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CASE REPORT

Intranuclear inclusions consistent with a *Nucleospora* sp. in a lymphoid lesion in a laboratory zebrafish, *Danio rerio* (Hamilton 1822)

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1 | INTRODUCTION

The zebrafish is an important model for biomedical research. Compared to other fish species, our understanding of diseases affecting zebrafish is rather new, but rapidly expanding. The potential for background diseases to compromise research endpoints has been well documented in rodents and is receiving increasing attention in zebrafish (Kent, Harper, & Wolf, 2012). The Zebrafish International Resource Center (ZIRC) offers a diagnostic pathology service for zebrafish in collaboration with the Oregon Veterinary Diagnostic Lab (OVDL). Over the past 20 years, the most frequently diagnosed pathogen of laboratory zebrafish is the microsporidian parasite *Pseudoloma neurophilia*, which causes reduced fecundity, weight loss and altered behaviour (Kent, Sanders, Spagnoli, Al-Samarrie, & Murray, 2020; Midttun, Vindas, Nadlers, Øverli, & Johansen, 2020). A second microsporidium, *Pleistophora hyphessobryconis*, which was well-described in other species as the agent responsible for Neon Tetra Disease, was first recognized in zebrafish in 2014 (Sanders et al., 2010). Here, we describe lesions and structures suggestive of a *Nucleospora* sp. in a laboratory zebrafish. *Nucleospora* species are intranuclear microsporidia that infect the nuclei of proliferating lymphoid cells in salmon and other fishes (Kent, Shaw, & Sanders, 2014) and histological sections revealed an infection consistent with these descriptions.

2 | BACKGROUND

This case report is from a submission from the University of Utah Centralized Zebrafish Animal Resource (CZAR). Zebrafish (*Danio rerio*) were maintained in accordance with approved institutional protocols under the supervision of the Institutional Animal Care and Use Committee (IACUC) of the University of Utah, which is fully accredited by the AAALAC. Description of this facility and health and biosecurity protocols are described by Hobbs, Shankaran, and James (2016). This facility is typical of a large, centralized facility with recirculating systems. The facility supports research for 20 investigators, 150 users and has 110 racks (another 19 at a second location on campus), and about 6,000 tanks. The facility has five separate recirculating systems, but fish are interchanged between systems. Water system parameters are standard for zebrafish in laboratories (Cockington, 2020; Hobbs et al., 2016, Hammer 2020). Water in tanks are maintained at approximately 28°C, 550 µS/cm conductivity and at pH 7.2. Approximately 10% of system water is replaced daily with RO water and automatically adjusted with Instant Ocean Sea Salts (Blacksburg, VA) and sodium bicarbonate (Pentair) (Minneapolis, MN). Recirculating water is filtered and UV treated before returning to tanks. Depending on the age of fish, they are fed commercial diets (e.g. Aquatox, Gemma or Zeigler). Live feeds, brine

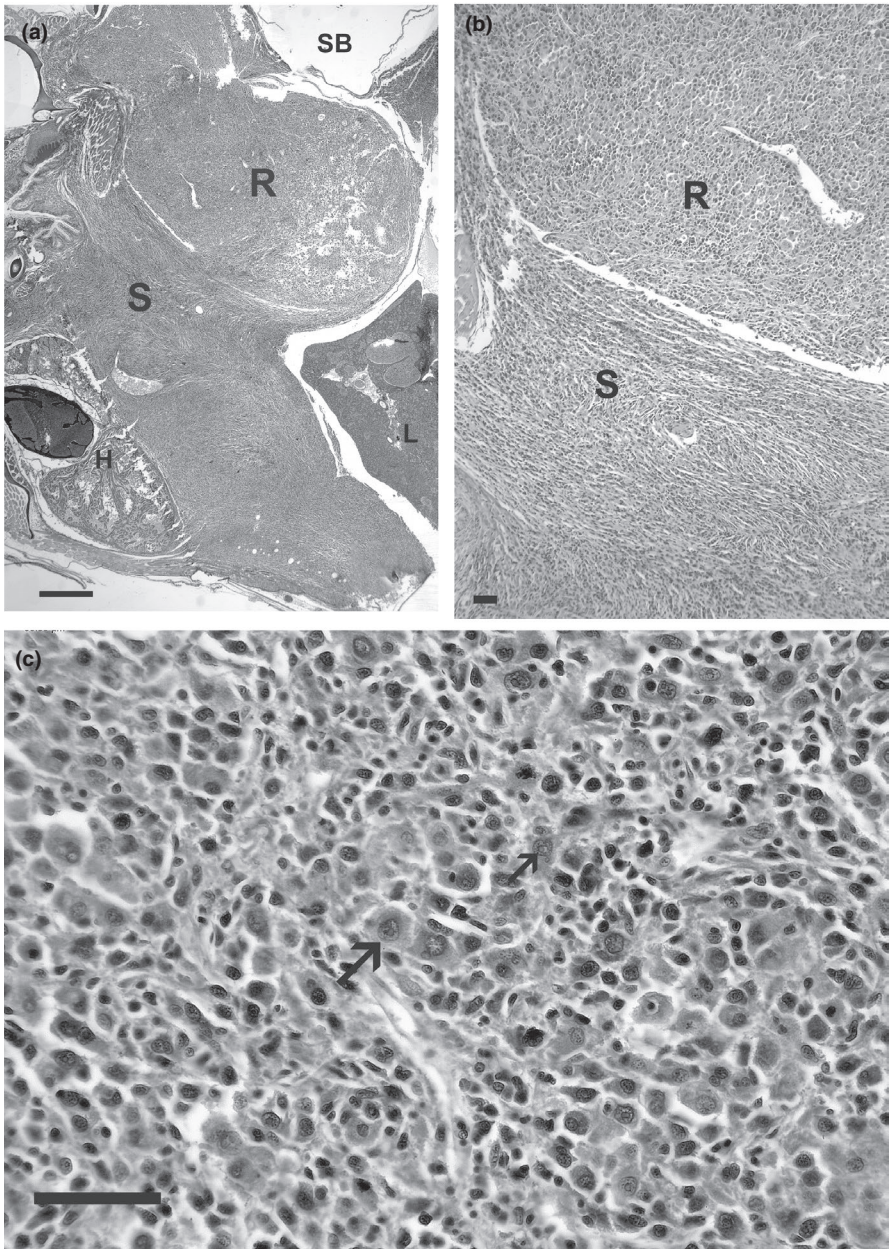

FIGURE 1 Lymphoid neoplasm in anterior coelom of a zebrafish. Haematoxylin and eosin. (a) Low magnification showing two major regions of tumour; spindle (S) and round (R) cells. H = heart, SB = swim bladder. L = liver. Bar = 0.5 mm. (b) Higher magnification of two regions of tumour. Bar = 50 μm. (c) ×400 of round cell region, with intranuclear inclusions (arrows). Bar = 10 μm

shrimp nauplii hatched in house and saltwater rotifers (*Brachionus plicatilis*) from Reed Mariculture, Campbell, California, are fed to larval and juvenile fish.

New lines of fish are introduced through a quarantine system in which only chlorine, and then, Ovadine (Western Chemical, Inc.) treated embryos may come out of the quarantine room and go into the main facility nursery. Embryos are treated at 24 hr post-fertilization (hpf) with 30 ppm chlorine twice for 5 min each, with a 5-min embryo water (E3) rinse in between, followed by three additional rinses in E3 for 5 min each. Embryos are then held overnight in E3. At 48 hpf, embryos are treated for 2 min in 12 ppm Ovadine followed by two rinses in E3 for 2 min each, then a final 5-min rinse in E3. Health monitoring entails sentinel fish receiving effluent water from individual systems as well as examination of moribund fish. Most zebrafish facilities maintain only zebrafish, but occasionally other species are maintained. At CZAR, in 2014, an investigator imported *Danionella translucida* from a public aquarium, and some of these fish were held in the CZAR quarantine facility. Embryos were ultimately treated with Ovadine, and eventually, they were transferred from quarantine into the main facility.

In 2019, we evaluated 130 fish from five separate systems in the facility by histopathology through the ZIRC/OVDL diagnostic programme. Fish represented various lines, including *AB, TL, TU and WIK, and included both sentinel fish and those exhibiting clinical signs. Ages ranged from around 2 months to over 2 years. Fish were preserved in Dietrich's fixative and process into midsagittal histologic sections using our standard procedure (Kent & Sanders, 2020). They exhibited a variety of "typical" diseases and pathogens found in zebrafish (Kent & Sanders, 2020), including spermatic seminomas, ultimobranchial adenomas, egg-associated inflammation and

fibroplasia, non-specific gill hyperplasia, coelomic spindle cell sarcomas, *Myxidium streisingeri*, mycobacteriosis, *Pseudoloma neurophila*, hepatic megalocytosis and nephrocalcinosis.

2.1 | Lymphoproliferation with intranuclear inclusions—*Nucleospora* sp.?

One adult female fish collected on 17 June 2019 from the CZAR System 4 presented with a distended abdomen with multiple lumps. The age and strain of this fish were unknown. Histopathology revealed a large neoplasm (about 5 mm diameter) within the cranial aspect of the coelom (Figure 1). It was just caudal to the pharynx, surrounding the caudal aspect of the heart and infiltrating into the surrounding skeletal muscle. The neoplasm was composed primarily of short, intersecting ribbons and bundles of spindle-shaped cells admixed with a round cell population composed primarily of lymphocytes (Figure 2). Spindle-shaped cells had distinct cell margins and a moderate amount of lightly basophilic cytoplasm. Nuclei were ovoid to spindle-shaped with densely packed chromatin and had inconspicuous nucleoli. Within the centre of the mass, the spindle cell and lymphocyte population transitioned into sheets of large round cells with foamy, lightly eosinophilic cytoplasm and distinct cell margins. Within this round cell population, mitotic figures were frequent (approximately 2–3 mitotic figures per 400× magnification field). There was also severe nuclear and cellular pleomorphism with frequent karyomegaly and multinucleate cells (Figure 2). Admixed with these round cells were large numbers of lymphocytes, plasma cells and macrophages. Nuclei of the round cells were generally round with coarsely stippled chromatin. Frequently, these nuclei contained a central, well-demarcated, nearly perfectly circular structure that displaced adjacent chromatin and had an amphophilic, glassy appearance. These structures were usually 1–2 μm, but occasionally as large as 4 μm in diameter. There was no evidence of *P. neurophilia* or *P. hyphessobryconis* in central nervous system, skeletal muscle or other tissues. None of the other numerous fish examined by histology from this facility showed inclusions as described in this fish.

2.2 | Special stains

These nuclear inclusions were suggestive of *Nucleospora salmonis* (Elston, Kent, & Harrell, 1987; Morrison, MacConnell, Chapman, & Westgard, 1990). Hence, two special stains that differentiate microsporidia were applied. For light microscopy, additional sections were stained with either Luna or Warthin–Starry as these stains highlight microspordian spores (Kent, Rantis, Bagshaw, & Dawe, 1995; Peterson, Spitsbergen, Feist, & Kent, 2011). Consistent with microsporidia, inclusions stained red with Luna and black with Warthin-Starry (Figure 2b,c).

Microsporidia contain chitin, and hence, fluorescent stains for chitin are commonly used to highlight their spores. Sections were, therefore, also stained with Fungi-Fluor® (Polysciences) as described by Kent and Bishop-Stewart (2003) and examined with a fluorescent microscope using a DAPI filter. Most of the inclusions showed increased fluorescence, suggesting the presence of chitin in presporogonic forms. Strongly fluorescing structures consistent with microsporidian spores (about 1–2 μm in length) were observed throughout the tumour region (Figure 2), but were rare.

2.3 | Electron microscopy

Microsporidian spores, particularly very small ones such as *Nucleospora* spp., are difficult to discern by light microscopy.

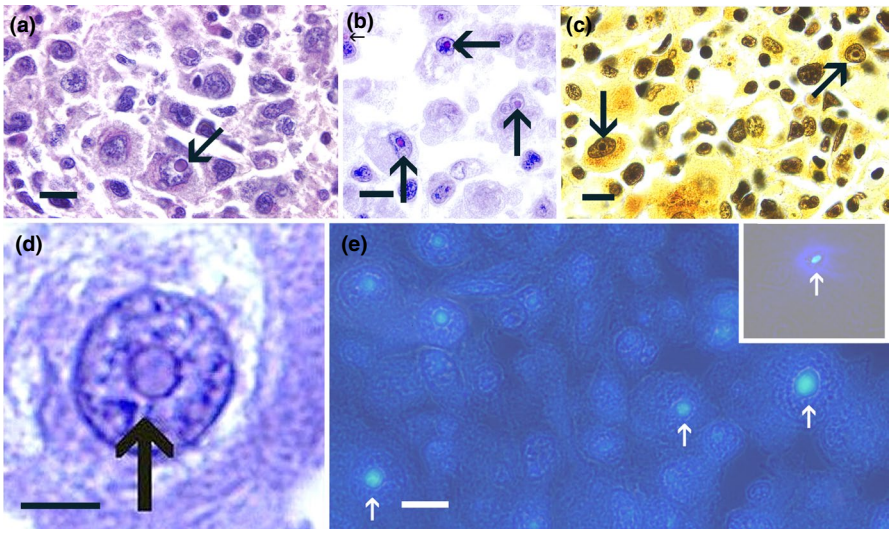

FIGURE 2 High magnification of intranuclear inclusions in round cell region of zebrafish tumour. (a) Arrows = inclusions. H&E. Bar = 10 μm. (b) Red-staining inclusions (arrows) with Luna stain. Bar = 10 μm. (c) Black staining inclusions (arrows) with Warthin–Starry. Bar = 10 μm. (d) High magnification of inclusion. Note nearly perfectly circular structure that displaces adjacent chromatin and has an amphophilic, glassy appearance. Bar = 5 μm. (e) Fungi-Fluor stain, with numerous fluorescing intranuclear inclusions. Insert shows highly fluorescent putative spore. Bar = 10 μm

Therefore, sections we also examined with Correlative Light Electron Microscopy (CLEM), a useful technique to examine structures observed in histological slides with transmission electron microscopy (Dobbie, 2019). One of us (L. Weiss) routinely employs this technique at his institution (Einstein Analytical Imaging Facility) following the general protocol used at their laboratory. The areas of interests are located by observation of sections stained with H&E and then matched with regions on unstained slides. Matching areas on the unstained paraffin slides were marked with a diamond scribe. The slides were deparaffinized, rehydrated and processed for EM as follows. Sample was fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide in buffer, and en bloc stained with 2% aqueous uranyl acetate, dehydrated through a graded series of ethanol and embedded in Lx112 resin using an inverted BEEM capsule over the area of interest. After polymerization, the BEEM capsule was popped off the slide using gentle heating and ultrathin (70 nm) sections of the scribed area were cut on a Leica EM Ultracut UC7, stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1400 Plus transmission electron microscope. Images were collected using Digital Micrograph software and a Gatan Orius camera.

Electron dense structures corresponding to the inclusions seen in the nuclei as described above were observed in the proliferating cells (Figure 3). The specimen was preserved in Dietrich's, which contains acid, and overall quality of both host cells and putative parasites was suboptimal. Nevertheless, the inclusions were consistent with *Nucleospora* sp.; development stages were comparable to *Nucleospora* species; figure 4 in Elston et al. (1987), figure 8 in Mullins, Powell, Speare, and Cawthorn (1994) and figure 8 in Chilmonczyk, Cox, and Hedrick (1991). One or two dark structures consistent with parasite nuclei were observed, and the regions corresponding to parasite cytoplasm usually contained numerous electron lucent inclusions.

2.4 | Molecular identification

An 18 gauge needle was used to obtain a core specimen from the paraffin block within the infected lesion. We have used this technique previously to target mycobacteria in focal lesions for PCR (Meritet, Mulrooney Kent, & Loehr, 2017) with about 50% success (Mason et al., 2016). The specimen was deparaffined and transferred to alcohol, and PCR amplification of rDNA was attempted using two sets of universal microsporidian primers, 530F/1492R and 530F/580R (Gosh & Weiss, 2009). These primers are routinely used to obtain sequence of novel microsporidia, including members of the Enterocytozooidae (Diamant et al., 2014; Freeman & Sommerville, 2009; Jones, Prosperi-porta, & Kim, 2012). Unfortunately, no product was amplified with either primer sets.

Additional fish were examined from this tank, and only one fish exhibited haemopoietic neoplasm/lymphosarcoma throughout coelom and infiltrating kidney. Similar to the fish with putative *Nucleospora*, the proliferating cells were large and blast-like, but did not contained structures consistent with the intranuclear inclusions.

3 | DISCUSSION

Light microscopy with H&E and special stains were all consistent with a diagnosis of *Nucleospora*. The nuclear inclusions exhibited morphologic consistencies with other descriptions of presporogonic stages of *Nucleospora* spp. as described previously. With H&E, they appear as variable sized, essentially perfectly spherical inclusions with a distinct, dark staining margin (Elston et al., 1987; Morrison et al., 1990). Consistent with reports by Peterson et al. (2011), the inclusions stained red with Luna and were dark brown to black with Warthin–Starry stain (Kent et al., 1995). Application of Fungi-Fluor revealed occasional, highly fluorescent structures identified as mature spores. Interestingly, the round inclusions consistently exhibited fluorescence, but not as intense as the spores, suggesting the presence of more diffuse chitin in sporogonic forms. The pathologic presentation, with infections in the nuclei of immature, proliferating lymphoid cells suggestive of neoplasia as seen with *N. salmonis* (Morrison et al., 1990) also supports our diagnosis. Dietrich's fixative, or similar fixatives with acid and alcohol added to formalin, is recommend for small fish histology (Kent et al., 2020; Wolf et al., 2015), but they are less optimal for electron microscopy. Nevertheless, morphology was adequate to demonstrate that the inclusions were consistent with presporogonic stags of *Nucleospora* sp. as noted above, in which multiple nuclei could be discerned within the inclusions. Even more definitive diagnosis would have been achieved with visualization of fully formed spores by CLEM, but as demonstrated with the Fungi-Fluor® stain, these stages were very rare. More confirmatory diagnosis of *Nucleospora* sp., or a related microsporidium, could also have been achieved with rDNA sequence. While this can be accomplished, it is often difficult to amplify DNA

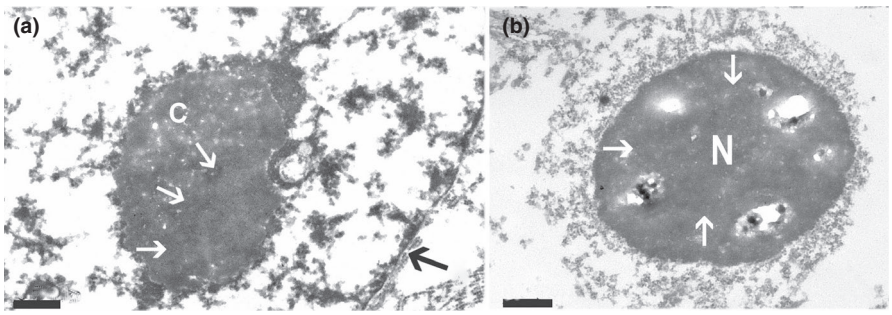

FIGURE 3 Correlative Light Electron Microscopy of inclusions. (a) Putative parasite within host cell nucleus. C = vacuolated parasite cytoplasm. White arrows demark parasite nucleus. Black arrow = host cell nuclear membrane. (b) Parasite with central nucleus (N). White arrows demark parasite nucleus Bar = 0.5 μm

from formalin-fixed paraffin-embedded (FFPE) specimens. There are a few reports of obtaining microsporidian rDNA from FFPE tissues (Gosh & Weiss, 2009; Tosoni et al., 2002), but often, as seen here, this approach is unsuccessful (Cali et al., 1998). The previous studies used tissues initially preserved in 10% formalin, whereas our specimen was preserved in Dietrich's solution. The very low pH of this preservative (which contains 2% glacial acidic acid) likely caused profound DNA degradation as the specimen was held in Dietrich's for many days before processing.

The source of the infection in this facility is unknown, particularly because the laboratory has a rigorous quarantine procedure, and only introduces chlorine disinfected embryos into its main facility (Hobbs et al., 2016). Nevertheless, there are potentials for breaches in biosecurity resulting in introduction of pathogens. *Nucleospora salmonis* was the first recognized species in the genus, but since then members of the genus have been described in several other fresh and seawater fishes. Pertinent to this report, *Nucleospora* spp. have been reported in Nile tilapia (*Oreochromis niloticus*) from fish farms (Rodrigues, Francisco, David, da Silva, & Júnior, 2017) and killifish *Nothobranchius rubripinnis* from the pet fish trade (Lom & Dyková, 2002). Many researchers still acquire zebrafish from the pet store trade, and there is a record of introduction of *Danionella translucida* from a public aquarium into this facility, but this was over 5 years ago. Live feeds are another potential source of the infection. The possibility of an aquatic arthropod as the source might also be considered as members of the Enterocytozooidae infect crustaceans, including *Desmozoon lepeophtheri/Paranucleospora theridion* which infects both fish and the parasitic copepod *Lepeophtheirus salmonis* (Freeman & Sommerville, 2009; Nylund, Nylund, Watanabe, Arnesen, & Karlsbakk, 2010. Moreover, whereas only chlorine or iodine-treated embryos are allowed into the facility, microsporidian spores have be shown to be quite resistant to both of these disinfectants (Ferguson, Watral, Schwindt, & Kent, 2007; Shaw, Kent, & Adamson, 1999). It would be expected that few pathogens would occur in laboratory zebrafish, particularly considering that they are reared in water that starts as pathogen-free. However, most of these facilities support multiple investigators that may bring in fish from various sources, including pet stores (Kent & Sanders, 2020), and at times, they may override or ignore biosecurity protocols set forth by attending veterinarians and laboratory managers. These all provide possible explanations for introduction of two serious bacterial pathogens into main facilities that had rigorous protocols for quarantine and fish introductions (Hawk et al., 2013; Mason et al., 2016).

In conclusion, examination of multiple fish with a given condition is desired, but we only found the inclusions in one fish. Therefore, here we demonstrate our approach to confirm a diagnosis of a novel finding with just one fish in a paraffin block; starting with histopathology, then adding special stains and electron microscopy on the same tissue. Here, we used CLEM, which allows for precise targeting of pathogens or lesions seen by histology for electron microscopy. The integrity of tissues at this level is compromised by preservation in formalin, and even worse with Dietrich's solution, but the advantage is that very small, localized structures can be captured for electron microscopy, and we have recently used this approach with other cases.

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CONFLICT OF INTERESTS

There is no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All data are included in the present manuscript.

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