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OCCURRENCE OF A MYXOZOAN PARASITE *MYXIDIUM STREISINGERI* N. SP. IN LABORATORY ZEBRAFISH *DANIO RERIO*

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Abstract

Over several years of screening diagnostic cases, the Zebrafish International Resource Center Health Services have encountered a myxozoan parasite of the ducts associated with the kidney in zebrafish *Danio rerio* from an average of 21% of facilities submitting specimens over 5 yr. The parasite is coelozoic and is associated with no appreciable histological changes. Plasmodia bear ovoid spores with 3 sutural ridges. Spores are consistent with the genus *Myxidium*, but are distinct from any known species, and are thus described as *Myxidium streisingeri* n. sp. Phylogenetically, this parasite is a member of the polyphyletic urinary bladder clade, which is consistent with the site of infection. The common occurrence of a myxozoan in this closed husbandry system is unexpected because these parasites are known to have complex life cycles, alternating between a vertebrate and invertebrate host. It may be that biofilters provide habitat for suitable invertebrate hosts or perhaps *M. streisingeri* n. sp. can be transmitted directly. Future control of this parasite in zebrafish research laboratories depends on a better understanding of this life cycle.

The use of zebrafish *Danio rerio*, as a model in biomedical research has greatly expanded in the last 20 yr. Studies focus on many topics including human disease (Phillips and Westerfield, 2014), toxicology (Dai et al., 2014), and behavior (Gerlai, 2014), to list a few recent examples. Hundreds of articles have been published based on zebrafish as a model for bacterial diseases, but only rarely have zebrafish been used as a study animal for parasitic infections (King and Cable, 2007; Coyne et al., 2011; Tysnes et al., 2012; Gratacap and Wheeler, 2014). Instead, biosecurity has been the focus, emphasizing identification and minimizing the impact of parasites in zebrafish used in research (Kent et al., 2009). These underlying infections may cause mortalities or confound experimental results and therefore it is important to recognize and minimize their impacts. Studies with immunological endpoints might be particularly impacted by a preexisting parasite infection. The Zebrafish

Disease Manual (Kent et al., 2012a) lists 6 diseases of laboratory zebrafish associated with parasites other than bacteria and viruses: velvet disease (*Piscinoodinium* sp.), white spot (*Ichthyophthirius multifiliis*), microsporidiosis (*Pseudoloma neurophilia* and *Pleistophora hyphessobryconis*), capillariasis (*Pseudocapillaria tomentosa*), and renal myxosporidiosis. In comparison, in a study of wild zebrafish, Smith et al. (2011) identified 23 species of parasite, including 11 species of digenean metacercaria, 1 cestode, and 2 myxosporeans, all of which likely require other hosts.

Laboratory zebrafish are typically housed on recirculating systems with a dedicated reverse osmosis water or straight dechlorinated city water, limiting the breadth of infectious diseases in general in these systems, and severely limiting any parasites that may require an intermediate host. Thus, pathogens of the laboratory zebrafish are limited by controlled husbandry settings, minimal exposure, and lack of intermediate hosts and reservoirs. Indeed, 5 of 6 of the parasites of laboratory zebrafish mentioned above can be transmitted directly, with the sixth species, a myxozoan, having an unknown life cycle.

Over a 5 year span (2006-2010), the zebrafish diagnostic service has encountered renal myxosporidiosis in diagnostic cases from an average 21% facilities submitting specimens per year (Kent et al. 2012a), and these infections have been noted by other authors as well (Harper and Lawrence, 2011; Kent et al., 2012b). Myxozoans are complex multicellular organisms that parasitize mostly fish, but also amphibians, reptiles, birds and mammals (Lom and Dyková, 2006). Most of our understanding of myxosporean parasitism focuses on a handful of pathogenic species (Kent et al., 2001), but well over 2,000 species have been described, with Lom and Dyková estimating 2,180 in 2006, and likely more than 100 others described since then. The mode of transmission of the zebrafish myxozoan is not known. However, conventional wisdom suggests that myxozoans have complex life cycles involving a vertebrate and invertebrate host, thus both hosts would need to be present, making this finding of a myxozoan in a controlled setting such as a zebrafish facility unexpected. Invertebrates such as oligochaetes have been observed in zebrafish systems (Kent et al., 2012a). Alternatively, this particular species could possibly be transmitted directly as reported for *Enteromyxum leei* in sea bream *Sparus aurata* (see Diamant, 1997).

The zebrafish myxozoan occurs in the mesonephric ducts and the lumen of the kidney tubules (Kent et al., 2012a). Holzer et al. (2004) first noted an affinity of freshwater myxozoan species from the urinary system regardless of spore morphology and this was later described as the freshwater “urinary bladder clade” by Fiala (2006). Subsequent additions of other species from distinct geographic localities supports the affinities of myxozoans for the urinary system of freshwater fishes (Whipps, 2011). Thus, there is some consistency in tissue specificity within lineages of myxosporeans regardless of spore type. Given this general trend, we expect the zebrafish myxosporean to also fall within this lineage. Here we describe this novel myxozoan and discuss its distribution in zebrafish facilities.

MATERIALS AND METHODS

For histology, fish were preserved whole. Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222), an incision was made in the abdomen, and the fish were preserved for at least 72 hr in Dietrich's fixative. Fish were then processed for histology, embedded in paraffin, and sectioned along sagittal planes. Sections were stained with either hematoxylin and eosin or Giemsa.

For wetmount preparations, zebrafish from a laboratory in Baltimore Maryland were shipped alive to the SUNY-ESF Fish Disease Laboratory in Syracuse, New York. Fish were held in aquaria until they could be examined for parasites (approximately 1 day to 2 wk). Fish were euthanized with 250 mg/L of tricaine methanesulfonate (Argent Laboratories, Redmond, Washington), buffered with sodium bicarbonate. Zebrafish mesonephric ducts were removed and placed on a microscope slide in a drop of saline and compressed under a cover slip. Squash preparations were examined with a Nikon 80i compound microscope (Nikon Instruments Inc., Melville, New York) with Nomarski and phase objectives. Images were captured with the 3Mp IDEA digital camera and analyzed with photomicrography software (Diagnostic Instruments Inc., Spot RT Software 4.6 Sterling Heights, Michigan). Measurements were made for each relevant spore dimension following the guidelines of Lom and Arthur (1989). The number of polar filament coils was noted where possible.

Infected mesonephric ducts were recovered from microscope slides and processed for DNA extraction. Tissues from 3 hosts were used for DNA extraction with the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions (Qiagen Inc., Valencia, California). PCR was performed in 50 μ L reaction volumes in Quick-Load® Taq 2X Master Mix (New England Biolabs, Ipswich, Massachusetts), 0.5 μ M of each primer and 3 μ L of template DNA. A first round amplification targeting the small subunit ribosomal DNA (SSU rDNA) used primers 18E and 18R (Whipps et al., 2003), followed by a second round of PCR with 18E and MyxospecR (Fiala, 2006) or Myxgen3F (Kent et al., 2000) and 18R. Amplifications were performed on a C1000™ Thermal Cycler (BioRad Laboratories, Hercules, California) with initial denaturation at 95 C for 3 min, followed by 35 cycles of 94 C for 30 sec, 56 C for 45 sec, 68 C for 90 sec, and a final extension at 72 C for 7 min. Product amplification was evaluated by observation on a 1% agarose gel and the remainder of the sample purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia). DNA was quantified using a DNA spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Direct sequencing was performed using the primers 18E and MyxospecR. In the 3 prime region of the SSU, direct sequencing yielded data (ambiguous base calls, indels) suggesting distinct copies of the SSU were present in the sample, so the amplified product (Myxgen3F/18R) from one infected host was cloned using the QIAGEN PCR Cloning Plus kit (Qiagen Inc.) and 5 clones were sequenced with plasmid primers T7 and SP6. Sequencing reactions were carried out with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, California).

Sequences were assembled in BioEdit (Hall, 1999) and verified as myxozoan by GenBank BLAST search. To evaluate the phylogenetic relationships of the novel species, an

alignment was created with Clustal X version 1.8 (Thompson et al., 1997) with default parameters (penalty for gap opening = 10, gap extension = 0.2), using sequences from BLAST results. Outgroups were selected based on earlier analyses (Whipps, 2011). Parsimony analyses (MP) were conducted in PAUP*4.01 (Swofford, 1998) using a heuristic search algorithm with 10 random additions of sequences and tree bisection-reconnection (TBR) branch swapping. Bootstrap values were calculated with 100 replicates using a heuristic search algorithm with simple sequence addition and TBR branch swapping. Trees were initially examined in TreeView X (Page, 1996) and edited and annotated in Adobe Illustrator (Adobe Systems Inc., San Jose, California).

DESCRIPTION

Myxidium streisingeri n. sp

(Figs. 1, 2, 3)

Diagnosis—Small disporous plasmodia (mean 13.6 μm , range 5.3 – 15.4 μm) coelozoic in the collecting ducts and mesonephric ducts. Majority of plasmodia with presporogonic stages, rarely mature spores. Mature spores ovoid in valvular view. Three ridges observed in sutural view, straight to slightly sinuous. Polar capsules round, oriented to opposite ends of spore with polar filament coiled 4, rarely 5 times. Mean spore dimensions in micrometers with number of measurements, standard error, and range in parentheses as follows: spore length 8.3 (n=19, ± 0.12 , 7.4-9.3); spore width 5.2 (n=16, ± 0.08 , 4.5-5.6); thickness 4.2 (n=3, ± 0.4 , 3.6-4.9); polar capsule diameter 3.0 (n=23, ± 0.05 , 2.5-3.5).

Taxonomic summary

Type host—*Danio rerio* (Hamilton, 1822) (Teleostei, Cyprinidae).

Site of infection—Coelozoic in collecting ducts of kidney and mesonephric ducts.

Prevalence—13 of 19 (68%) fish examined by wetmounts, 6 of 14 by histology.

Locality—Baltimore, Maryland. It is worthwhile noting that as a parasite of laboratory fishes, this potentially occurs in any laboratory that houses zebrafish, and likewise could go locally extinct in any of these facilities.

Specimens deposited—Deposited at the Harold W. Manter Laboratory collection (HWML), University of Nebraska State Museum, Lincoln, Nebraska. Zebrafish tissue sections stained with Giemsa (HWML 75041) and H&E (HWML 75040). GenBank accession numbers for partial SSU rDNA sequences from 5 unique clones: KM001684-KM001688.

Etymology—Named in honor of George Streisinger, formerly at the University of Oregon, a pioneer in the use of zebrafish as a model organism.

Remarks

The myxozoan encountered here bears spores consistent with species of the genus *Myxidium*. That is, polar capsules are located at opposite ends of the spore and oriented outward, with the sporoplasm between them, and the suture runs longitudinally. In the synopsis by Eiras et al. (2011), only 15 of the 232 nominal *Myxidium* species bear spores with any overlap in length and width to *M. streisingeri* n. sp. and none look superficially similar to our species, having either many more valve ridges or a tapered spore. To the best of our knowledge, the only species that bears superficial similarities to *Myxidium streisingeri* n. sp. is *Zschokkella costata* Kaschkowky 1965, which infects *Rutilus rutilus* in Russia. Shulman (1966) provides a description of this species and line drawings, citing spores slightly longer (9.0-10.5 µm) and wider (5.0-6.5µm) than *M. streisingeri* n. sp. Furthermore, *Z. costata* has 6 longitudinal ridges versus the 3 we observed for *M. streisingeri* n. sp. In both species, the polar capsules are found at opposite ends of the spore, oriented toward the poles or slightly away from the poles at opposite sides of the spore. Phylogenetic analysis yielded a single most parsimonious tree (Fig. 4). *Myxidium streisingeri* n. sp. is sister to a clade of species that infect the urinary bladder, kidney ducts, or kidney, including *Acauda hoffmani* (renal tubules), *Chloromyxum* sp. (renal tubules), *Hoferellus gilsoni* (urinary bladder), *Myxidium giardi* (kidney), *Myxobilatus giardi* (renal tubules), *Ortholinea orientalis* (kidney ducts), and *Zschokkella* sp. (urinary bladder).

DISCUSSION

Myxidium streisingeri n. sp. was observed in 12-30% of facilities submitting research cases to the Zebrafish International Resource Center (ZIRC) between 2006 – 2010 (Kent et al., 2012a). This represented a range of 3-10 facilities that submitted to ZIRC having an infection on a given year between 2006-2010. This high prevalence may at first appear unusual as essentially all myxozoans with known life cycles require an invertebrate alternate host, and most zebrafish research facilities use pathogen-free water. Many laboratories still purchase zebrafish directly from pet stores, and these fish are often reared in ponds. This provides a logical original source of the infection to zebrafish laboratories. However, this lack of biosecurity alone doesn't completely explain our observations. For example, if introductions from fish in outdoor ponds are so common, one might expect more than 1 myxozoan species and perhaps many other parasites in addition to myxozoans. Furthermore, we have detected the infection in several populations that were reared from eggs in these facilities, indicating that the life cycle of this myxozoan is being perpetuated in these facilities.

A hallmark discovery in the study of myxozoans was the life cycle of *Myxobolus cerebralis* by Wolf and Markiw (1984) who showed this species must cycle between the vertebrate fish host and an invertebrate annelid host. Similar life cycles are reported for other myxozoans and it is reasonable to suspect *M. streisingeri* n. sp. also uses an annelid second host. Zebrafish laboratories are generally maintained as large, recirculating units with biological filters. These nutrient rich filters provide an excellent environment for a variety of invertebrates, with bryozoan and oligochaete worms commonly found in these systems (Kent et al., 2012a). Whereas we have not identified the alternate host for *M. streisingeri* n.

sp., it is likely an oligochaete, based on our knowledge of the life cycles for other freshwater myxozoans (Atkinson et al., 2007). Alternatively, *M. streisingeri* n. sp. could be transmitted directly, as has been reported for *E. leei* (see Diamant, 1997), but this is unusual for myxozoans.

As with most coelozoic myxozoans, *M. streisingeri* n. sp. is confined to the lumen (Figs. 1A, 3A) and is not associated with appreciable histopathological changes, even in heavy infections. As with other laboratory animals, these infections should still be considered as an underlying cause of non-protocol induced variation in research (Kent et al., 2012b). The specific site of infection was coelozoic in the renal system and this is phylogenetically consistent with other members of what are considered the urinary bladder clade defined by Fiala (2006). This group is made up of representatives from several genera (Fig. 4), some of which, like *Chloromyxum*, *Myxidium*, and *Zschokkella* have many more representatives elsewhere in the myxozoan tree of life (Bartosová et al., 2009; Whipps, 2011; Heiniger and Adlard, 2014).

It would seem that the ultimate spore type for these urinary system myxozoans is plastic, the group having no apparent synapomorphies. With regard to *M. streisingeri* n. sp., the spores are consistent with the genus *Myxidium*, having polar capsules oriented in opposite directions. The diminutive, elliptical spores with 3 valve striations are less consistent, but as Heiniger and Adlard (2014) point out, there are many ambiguous characters within *Myxidium*. Superficially, *M. streisingeri* n. sp. is similar to *Z. costata*, both having similar shaped spores and valvular striations. It is worthwhile pointing out that neither *Myxidium* nor *Zschokkella* is monophyletic, and that the distinction between the 2 genera is somewhat ambiguous (Diamant et al., 1994; Heiniger and Adlard, 2014). Synonymy of these genera would do little to resolve this problem, as this new group would remain paraphyletic. Given these issues, *M. streisingeri* n. sp. is aptly placed in the genus *Myxidium* until additional characters are found or a major taxonomic revision can be proposed.

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Figure 1. Plasmodia and spores of *Myxidium streisingeri* n. sp. in wet mount. Nomarski. (A) Mesonephritic duct with numerous plasmodia. (B) Plasmodium attached to wall of mesonephritic duct. (C) Plasmodium with sporogonic stages. (D) Valvular view of spore with sporoplasm distinct between polar capsules. (E) Valvular view of spore. (F) Sutural view of spore. (G) Same spore in showing 3 sutural ridges. (H) Two spores at different angles, showing sutural ridges and valvular surface. Bar = 5 µm.

Figure 2.
Line drawing of *Myxidium streisingeri* n. sp. (A) Valvular view. (B) Sutural view. Bar = 5 μm.

Figure 3.

Histological sections of zebrafish infected with *Myxidium streisingeri* n. sp. (A) Plasmodia in mesonephric duct. (B) Plasmodia attached to duct wall. (C) Spore within plasmodium showing orientation of polar capsule toward pole of spore (arrow). (D) Giemsa stained section showing spore with distinct sutural ridges (arrowhead). Bar = 10 μm.

Figure 4.

Phylogenetic tree generated from parsimony analysis of small subunit ribosomal RNA gene sequences for *Myxidium streisingeri* n. sp. Genbank accession numbers are listed adjacent to species names. Support values in percent units at branching points, values under 50% not shown.