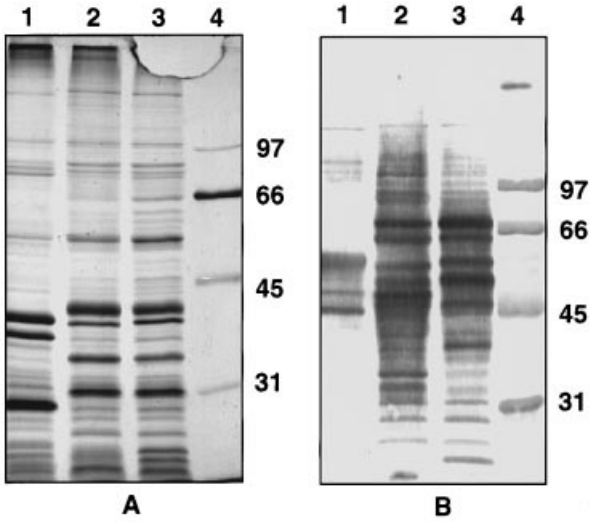

Fig. 1. (A) Silver-stained polyacrylamide gel showing identical protein banding patterns for spores of avian and human isolates of *Encephalitozoon hellem*, while the closely related microsporidian, *E. cuniculi*, shows a different protein profile. (B) Western blot analysis using rabbit anti-*E. hellem* antiserum and horseradish peroxidase–Protein G on spores of MU5 and human isolates of *E. hellem*. These show antigenically similar patterns, while *E. cuniculi* antigens show a limited cross-reactive but unique antigenic pattern. In both panels, Lane 1, *E. cuniculi*; Lane 2, MU5 isolate; Lane 3, *E. hellem* human isolate; Lane 4, MW markers.

DISCUSSION

In this report, microsporidia are identified for the first time in cloacal swabs and droppings from a live, clinically normal peach-faced lovebird. Previously, microsporidia have only been diagnosed in these hosts at post-mortem. Parasite spore shedding was documented to persist for at least 2 months. These parasites were established in tissue culture.

The protein and antigenic profiles of the MU5 avian isolate and the human isolate were very similar to each other and distinct from that of *E. cuniculi* (Fig. 1 A and B). These data strongly suggest that the MU5 avian isolate is the parasite species, *E. hellem*. Minor antigenic variation, as seen in the present study between the MU5 and the human isolate, has been reported previously for both *E. hellem* (Mathis *et al*. 1999) and *E. cuniculi* isolates from various host species (Mathis *et al*. 1997).

Ribosomal RNA gene sequencing, a tool used frequently to establish the species identity of various microsporidia, showed that the avian isolate is essentially identical to several previously sequenced *E. hellem* isolates of human origin. Comparisons of the MU5 sequence were made with sequence of 4 *E. hellem* isolates that had been sequenced up to the 3′ end of the SSU rRNA gene but did not include the ITS region. These 4 sequences (L19070|EZOSRRNAX, L39108|EZORGOB, AF118142, AF118143) (Visvesvara *et al*. 1994; Baker *et al*.

Table 1. Sequence comparisons of the 3′ end of the SSU and a portion of the ITS regions of the rRNA gene among previously reported human *Encephalitozoon hellem* isolates, the MU5 avian isolate and the 1537r primer used to amplify the 3′ end of the SSU rRNA gene

Isolate	Aligned sequences from bases 1294 to 1340
	1294 1314* 1325 1335
MU5	GAACCAGGCAGGATCAGTATGTTGATTTGATTTATTT------G----TGGGGATT
L29557 | EZORGLSA	●●●●●GC●●●●●●●●GT●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
L13331	●●●●●GC●●●●●●●●GT●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
AF110327	●●●●●GC●●●●●●●●GT●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
AF110328	●●●●●GC●●●●●●●●GT●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
L19070 | EZOSRRNAX	●●●●●TT●●●●●●●●TA●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
L39108 | EZORGOB	●●●●●TT●●●●●●●●TA●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
AF11842	●●●●●TT●●●●●●●●TA●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
AF11843	●●●●●TT●●●●●●●●TA●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
primer 1537r†	GAACCATTAGCAGGATCATAA

* Indicates the end of the SSU portion of the gene at base 1314.
† Reverse primer is reversed and translated to forward sequence.

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1995; Mathis *et al*. 1997) differ from the MU5 isolate in that they contain TT and TA substitutions for GC and GT at base positions 1300–1301 and 1312–1313 (Table 1). Upon reviewing how these DNAs were sequenced, it was found that these isolates were amplified using a primer (1537r) that was originally designed based on a universal eukaryotic primer and the sequence of the microsporidian, *Vairimorpha necatrix* (Vossbrinck *et al*. 1987). Thus, the terminal 21 bases of sequence reported for these isolates are that of the primer used in the PCR amplification reaction and may not be the true sequence of *E. hellem*.

When aligning the same bases in 4 other *E. hellem* isolates of human origin that included sequences at the 3′ end of the small subunit in association with the ITS region (L29557|EZORGLSA, L13331, AF110327, AF110328) (Vossbrinck *et al*. 1993; Katiyar *et al*. 1995; Mathis *et al*. 1999), the sequences were GC and GT respectively at bases 1300–1301 and 1312–1313 (Table 1). That sequence pattern is identical to the bases identified in the MU5 avian isolate. Therefore, it is probable that the accurate sequences for this typically conserved portion at the 3′ end of the SSU rRNA gene of *E. hellem* are GC and GT bases at positions 1300–1301 and 1312–1313, respectively.

The ITS region of the ribosomal RNA gene has been used to classify the closely related microsporidian, *E. cuniculi*, as 3 different strains based on a 4 base sequence that is repeated 2, 3 or 4 times (Didier *et al*. 1995*b*). Recently, heterogeneity has also been reported in the ITS region of *E. hellem*, grouping various isolates into 3 genotypes (Mathis *et al*. 1999). Our avian isolate has 100% homology with genotype 1, the most frequently reported human genotype (L29557|EZORGLSA, L13331), in contrast to genotype 2 (AF110327) and genotype 3 (AF110328) (Table 1). It has also been reported that the ITS sequence from microsporidia in an Indonesian psittacine bird, a yellow-streaked lory, is genotype 1 (Mathis *et al*. 1999).

Birds have not yet been described as natural hosts for any microsporidian species. However, *E. hellem* has been identified with increasing frequency in a variety of psittacine hosts (Black *et al*. 1997; Pulparampil *et al*. 1998; Suter *et al*. 1998), and also in one non-psittacine host, an ostrich (Snowden & Logan, 1999). These data, along with the new observation of parasites in an asymptomatic lovebird host, suggest that *E. hellem* may actually use birds as their natural hosts, and that only severely immunocompromised humans become infected in unusual circumstances as accidental parasite hosts. The infected lovebird that persistently shed spores was also positive for PBFD virus. It has been reported that this virus is immunosuppressive to avian hosts since infected birds often have co-infections with additional avian pathogens (Latimer, Niagro & Campagnoli, 1993). The relationship between microsporidian and PBFDV infections requires further investigation.

The identification of a clinically normal pet bird that sheds *E. hellem* spores raises potential concern for zoonotic transmission of the parasite. A number of issues must be addressed. Further comparisons of avian and human isolates of *E. hellem* are needed to determine if the parasites are identical. Epidemiological studies of microsporidia in asymptomatic pet birds are also needed to determine the prevalence of the parasites in various species of pet birds and to determine spore shedding patterns within those birds. Additional studies are needed to determine the host range of these parasites in various psittacine and soft-billed pet bird species and in wild bird populations. Finally, the zoonotic risks of this parasite in severely immunocompromised humans need to be clarified.

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