Vol. 108: 37–44, 2014
doi: 10.3354/dao02690

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published February 4

In vitro growth of the microsporidian *Heterosporis saurida* in the eel kidney EK-1 cell line

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ABSTRACT: *Heterosporis saurida* is an intracellular microsporidian that infects lizardfish *Saurida undosquamis*. Although some attempts have been introduced to clarify microsporidian host–pathogen interactions, development of novel strategies to combat fish diseases is still needed. Here we present an *in vitro* cultivation model for fish microsporidia based on an eel kidney cell line (EK-1), which is susceptible to infection by *H. saurida*. Spores were isolated from infected lizardfish and used to inoculate EK-1 cells. *H. saurida* were propagated in the eel kidney EK-1 cell line and detected by immunofluorescence. Developmental stages of *H. saurida* were seen in EK-1 cells by transmission electron microscopy. Identity of the parasite was confirmed by partial sequencing of the 16S rDNA gene. Our cell culture model provides a valuable means to explore molecular and immunological events and will facilitate development of effective treatment strategies.

KEY WORDS: Fish · Microsporidia · *Heterosporis saurida* · Disease · Cell culture · EK-1 cell line

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INTRODUCTION

Microsporidia are obligate intracellular parasites of all major animal groups, including fish. Microsporidian infections in fish are deleterious and endanger the lives of the hosts. Outbreaks can reach epizootic levels, especially under hatchery conditions, which favor transmission (Lom & Dykova 1992, Schmahl & Benini 1998). High mortality rates caused by *Heterosporis saurida* have contributed to declines in production of economically important fish, and subsequent financial losses in aquaculture industries (Al-Quraishy et al. 2012). Microsporidia were originally identified as fungi, but they were reclassified as protozoans by the end of the 19th century, an assignation that was accepted for over 100 yr until molecular techniques and phylogenetic analyses placed microsporidia back into the Fungi (Nageli 1857, Pasteur 1870, Hirt et al. 1999, Keeling et al. 2000, Keeling & Fast 2002).

Although microsporidia are obligate intracellular pathogens, they have an extracellular infective spore phase of development. This spore contains a hollow polar filament, which distinguishes microsporidia from all other organisms and plays a crucial role in host cell invasion. The spore extrudes the filament, through which it then injects its sporoplasm and nucleus into the cytoplasm of the host cell. Intracellular proliferation follows, characterized by the developmental stages of merogony and sporogony (Monaghan et al. 2009). The phylum Microsporidia comprises over 1200 species in nearly 150 genera (Wittner 1999, Franzen & Müller 2001). Of these, 18 genera have members known to infect fish, and include *Glugea*, *Loma*, *Heterosporis*, and *Nucelospora*, which cause severe disease in economically important fish (Lom & Nilsen 2003, Sanders et al. 2012). Several *Heterosporis* species have been reported to infect fish: *H. anguillarum* in cul-

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tured eels in Japan (Hoshina 1951), *H. schuberti* in cultured ornamental cichlids and loricarid catfish from Germany (Lom et al. 1989), and *H. finki* in cultured angelfish *Pterophyllum scalare* from France (Michel et al. 1989). An undetermined species of *Heterosporis* was reported from cultured bettas *Betta splendens* in Thailand (Lom et al. 1993). Since 2000, *Heterosporis* has been confirmed in yellow perch *Perca flavescens* in the USA (Miller 2009). Most recently, *H. saurida* has been described from lizardfish *Saurida undosquamis* in the Arabian Gulf, Saudi Arabia (Al-Quraishy et al. 2012). These *Heterosporis* species, in particular, cause significant pathogenic effects on the host and hence have negative economic consequences in the fish trade (Al-Quraishy et al. 2012).

Interest in *in vitro* propagation of certain microsporidia has intensified recently because several microsporidia are found to have low host specificity (Rinder et al. 2000, Sutherland et al. 2000, Lores et al. 2003, Coyle et al. 2004, Kumar et al. 2013), and some genera have been identified as opportunistic pathogens of humans, especially patients with acquired immune deficiency syndrome (AIDS; Didier 2005). Microsporidia that parasitize humans have also been reported in domestic animals, which may represent reservoir hosts (Breitenmoser et al. 1999, Mathis et al. 1999, Rinder et al. 2000). The fact that microsporidia have been observed in fishes and can be cultured in mammalian cell lines, e.g. *Heterosporosis* sp. in the rabbit kidney epithelial cell line RK-13, supports the hypothesis that mammals may be natural reservoir hosts of fish microsporidia (Lores et al. 2003, Kumar et al. 2013).

Although cell and tissue cultures are essential tools for the propagation and study of obligate intracellular pathogens such as microsporidia, research is hindered by a lack of suitable cell cultures for propagation and study of fish microsporidia. *In vitro* cultivation of parasitic microsporidia not only provides information on the development of the parasite but also enables new approaches for disease control. *In vitro* propagation will also help to identify specific proteins that may enhance the invasive properties of the parasite and subsequently lead to the development of monoclonal antibodies that will aid in neutralizing parasitic invasion. Moreover, evaluation of functional antibodies and cell-mediated defensive systems against the parasites and screening of drugs in order to identify potential therapeutic agents can only be made in a cost-effective manner using *in vitro* culture (Visvesvara & Garcia 2002). Large numbers of parasites at specific stages can be produced in cultures, which allows time-series molecular studies to be performed (Didier et al. 1994, 1996, Deplazes et al. 1996, Hollister et al. 1996, Croppo et al. 1997, Mathis et al. 1997, Franzen & Müller 2001, del Aguila et al. 2001). Moreover, continuous culture over long periods of time may cause attenuation of strains, which potentially leads to development of vaccines (Visvesvara & Garcia 2002).

In this study, we present a cell culture model for *Heterosporis saurida* in eel kidney epithelial cells (EK-1). Development of this *in vitro* approach was essential for our planned research into the control of *H. saurida* using small interfering RNA (siRNA) technology, and will facilitate immunological and cell–pathogen interaction studies.

MATERIALS AND METHODS

Collection of *Heterosporis saurida* spores

Lizardfish were collected from 2 localities in Saudi Arabia: a fish market in Riyadh, and from fishermen in Al-Gezan on the Red Sea. Infection was determined by the occurrence of numerous cysts in the abdominal cavity, skeletal muscles, and the mesenteric tissues (Al-Quraishy et al. 2012). Using 2 sterile scalpels, cysts were carefully opened and spores were released into a Petri dish and collected in 2 ml microcentrifuge tubes. Released spores were then kept in distilled water at 4°C until used for PCR and *in vitro* studies.

Cell line and growth conditions

Eel kidney epithelial cells (EK-1; Chen et al. 1982) were grown at 26°C and maintained in L-15 (Leibovitz) medium with L-glutamine (Sigma Aldrich), and 10% fetal bovine serum (FBS, Invitrogen). Penicillin and streptomycin (Sigma Aldrich) were added at 100 IU ml^{-1} and 100 µg ml^{-1}, respectively.

Preparation of spores

Microsporidian spores were treated with 10000 U ml^{-1} penicillin and 10000 µg ml^{-1} streptomycin in L-15 medium for 2 h at 37°C to inactivate bacteria. Spore pellets were washed twice with L-15 medium, resuspended in L-15 medium and counted using a hemocytometer to achieve a concentration from 1×10^5 to 1×10^7 spores ml^{-1}.

In vitro infection of EK-1 cells with *Heterosporis saurida* spores

EK-1 cells were sub-cultured and seeded in 24-well plates in duplicate. Six wells were infected with different spore concentrations (1×10^5, 1×10^6, and 1×10^7 spores ml^{-1}). The remaining wells received no spores and were used as no-spore controls. L-15 medium supplemented with 10% FBS was added to the cells, and 24 h after seeding, 100 µl of the spore suspensions were added to each cell line monolayer. At 2 d post-infection (p.i.), the medium was removed from each well and the well was rinsed 3 times with fresh medium. The medium was replaced twice a week and the cell cultures were observed every day under an inverted microscope. The whole procedure was repeated 3 times to assess *in vitro* growth of spores.

Re-infection of EK-1 cells

At 3 wk p.i., monolayers were trypsinized, harvested, and centrifuged. Pellets were re-suspended in L-15 medium and used to infect new cultures of EK-1 cells, maintained as described previously.

Production of *Heterosporis saurida* spores in EK-1 cells

EK-1 cells were grown in two 24-well plates, and 1×10^7 spores of *H. saurida* were added to each of the wells. Six wells on each plate received no spores and were used as no-spore controls. The cells were predominantly epithelial-like and were grown at 26°C in L-15 supplemented as described above. At 2 d p.i., cells were washed 3 times with phosphate-buffered saline (PBS) to remove non-attached spores from the wells. Cells were lysed to release the spores, centrifuged, then washed with medium supplemented with 10% FBS. Pelleted spores were re-suspended in PBS. Spores were counted in duplicates from 4 wells of infected cells using a hemocytometer, and numbers of spores were recorded every week for 3 wk.

Transmission electron microscopy (TEM)

Cultures were interrupted at 12, 24, 48, 72 h, and 1 wk p.i. EK-1 cells were washed with serum-free L-15 medium, trypsinized, and harvested in 10 ml sterile glass tubes. Cell pellets were washed twice with PBS, pH 7.2, and fixed in 5% glutaraldehyde (Sigma Aldrich) for 2 h at 4°C. Cells were washed twice with 0.2 M phosphate buffer pH 7.4, then incubated overnight in the same buffer at 4°C, then post-fixed in 1% osmium tetroxide (Sigma Aldrich) at 4°C for 2 h. After several steps of dehydration, the cells were embedded in Epon, and semi-thin sections were cut and then stained with toluidine blue (Sigma Aldrich). The sections were contrasted with uranyl acetate and lead citrate (Sigma Aldrich) and then examined using an electron microscope (Zeiss EM109).

Immunofluorescence assay for detection of *Heterosporis saurida* in EK-1 cells

Inverted phase contrast with fluorescence attachment and confocal microscopes were used to monitor living and fixed cultures. Living cultures were observed daily by phase contrast microscopy. Cell cultures were fixed in cold acetone/ethanol (1:1) for 10 min at 4°C, air dried, and washed 3 times with PBS. Non-specific binding was blocked with 1% bovine serum albumin (Sigma Aldrich) for 30 min. After washing for 3 times for 5 min with PBS, the fixed cells were then incubated with rabbit antiserum containing polyclonal antibodies against microsporidia (Medicago; 1:50 dilution) for 1 h at 37°C. The cells were washed 4 times, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:400) for 45 min at 37°C (Sigma Aldrich). One microliter of propidium iodide (1 mg ml^{-1}; Sigma Aldrich) was added to counterstain the nuclei. The control cells were incubated and covered with the diluted stain for 1 to 5 min. The wells were then washed 3 times with PBS for 10 min each and mounted with mounting medium (Invitrogen).

PCR detection of *Heterosporis saurida* in infected cells

At 1 wk p.i, cells were harvested and washed with PBS, and then DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). PCR primers HG4F (5′-GCG GCT TAA TTT GAC TCA AC-3′) and HG4R (5′-TCT CCT TGG TCC GTG TTT CAA-3′) were used according to Gatehouse & Malone (1998). PCR amplicons were cloned into the pDrive cloning vector (Qiagen) and sequenced (LGC Genomics). An NCBI-BLAST similarity search was performed.

RESULTS

Prior to being added to EK-1 cells, spores were ovoid and phase-bright, and cells had the ordinary phase-contrast appearance of epithelial cells in culture (Fig. 1A). The highest infection rate in EK-1 cells was obtained when 1×10^7 ml^{-1} of *Heterosporis saurida* was used, whereas lower infections were observed with 1×10^6 and 1×10^5 ml^{-1} *H. saurida*. The majority of EK-1 cells were infected at 24 h p.i.

Successful infection of *Heterosporis saurida* was clearly visible by phase contrast microscopy in EK1 cultures 48 h p.i. (Fig. 1B). Spindle-shaped structures, meronts, sporonts, and mature spores of *H. saurida* spores were observed in the cytoplasm. Cytopathic effects were evident within 48 h, with respect to controls, viz. hypertrophy of the nucleus and cytoplasm.

Transmission electron microscopy showed a variety of microsporidian developmental stages. Spores were observed in the host cell cytoplasm, and all stages were present near the nucleus from 12 h p.i., which was evidence for replication of the parasite in EK-1 cells (Fig. 2). Developmental stages were seen in the host cell cytoplasm. Sporogonial stages were monosporoblastic or disporoblastic. The spores had a clear, thin, electron-dense exospore and a thick, electron-lucent endospore (Fig. 2B). Weekly spore counts showed that the number of *Heterosporis saurida* increased with time (Fig. 3). With prolonged incubation time, destruction of the cell monolayer occurred and cell-free areas appeared. At 3 wk p.i., cells detached and lysed, and *H. saurida* spores were released into the medium.

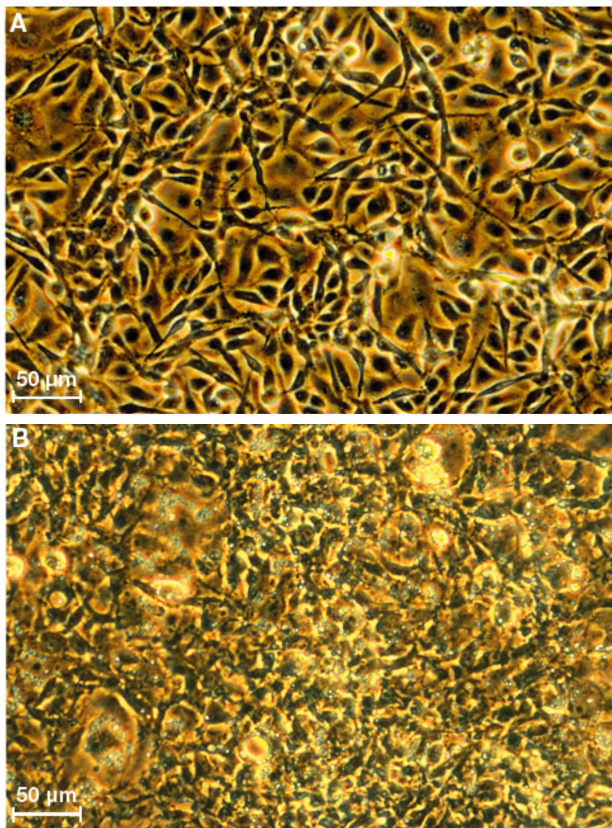

Fig. 1. Phase contrast images showing eel kidney epithelial cell line EK-1. (A) Non-infected control and (B) infected with *Heterosporis saurida*

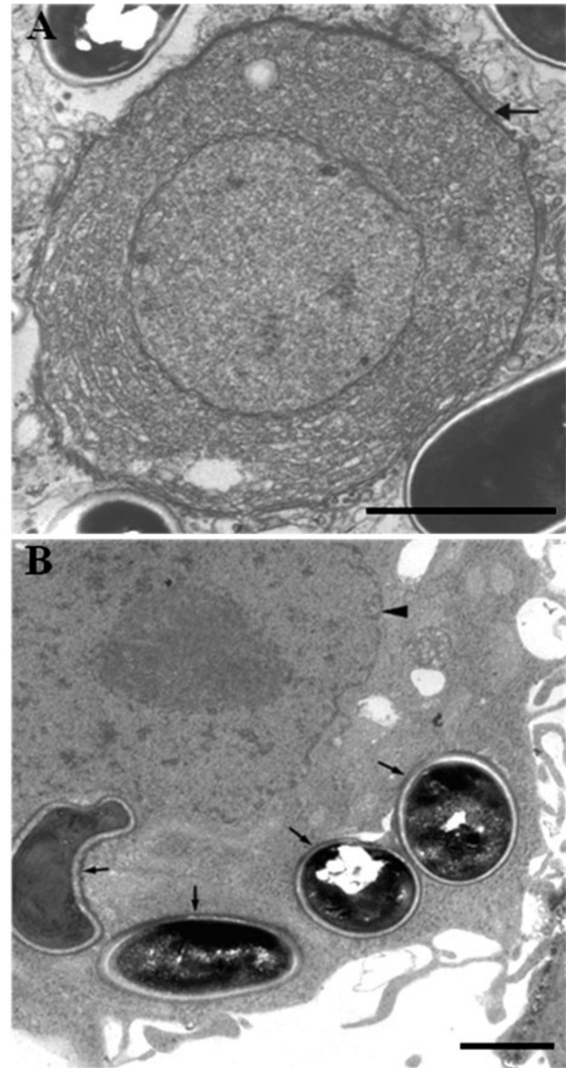

Fig. 2. *Heterosporis saurida*. Transmission electron microscope images showing developmental stages in eel kidney epithelial cells. (A) Infected cell showing meront (arrow) with endoplasmic reticulum and ribosomes bound by a plasma membrane in direct contact with cell cytoplasm. Scale bar = 0.5 µm. (B) Detail of infected cell showing sporonts, which were elongated and oval in shape with dense cytoplasm and no discernible internal structures. Mature spores (arrows) showed that the spore wall consisted of a dense exospore and a lucent layer endospore; arrowhead indicates the host cell nucleus. Scale bar = 1 µm

We observed the complete microsporidian life cycle, with successful infection of *Heterosporis saurida* in EK-1 cells and evident signs of infection seen 2 d p.i. Spindle-shaped structures, meronts, sporonts, and mature spores of *H. saurida* were evident in the cytoplasm. Production of new spores was observed by 72 h at 26°C; the *H. saurida*-infected cell suspension successfully re-infected new EK-1 cell cultures. Strong fluorescence was observed upon addition of the antimicrosporidia polyclonal antibody to *H. saurida* spores in EK-1 cells. The parasite was observed in the host cell cytoplasm near the nucleus (Figs. 4 & 5).

A 1100 bp PCR fragment of *Heterosporis saurida* from both spores and EK-1 infected cells was amplified (Fig. 6), and the NCBI BLAST search demonstrated that the amplicons had 100% similarity with *H. saurida* (JF745533).

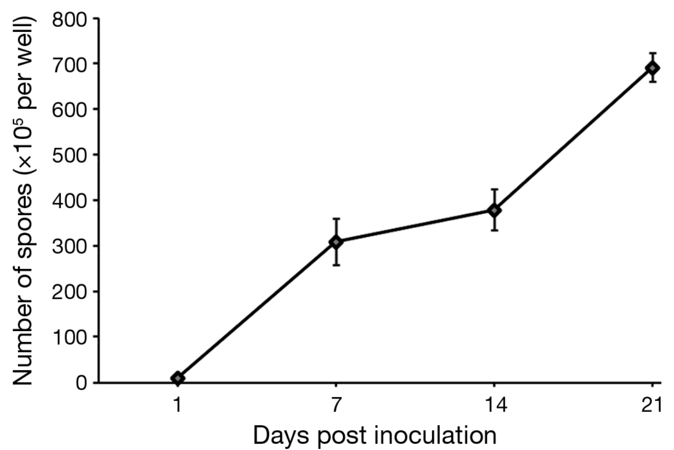

Fig. 3. *Heterosporis saurida*. Production of spores in eel kidney epithelial cells; spore-production peaked in the third week. Data are mean (± SD) numbers of spores in 4 wells of infected EK-1 cells

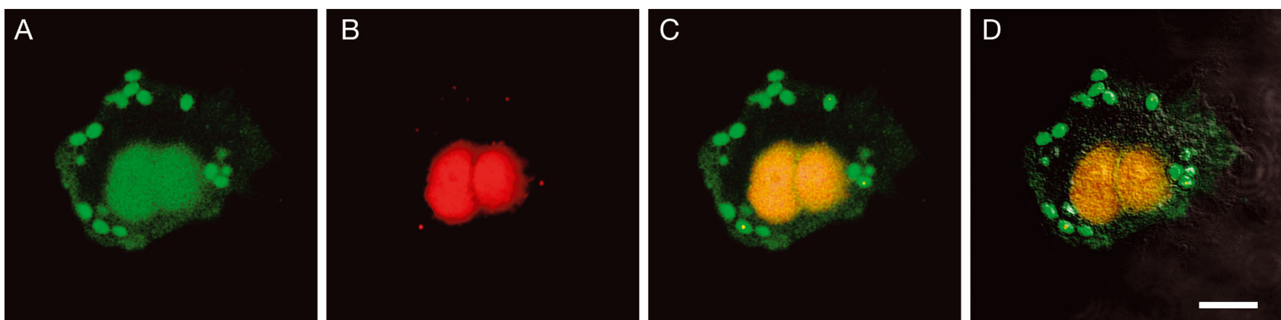

Fig. 4. Immunofluorescence assay of *Heterosporis saurida* in EK-1: confocal microscopy view collected from (A) green, (B) red, and (C,D) merged channels. *H. saurida* spores (in green) are stained with fluorescein isothiocyanate (FITC)-conjugated with goat anti-rabbit IgG after an incubation step with polyclonal antibodies raised against microsporidia. The nuclei (in red) are counterstained with propidium iodide. Scale bar = 1 µm

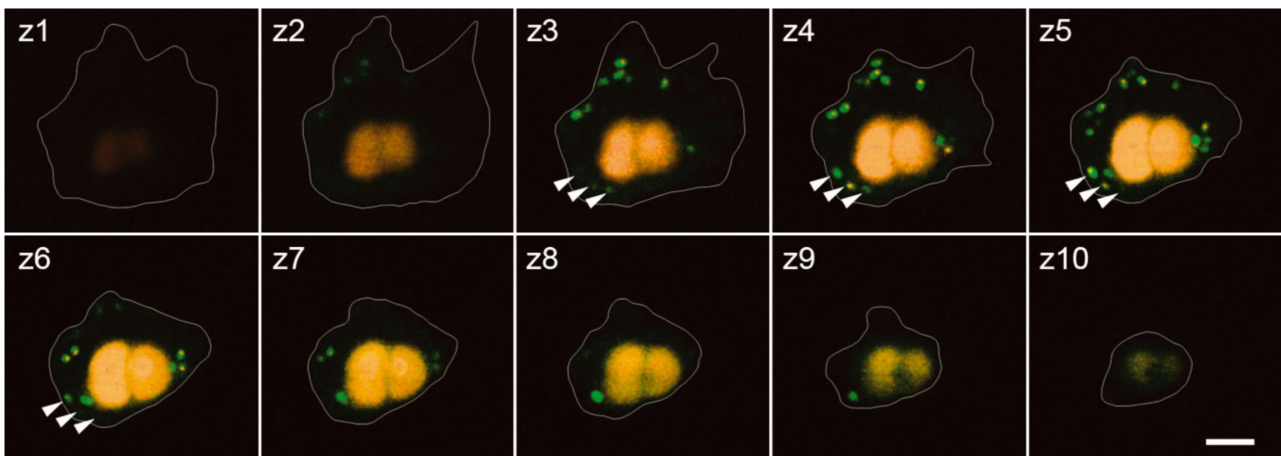

Fig. 5. *Heterosporis saurida*. *z*-axis series of confocal images showing the spores (white arrowheads) in the host cell cytoplasm near the nucleus. Scale bar = 1 µm

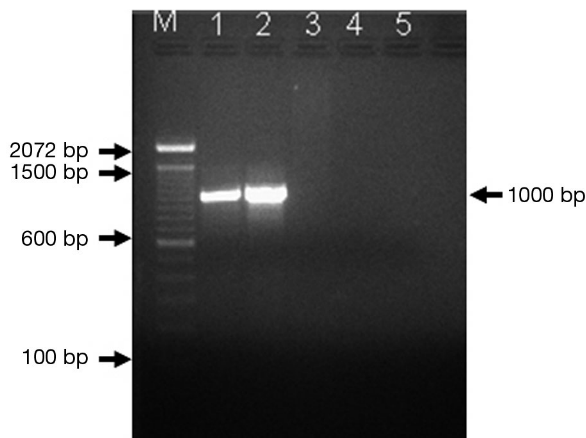

Fig. 6. *Heterosporis saurida*. Agarose gel electrophoresis of amplified DNA showing an 1100 bp PCR fragment of *H. saurida* from both spores and EK-1 infected cells by primers HG4F and HG4R. Lanes: M: DNA ladder (100 bp, Invitrogen); 1: *H. saurida* spores; 2: EK-1 cells infected with *H. saurida*; 3: control EK-1 cells; 4: negative control; 5: non-template control

DISCUSSION

Cellular and molecular studies of the microsporidian *Heterosporis saurida* have been hampered due to the lack of cell culture systems for this pathogen. Molecular and ultra-structural studies of *H. saurida* have been limited to *in vivo* and gene sequence analyses (Peyghan et al. 2009, Al-Quraishy et al. 2012). A cell culture model will offer a valuable means to explore molecular and immunological events, which helps in the development of effective therapeutic agents. Ideally, *H. saurida* would be cultured in a lizardfish cell line, but such a cell line has not yet been developed (Kumar et al. 2013). Although efforts have been made, e.g. by Kumar et al. (2013), lizardfish microsporidia have not been cultured successfully in other fish cell lines prior to this study. Herein, we investigated the capacity of eel kidney EK-1 cells to propagate *H. saurida*. We found that EK-1 cells supported development of *H. saurida in vitro*, thereby providing a fish cell culture model that enables further studies of the pathogen at the cellular level.

Currently, *in vitro* culturing of fish microsporidia using fish cell lines has not shared the success attained with other *in vitro* systems (Monaghan et al. 2009). Although cell lines from many fish are available, failure to culture microsporidia is probably due to a lack of specific differentiated cell and tissue types from fish (Monaghan et al. 2009). A variety of trials have been undertaken in this area of research. Short-term, primary cultures have been used to study the phagocytic and respiratory burst capabilities of phagocytes in response to fish microsporidia, and relatively long-term primary culture of salmonid leukocytes has been used to culture *Nucleospora salmonis* (Wongtavatchai et al. 1994, 1995, Desportes-Livage et al. 1996). Different fish cell lines were tested for their capacity to support the growth of fish microsporidia. The chinook salmon embryo cell line CHSE-214 supported the growth of spores of *Glugea* sp. initially, but development stopped after 48 h (Lores et al. 2003). *Pseudoloma neurophilia* spores were collected from infected zebrafish *Danio rerio* and inoculated onto cultures of channel catfish ovary (CCO), zebrafish caudal fin fibroblast (SJD1), epithelioma papulosum cyprini (EPC), and fathead minnow epithelial (FHM) cell lines, which were then incubated at 28°C. Aggregates of 8 or more spores per cell were observed but further development did not occur (Watral et al. 2007). The most promising *in vitro* fish microsporidia system utilizes the eel *Anguilla japonica* epithelial cell line (EP-1). This line was developed from infected tissues of eel elvers and can be infected with *Pleistophora anguillarum* (now *Heterosporis anguillarum*) (Kou et al. 1995, Lom et al. 2000, Monaghan et al. 2009).

Kumar et al. (2013) demonstrated an *in vitro* culture model for *Heterosporis saurida* in the mammalian cell line RK-13. Our findings suggest that mammals may act as a reservoir or incidental hosts of fish microsporidia, and that the fish microsporidia could be transmitted from fish to mammals, which further demonstrates the low host specificity in cell culture. However, the relationships between human and fish microsporidia require further investigations, which would benefit greatly from *in vitro* microsporidia cell culture systems to facilitate molecular and immunological studies (Didier et al. 1994, 1996, Deplazes et al. 1996, Hollister et al. 1996, Croppo et al. 1997, Didier 1997, Mathis et al. 1997, Franzen & Müller 2001, del Aguila et al. 2001). In addition, chemotherapy testing and development for microsporidiosis would be facilitated by the establishment of such cell culture models (Didier 1997).

Ultrastructural examination of EK-1 infected cells showed that the structure of *Heterosporis saurida* was similar to the parasite in naturally infected lizardfish (Kumar et al. 2013). Except for the anterior end where the central zone of the anchoring disc contacted the wall, the spore wall was thick and consisted of an electron-lucent endospore and a thin electron-dense exospore (Fig. 2B) (Al-Quraishy et al. 2012). All developmental stages of *H. saurida* were observed in the EK-1 cells. Additionally, *H. saurida* spores were

detected in EK-1 cells using anti-microsporidia polyclonal antibodies. The identity of the microsporidian spores was confirmed by PCR amplification and sequencing of the amplicons.

In this study, we have introduced a system for *in vitro* growth of *Heterosporis saurida* that allows research on this pathogen for the first time in a fish cell line, thereby expanding the fish cell line panel available for *in vitro* cultivation of microsporidia. Investigating cellular and molecular factors determining the interactions between *H. saurida* and fish cells on a cellular level will now be possible. Our cell culture model provides a cellular tool for analyzing this pathogen that allows rapid development of, and increase in, molecular knowledge on this fish pathogen, eventually facilitating the development of novel treatment methods.

Acknowledgements. We thank O. Haenen, National Reference Laboratory of Fish and Shellfish Diseases of the Netherlands, M. Glösmann (VetCORE) for providing help taking the confocal images, and M. W. Stock for technical assistance. This work was funded by King Saud University, National Program for Science and Technology (11-BIO-1899-02).

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Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: July 4, 2013; Accepted: October 22, 2013
Proofs received from author(s): January 10, 2014