

Detection of the microsporidian *Nucleospora cyclopteri* in tissue samples from lumpfish (*Cyclopterus lumpus*) using *in situ* hybridization (ISH)

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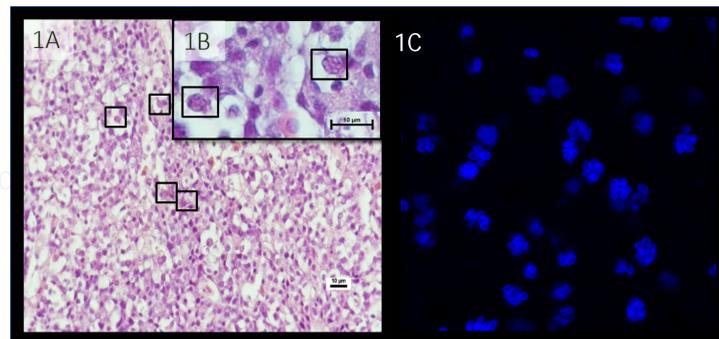
Lumpfish (*Cyclopterus lumpus*) is increasingly used as a cleaner fish to control infections of salmon lice in the Norwegian aquaculture industry. However, lumpfish is itself infected by a number of pathogens with mostly unknown impact on the fish health. One of these is the microsporidian parasite *Nucleospora cyclopteri* that contributes to mortality in both wild and farmed lumpfish. This parasite develops within the nuclei of leucocytes and induces a systemic infection with swollen kidneys (renomegaly) (Figure 1) as the most prominent macroscopical sign. To enable detailed studies of the distribution of pathogens, their localization and impact in various tissue types, methods that are both highly sensitive and specific for the particular pathogen are needed. Traditional staining methods, such as Hematoxylin-Eosin (HE), Gram-Twort and Calco-Fluor-White (CFW) are able to stain microsporidian spores. However, pre-sporogonial stages that dominate in many samples are not stained by these methods. In addition, most traditional staining methods are non-specific and stain bacteria and fungi as well as microsporidia.

Detection of *Nucleospora cyclopteri* in routine diagnostics



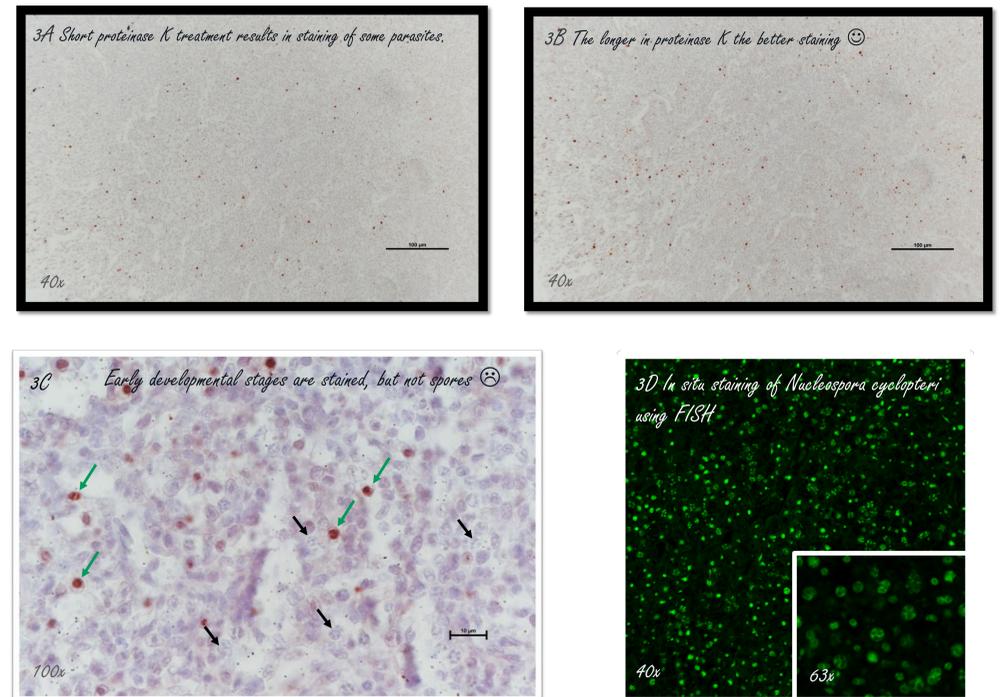
Figure 1. Dissection of lumpfish showing swollen kidney, renomegaly, and nodules indicating infection with *Nucleospora cyclopteri*.

Figure 2. Microsporidia are tiny parasites, difficult to detect by routine examination of tissues stained with HE, at 40X magnification (Fig 1A). Even at 100x magnification (Fig. 1B) they might be difficult to detect, and these analyses are time consuming. Staining with calco-fluor white (Fig. 1C) improves detection, but only stains spores (chitin wall) and is non-specific to the pathogen in question.

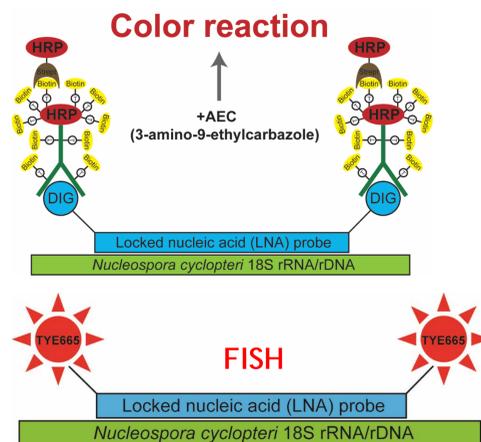


In situ hybridization- optimization and results

To facilitate penetration of the probe and thereby its contact with the DNA/RNA of the parasite, proteinase K -treatments of different duration were applied. A larger proportion of the parasites were stained at 60 min exposure compared to 30 minutes. At 2 and 3 hours the unspecific background stain increased. Figure 3 shows the difference in staining for the DIG-labelled probe after 30 mins (3A) and 60 mins (3B) exposure (both images at 40X magnification). With this probe, the method seem to specifically stain early developmental stages of *Nucleospora cyclopteri* (green arrows in figure 3C), while mature spores are stained to a lesser degree (black arrows in figure 3C). The application of a fluorescence probe (FISH) (fig 3D, 40X and E, 63x) improved staining considerable and this probe also seemed to result in staining of spores.



In situ hybridization method



- Two locked nucleic acid (LNA) modified oligonucleotide probes labelled with either digoxigenin (DIG) or the fluorescent dye TYE665 were designed to specifically target *Nucleospora cyclopteri* 18S rDNA/rRNA for *in situ* hybridization (ISH) and fluorescence *in situ* hybridization (FISH), respectively.
- Both probes were tested on paraffin-embedded tissue sections from parasite-positive lumpfish as determined by real-time PCR.
- Several parameters were varied, tested and optimized ISH/FISH protocols were developed.
- The infections were confirmed by light microscopy and compared with neighbouring sections stained with traditional staining methods.

Conclusions

- Specific *in situ* hybridization methods for the detection of *Nucleospora cyclopteri* have been developed.
- DIG-labelled probes were shown to stain mostly early developmental stages and not microsporidian spores, while the FISH probes also seemed to stain microsporidian spores, suggesting better permeability of the latter method over the streptavidin-biotin antibody-based DIG-system.

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The Norwegian Veterinary Institute's activities encompass the entire chain from plants, via animal feed, fish, animals and food for human consumption.

