# Comparative study of immunohistochemical methods to diagnose mycobacteriosis in swordtail *Xiphophorus helleri*

S. Gómez *, J. A. Navarro, M. A. Gómez, J. Sánchez, A. Bernabé

Histología y Anatomía Patológica, Facultad de Veterinaria, Universidad de Murcia, Apartado 4021, E-30071 Murcia, Spain

ABSTRACT: A comparative immunohistochemical method employing avidin-biotin-peroxidase complex and using polyclonal and monoclonal antibodies in paraffin-embedded sections was tested for its ability to detect antigens which cause mycobacteriosis in swordtail *Xiphophorus helleri*, a freshwater fish. By using the polyclonal antibodies anti-*Mycobacterium bovis* (BCG strain) and anti-*M. paratuberculosis*, we were able to find the mycobacterial antigens in tissues of the fish. The monoclonal anti-*M. avium* detected a positive reaction only in cells of kidney granulomas. The use of bleaching to eliminate confusion between melanin and chromogen end-product during the diagnosis of fish mycobacteriosis was also studied.

KEY WORDS: Fish mycobacteriosis · Swordtail

## INTRODUCTION

Mycobacterium-caused fish diseases have been reported since the end of the last century (Bataillon et al. 1897) and are generally referred to as fish tuberculosis. This condition has been diagnosed in both freshwater and marine fish, and various species of the genus *Mycobacterium* have been isolated in both cases, including *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. chelonae* and *M. fortuitum* (Bragg et al. 1990, Noga et al. 1990, Lansdell et al. 1993). Fish can be affected irrespective of whether they are in their natural environment (Dalsgaard et al. 1992) or cultured (Hedrick et al. 1987, Colorni 1992). Mycobacteriosis of fish potentially are pathogenic to fish handlers (Suh & Hoffman 1992).

Immunohistochemical techniques have proved to be very useful in animal pathology. The ability to detect antigen in fixed tissues may be especially benefical in cases where the diseased animals are long distances from the diagnostic laboratories. Immunohistochemical staining may also be an efficient means of detecting organisms which are difficult to diagnose by viral isolation or bacterial culture. These organisms may be detected in formalin-fixed sections (Haines & Clark 1991). This has prompted their increasing use in fish infectious pathology (Hoffmann et al. 1989, Evensen & Rimstad 1990, Jansson et al. 1991).

This work describes the use of 3 antibodies and the avidin-biotin complex in paraffin-wax-embedded sections for the immunohistochemical study of fish mycobacteriosis.

## MATERIALS AND METHODS

**Samples.** Four aquarium-maintained specimens of swordtail fish *Xiphophorus helleri* Heckel were used. The specimens were sent to our laboratory previously fixed in formalin for diagnostic purposes.

**Histological techniques.** Paraffin blocks were sectioned 5 μm thick and stained with hematoxylin and eosin (H&E). Adjacent sections were stained with periodic acid-Schiff (PAS), Ziehl Neelsen (ZN) and using immunohistochemical techniques. Because of the small size of examined fish, complete sagittal sections were obtained.

*E-mail: serafing@fcu.um.es

**Immunohistochemical procedures.** Immunohisto-
logical tests for mycobacterial antigens were performed
in 2 group of samples (A and B) using an avidin-biotin-
peroxidase method. In this study we used the poly-
clonal antisera rabbit anti-*Mycobacterium bovis*, BCG
strain (Dako), and anti-*M. paratuberculosis* (Dako); the
monoclonal anti-*M. avium* (Chemicon); and 2 biotyni-
lated secondary antibodies, swine anti-rabbit immuno-
globulin (Dako) and rabbit anti-mouse immunoglobu-
lin (Dako).

*Group A:* Sections were deparaffinized, rehydrated
and treated with 1.5% methanolic hydrogen peroxide
for 20 min to eliminate the endogenous peroxidase
activity. Then they were treated with 3 M urea solution
for 5 min to enhace the sensivity of the antigen detec-
tion. Blocking was carried out with 1:100 normal serum
of the species in which the secondary antiserum was
raised. Next, sections were incubated for 1 h with the
primary antisera, either the polyclonal antibodies or
the monoclonal antibody, diluted 1:500 to 1:1500. Sec-
ondary antibodies diluted 1:200 (Dako) were applied
for 30 min. The avidin-biotin complex (Vector Labora-
tories) was incubated for 30 min. All incubations were
performed at room temperature. Finally, the reaction
was developed with diaminobenzidine, counterstained
with haematoxylin, dehydrated and mounted with
Eukitt.

*Group B:* Concentrated hydrogen peroxide was used
to prevent tissue pigments from interferring with the
interpretation of results. After rehydration, the sections
were treated with 5% hydrogen peroxide for 16 h in
order to remove the melanin which was present in
some of the tissues, as well as to inhibit endogenous
peroxidase. All subsequent reactions were carried out
in the same way as for the samples of Group A.

Positive controls were carried out using previously
tested sections. The negative control was performed
by substitution of primary antibody by normal swine
and rabbit sera or phosphate-buffered saline (PBS).

## RESULTS

### Pathology

Histopathological examination revealed the pres-
ence of granulomas in liver, pancreas, digestive tract,
kidney, peritoneal serosa, eye and connective tissue of
all 4 fish in H&E-stained sections. The granulomas
consisted of epithelioid cells with few or no fibrous
tissue capsules, no central area of necrosis and varying
numbers of pigmented cells. A few granulomas with
central necrosis or a well-developed fibrous tissue
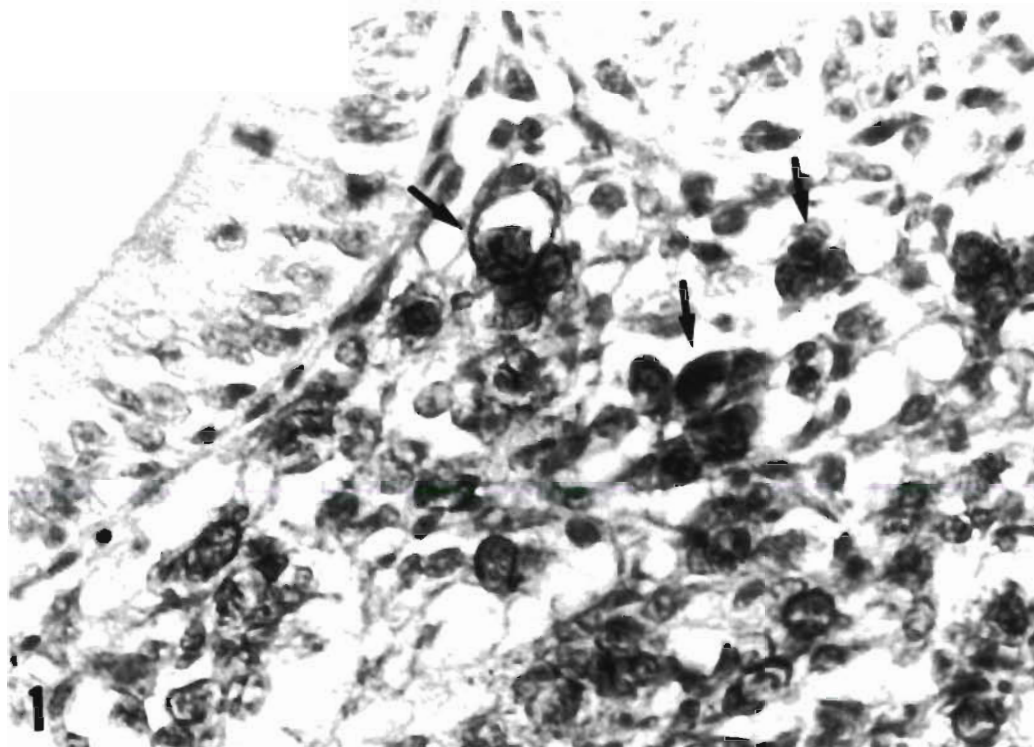capsule were seen in 2 fish. Granulomas were absent



Fig. 1. *Xiphophorus helleri* intestine. Positive immunostaining in macrophages. Section stained with polyclonal antiserum to *Mycobacterium bovis* (arrows). ×1017
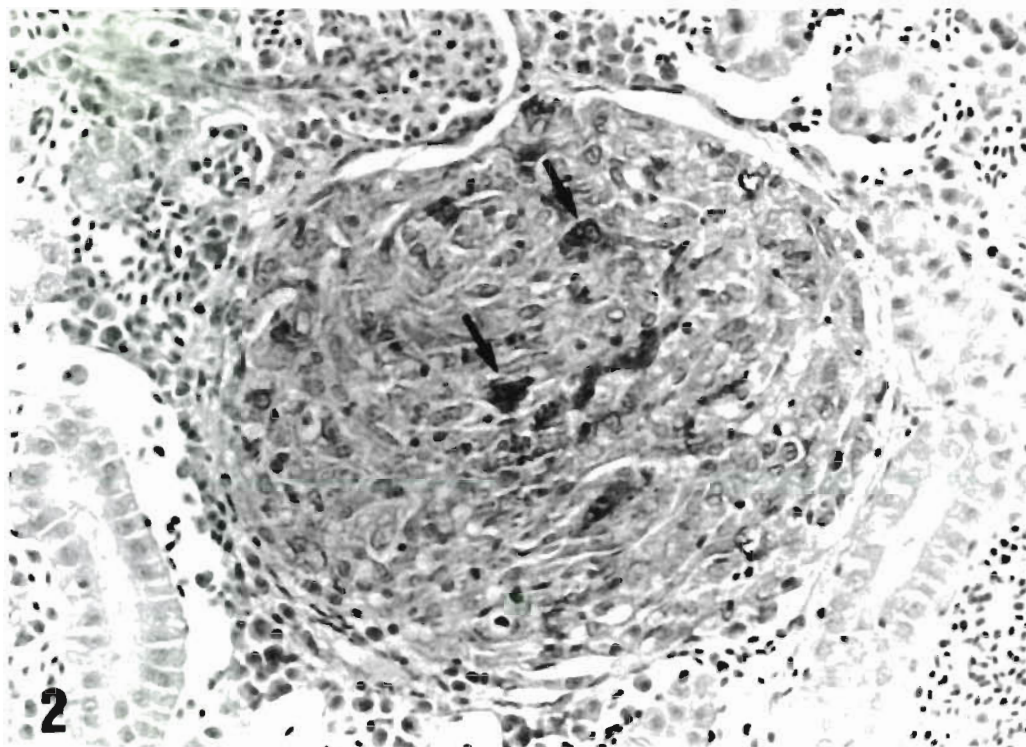
Fig. 2. *Xiphophorus helleri* kidney. Positive immunostaining in macrophages of a renal granuloma. Section stained with monoclonal antiserum to *Mycobacterium avium* (arrows). ×428

in the submucosa of the digestive tract, where they were replaced with a diffuse macrophagic infiltrate. PAS-positive reaction was observed in inflammatory cells. Sections stained by the ZN method revealed epithelioid cells and macrophages containing intra-cytoplasmic acid-fast bacilli.

## Immunohistopathology

*Group A:* Positive reactions indicating the presence of mycobacterial antigen in the epithelioid cells of granulomas and macrophages of the diffuse inflammatory intestinal effusion were obtained by the use of polyclonal antisera. The specific reaction was visible as a golden-brown cytoplasmic colour within phagocytic cells. In general it was difficult to identify a single bacteria because the reaction product coalesced to produce a diffuse staining of phagocyte cytoplasm (Fig. 1). The immunohistological method revealed positive areas in the liver section of a fish which had failed to reveal the presence of bacilli or showed only a small number of them when ZN stain was used.

With the monoclonal antibody, reactions were obtained only in epithelioid cells of kidney granulomas (Fig. 2).

*Group B:* In sections treated with 5% concentrated hydrogen peroxide for 16 h, no significant reaction was observed by applying polyclonal or monoclonal anti-bodies.

Because we used full sagittal sections, we could simultaneously observe all affected organs for reaction with antisera.

## DISCUSSION

Chronic inflammation, with development of prolifer-ative lesions, is characteristic of many fish diseases related to bacteria, fungi and parasites (Roberts 1989). This picture is commonly seen in tropical fish, and a differential diagnosis must be considered (Reichen-bach-Klinke 1971). In mycobacterially affected fish, 3 types of nodule, possibly representing different stages of development, have been observed (Hastings et al. 1982); the absence of mycobacteria in nodules of fish with marked clinical signs of disease has also been reported (Hastings et al. 1982, Gómez et al. 1993).

The immunohistochemical method used in the pre-sent study demonstrated the presence of mycobacterial antigens in tissues of affected fish. Using the poly-clonal antibodies, immunoreactivity was demonstrated either in phagocytic cells of nodules or in inflammatory

effusions. Since the number of epitopes recognized by the polyclonal antisera is also very large, and taking into account the existence of an antigenic community among the different species of mycobacteria, we consider it better to use polyclonal antisera. Both the anti-*Mycobacterium bovis* (BCG strain) and the anti-*M. paratuberculosis* serum proved to be effective in formalin-fixed paraffin-embedded material.

It is tedious to isolate certain bacterial species, but they may be readily demonstrated in previously formalin-fixed tissue samples using immunohistochemical stains. This enables the pathologist to associate the immunohistological stain, with the lesions present in the tissues demonstrating the infectious disease agent (Haines & Clark 1991). However, attempts to identify fish *Mycobacterium* species are often unsuccessful (Colorni 1992, Hatai et al. 1993).

The restricted reaction observed with monoclonal anti-*Mycobacterium avium* may be attributed to the possibility that the recognized epitope was only present in these lesions, which could indicate an infection caused by several species of mycobacteria in the same animal (Terver et al. 1984).

In other cases, the bleaching of melanin with a strong hydrogen peroxide solution apparently has not inhibited the immunoreactivity of certain pathogens of fish when the chromogen diaminobenzidine has been (Jansson et al. 1991), but some authors do not regard bleaching as totally necessary under the same conditions (Hoffmann et al. 1989). However, in the present study, no immunostaining was observed after the treatment with concentrated hydrogen peroxide solution, probably due to an induced loss of antigenicity.

The advantages of diaminobenzidine, the most commonly used chromogen, are that it produces very intense deposits, it is insoluble in aqueous and organic solvents and the stain is stable indefinitely. Diaminobenzidine deposits may be confused with melanin and hemosiderin; however, their color becomes readily distinguishable with minimal experience (Haines & Chelack 1991). The results obtained here confirm the validity of these immunohistochemical methods for diagnosing fish mycobacteriosis in formalin-fixed specimens.

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