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An ISH technique for the early detection of *Enterospora nucleophila*, an intranuclear microsporidian causing emaciative disease in gilthead sea bream

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Enterospora nucleophila is a microsporidian parasite causing serious emaciative disease in cultured gilthead sea bream (GSB), *Sparus aurata*, and closely related to *Enterocytozoon hepatopenaei* from shrimp. The parasite can be found within the nuclei of enterocytes and rodlet cells of the fish intestinal epithelium, and occasionally in the cytoplasm of phagocytes in more advanced infections. However, spores are the only stage that can be unmistakably identified but even this may require thorough histopathological examination and use of chitin fluorescent stains for a proper confirmatory diagnosis, due to their minute size and intranuclear location. In the absence of spores, the infection can be suspected from a remarkable hypercelullarity and the presence of altered nuclei in the epithelial layer. This results in a very poor correlation of disease signs with diagnosis of *E. nucleophila* infection, and to current uncertainty about its real impact in GSB culture.

This work describes the development and application of an *in situ* hybridization (ISH) technique as a powerful tool to overcome current diagnostic limitations for this species, and to decipher basic data on the infection and disease onset. We designed DIG-labelled oligonucleotide probes targeting unique regions of the (+) strand of *E. nucleophila* rDNA gene, and we developed an ISH protocol that results in good staining of infected host cells prior to the development of spores or other conspicuous stages.

On clinically infected samples, numerous ISH-positive cells are present which are not stained with fluorescent whiteners that bind to parasite spores. In contrast, poor staining of spores was obtained with the DNA probes, due to their limited penetration in these stages and the low number of target gene copies that they harbour. These aspects might be improved using specific steps (e.g., chitinase or more aggressive permeabilization strategies) but they are not necessary in a clinical diagnostic context.

Due to the relevance of *E. nucleophila* infections it is essential to decipher unknown aspects of the parasite's biology and course of infection using proper diagnostic tools. Their cryptic nature makes this a challenging task for microsporidians like *E. nucleophila*, which can benefit from the method hereby presented.

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