Comparison of immunohistochemistry and Ziehl-Neelsen for the detection of Mycobacterium infection in sea bass (Dicentrarchus labrax)

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Abstract
Ziehl-Neelsen (ZN) and immunohistochemistry were used to detect Mycobacteria in histological sections of internal organs from sea bass (Dicentrarchus labrax) intraperitoneally inoculated with Mycobacterium fortuitum. Both methods allowed for detection of advanced lesions caused by Mycobacteria, but immunohistochemistry allowed for their detection in early granulomas. Thus immunohistochemistry is suited to the study of Mycobacterium pathogenesis in fish and may improve the diagnostic capacity for these bacteria.

Mycobacterial infections are frequently described in wild and farmed fish. Mycobacterium marinum, M. fortuitum, and M. chelonae are the most frequently reported species (Gauthier et al., 2003). Mycobacteriosis in fish has been described as a systemic, chronic, progressive disease with granulomas scattered or grouped in virtually any tissue, but particularly in the spleen, kidney and liver (dos Santos et al., 2002). Acid-fast bacteria are easily detected by Ziehl-Neelsen in mature granulomas showing necrosis, but not in the early lesions (Gauthier et al., 2003; Sarli et al., 2003). A more efficient method to detect acid-fast bacteria in early lesions is immunohistochemistry (IHC). It allows for a preliminary screening between Mycobacterium and Nocardia, both acid-fast bacteria, even if isolation is generally required to identify Mycobacteria species (Schäperclaus, 1992). The present study demonstrates the improved role of IHC over ZN stain for the detection of Mycobacteria in early lesions.

In order to compare ZN and IHC in histological sections, we used sea bass (Dicentrarchus labrax) intraperitoneally inoculated with 10⁴ cfu/10g body weight of Mycobacterium fortuitum and euthanased at different times for a pathogenesis study. Spleen, kidney, liver, gut and peritoneum were sampled from 13 fish chosen according to the different grade of granulomatous lesions noticed in a previous study (Sarli et al., 2003). Among these, 5 euthanased at 1...
week post inoculation (p.i) and 7 at 2 weeks p.i. showed the presence of early granulomas. One fish euthanised at 6 week p.i. with advanced granulomatous lesions was used as control. The samples were fixed in 4% buffered formalin and then paraffin wax embedded. Four micron thick sections from each organ underwent Haematoxylin-Eosin (H-E), ZN and IHC to *Mycobacterium* spp. stains. H-E lesions were graded as follows: 1= granulomas without necrosis; 2= granulomas with initial necrosis; 3= granulomas with evident necrosis. ZN staining was performed according to a standard method (Mazzi, 1977). For IHC, the sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in 0.3% hydrogen peroxide for 30 min. Sections were then rinsed in Tris buffer, immersed in citrate buffer (2.1 g citric acid monohydrate/litre distilled water - DW), pH 6.0, incubated for 10 min in a microwave oven at 750 W, and allowed to cool to room temperature (RT) (approximately 20 min). The sections were pre-incubated for 10 min to RT with Protein Block Serum Free (Dako, Amsterdam, The Netherlands). The primary antibody anti-*Mycobacterium bovis* (polyclonal, 1:3000, Dako, Amsterdam, The Netherlands) was applied overnight at 4° C and was followed by a commercial streptavidin-biotin-peroxidase technique (LSAB Kit, Dako, Amsterdam, The Netherlands). In negative control sections, the primary antibody was replaced with Block Serum Free (Dako). Granulomas were observed with H-E in the peritoneum, kidney, liver and spleen. The
initial lesions (grade 1) were nodular aggregations of cells characterized by the abundant slightly eosinophilic cytoplasm of the macrophages and few lymphocytes at the periphery. These evolved from pyknosis and/or karyorrhexis of few centrally placed cells (grade 2) to a well appreciable centre of amorphous eosinophilic material surrounded by spindle and loosely arranged or strictly packed cells (grade 3); in some cases, these grade 3 granulomas were confluent.

Mycobacteria, stained with ZN, appeared as isolated or clustered intracellular (grade 1, 2, 3) or extracellular (grade 3) organisms.

Immunohistochemically, Mycobacteria appeared as brownish granules or clusters and were more evident than with ZN. In particular, in fish sacrificed at 1 and 2 weeks p.i. with early lesions (granulomas without necrosis, grade 1), acid-fast bacilli were rarely found with ZN, but in the same lesions, IHC showed a stronger and more striking positivity. The ZN and immunohistochemical positivity were localized in the cytoplasm of histiocyte cells. In aged lesions (granulomas with necrosis) found 6 weeks p.i. both IHC and ZN showed strong positivity, which was revealed in the cytoplasm of histiocytes and in necrotic debris in the centre of granulomas.

Bacteria such as Mycobacterium spp. and Nocardia spp. have been reported to induce granulomatous lesions in fish (Ribelin and Migaki, 1975) and both genera are ZN positive, but this method is poorly sensitive in small and early granulomas. In fact, there appeared to be a direct relationship between number of mycobacteria and severity of the lesions, as described by other authors (Gutiérrez Cancela and García Marín, 1993).

The IHC technique detected M. fortuitum in tissue sections with granulomas. Similar results have been reported for other mycobacteria and in different animals including man (Humphrey & Weiner, 1987; Gutiérrez Cancela & García Marín, 1993; Massone et al., 1990; Thoresen et al., 1994; Carabias et al., 1998).

This study shows the potential value of this immunohistochemical method for the detection of mycobacterial antigens in ZN negative or slightly positive granulomas as reported in other investigations and is considered a valuable adjunct to standard cultivation and ZN staining of tissue sections for the diagnosis of mycobacteriosis (Gutiérrez Cancela & García Marín, 1993; Thoresen et al., 1994).

The difference in sensitivity may be explained by the ZN technique detecting only “perfectly preserved” organisms, whereas IHC detects mycobacterial antigens, fragments and living or dead organisms, even with “defective” cell walls (Gutiérrez Cancela & García Marín, 1993).

In IHC, the positivity was due to the presence of mycobacterial antigens. While the ZN technique always stains both Mycobacterium and Nocardia (Schäperclaus, 1992), anti Bacillus Calmette-Guérin (BCG) antibodies cross-reacted with N. asteroides in only few cases (Harboe et al., 1979). In conclusion, the immunohistochemical method for mycobacterial diagnosis offers the advantage of improved, early etiological screening of
slides awaiting isolation. Moreover it allows for pathogenesis to be traced from early stages of infection.

References


Ribelin WE and Migaki G (1975). Fish pathology. The University of Wisconsin Press Ltd, pp. 82-84.

