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13. *Mycobacterium* group

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Table of contents

- 13.1. Aetiology of *Mycobacterium* group
- 13.2. Sampling
 - 13.2.1. Preparation and shipment of samples from fish
- 13.3. Diagnostic procedures for *Mycobacterium* group
 - 13.3.1. Primary cultivation of bacteria (choice of media and isolation of strain)
 - 13.3.2. Screening of pure cultures
 - 13.3.3. Identification of the strain
 - 13.3.3.1. API – Biochemical identification
 - 13.3.3.2. Mass spectrometry (MALDI-TOF)
 - 13.3.3.3. PCR
 - 13.3.4. Typing of the bacteria
 - 13.3.5.1. Genotyping
 - 13.3.5. *In vitro* susceptibility testing
- References
- Photos

13.1. Aetiology of *Mycobacterium* group

Fish mycobacteriosis, also known as “piscine tuberculosis” is usually a chronic progressive disease caused by several species of the genus *Mycobacterium* (Jacobs *et al.*, 2009) even if, on some occasions, septicaemias were reported in sturgeons infected by *Mycobacterium pseudoshotsi*. It is caused by a ubiquitous acid-fast-bacilli identified as non-tuberculous mycobacteria (NTB). The main NTB species affecting fish are *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae* which can be classified into 1) slow grower NTB mycobacteria such as *M. marinum* and 2) rapid grower NTB mycobacteria such as *M. fortuitum* and *M. chelonae* (Novotny *et al.*, 2004; Hashish *et al.*, 2018). *Mycobacterium marinum* is the most important fish pathogen, representing a significant threat to seabass culture in the Mediterranean (Toranzo *et al.*, 2005).

All *Mycobacterium* spp. infecting fish are also able to infect humans, resulting in local granulomatous inflammation usually at the extremities such as hands and fingers (Jacobs *et al.*, 2009; Aubry *et al.*, 2017).

Mycobacterium spp. are Gram-positive bacteria, aerobic, acid-fast, with the shape of non-motile rods, 0.2-0.6 µm in diameter and 1-10 µm long (Gauthier and Rhodes, 2009). The bacteria cell wall has a unique specific composition rich in mycolic acids (3-hydroxy long-chain fatty acids), essential for the survival of this genus (Marrakchi *et al.*, 2014).

Piscine mycobacteriosis is typically a chronic progressive disease that may not produce clinical signs or take a long time for clinical signs and mortality to develop. External clinical signs are usually non-specific, such as scale loss and dermal ulceration, ascites, pigmentary changes, lethargy and abnormal behaviour. Internally, fish may exhibit spleen, kidney and liver enlargement, and show characteristic grey/white nodules (granulomas) in these internal organs (Decostere *et al.*, 2004; Toranzo *et al.*, 2005).

13.2. Sampling

13.2.1. Preparation and shipment of samples from fish

Isolation and culture of bacteria from external surfaces and whole viscera are questionable due to the possible presence of contaminants (Rhodes *et al.*, 2004).

Tissue samples from fish are normally homogenized and then plated on appropriate culture media, including Middlebrook 7H10 or Lowenstein-Jensen media to enhance *Mycobacterium* spp. growth (Hashish *et al.*, 2018).

13.3. Diagnostic procedures for *Mycobacterium* group

Mycobacterium spp. are best visualized in tissue sections (such as smears from spleen and kidney) with the Ziehl-Neelsen acid-fast stain. The “acid-fastness” or resistance of the cell wall to acid-alcohol decolourization after staining with carbol-fuchsin is characteristic of *Mycobacterium* species (Toranzo 2004; Gauthier and Rhodes, 2009). A Quick TB stain kit is available for rapid staining (RAL Diagnostic, Martillac, France).

13.3.1. Primary cultivation of bacteria (choice of media and isolation of strain)

Culture continues to be an important diagnostic method for diagnosis of fish mycobacteriosis, however, in many cases; the isolation of the aetiological agent often fails due to the fastidiousness of the pathogen (Austin and Austin, 2012). Successful isolation has been achieved from homogenates of infected tissue (from kidney, liver or spleen) on standard mycobacterium media such as Petragnani, Löwenstain-Jensen, Middle-brook 7H10 and Dorset egg media. All fish mycobacteria are cultured at 20-30°C. Incubation for 2-30 days is suitable for the fast-growers such as *M. fortuitum* and *M. chelonae*, which typically show growth after seven days. *M. marinum*, a slow grower, requires longer incubation for visible growth and may require months (Frerichs 1993; Decostere *et al.*, 2004; Gauthier and Rhodes, 2009).

M. marinum is a strict aerobe and its preferred carbon sources are glycerol, pyruvate and glucose but ethanol can also be used. It grows in all media used for mycobacterial growth such as egg-based, broth or agar-based without any additives or only 2-5% oleic acid-albumin-dextrose-catalase and also ion blood-containing agar. After subcultures, some strains may grow on ordinary media but addition of 2-5% of carbon dioxide in the gas phase above the medium improves its growth. It has an optimal growth temperature of 30°C, whereas small colonies or no growth is observed at 37°C (Aubry *et al.*, 2017).

13.3.2. Screening of pure cultures

Culture-based detection and isolation of *Mycobacterium* spp. from skin or gills of fish is complicated by the existence of background microbiota, which competes with the *Mycobacterium* growth on the standard media, therefore, it is recommended to carry out an aseptic necropsy. Plating of tissue homogenate on a non-selective medium such as BHI is recommended in order to detect a non-mycobacterial infection or sample contamination (Rhodes *et al.*, 2004; Hashish *et al.*, 2018).

Mycobacterium spp. show high hydrophobicity and are very resistant to treatment with both acidic and basic chemicals in addition to benzalkonium chloride and hypochlorite. These substances have been used to assist the isolation of pure cultures of *Mycobacterium* spp. with a high content of microbiota although it can affect negatively the recovery of mycobacteria (Rhodes *et al.*, 2004).

13.3.3. *In vitro* susceptibility testing

For *in vitro* susceptibility testing two methods are available: (i) Etest and (ii) agar dilution method. The latter is the method recommended for antibiotic susceptibility testing of *M. marinum*. The agar dilution method can be performed on Mueller-Hinton agar (Difco, Serlabo, Bonneuil sur Marne, France) supplemented with 5% Middlebrook OADC (oleic acid, albumin, dextrose and catalase [OSI, Elancourt, France]) (Aubury *et al.*, 2000).

After the *in vitro* testing of 17 antibiotics, *M. marinum* has shown natural multidrug resistance to the anti-tuberculosis drugs: isoniazid, ethambutol, and pyrazinamide. Minocycline, doxycycline, clarithromycin, linezolid, sparfloxacin, moxifloxacin, imipenem, sulfamethoxazole, and amikacin may have moderate activity. Rifampin, rifabutin, tetracyclines (particularly minocycline), amikacin, imipenem, and clarithromycin appeared to be good candidates for testing *in vivo* efficacy (Aubury *et al.*, 2000; Aubury *et al.*, 2017).

13.3.4. Identification of the strain

For strain identification, the sole utilization of 16S rRNA sequences has proven to be insufficient or too conserved to study the relationship of close related organisms. In *M. marinum*, the combined analysis of the restriction enzyme map of at least two genes (i. e. 16S rRNA and *hsp65*) has proven to be a useful molecular tool for the detection of intraspecific variation (Aubury *et al.*, 2000; Ucko *et al.*, 2002).

13.3.4.1. API – Biochemical identification

Biochemical identification is based on phenotypic characteristics such as growth rate, colonial morphology, cord formation and pigmentation (Nagwa *et al.*, 2000).

In the case of *M. marinum*, colonies are typically smooth, white or beige when the media is kept in the dark and yellow to orange after exposure to light (photochromogenic). Photochromogenicity is due to the active production of beta-carotene mediated by the gene *ctrB* and can be inhibited by chloramphenicol. Biochemically, *M. marinum* does not show nitrate reductase production and cannot grow on a medium containing thiacetazone (Aubury *et al.*, 2017).

This traditional method of diagnosis requires that the pathogen needs firstly to be recovered on culture medium and identified by means of a battery of differential biochemical tests. This method, however, often fails to identify *M. marinum* conclusively. Not only may the morphology and biosynthetic capabilities of mycobacteria vary depending on culture conditions but also there are often strain variations that do not quite fit into the typical biochemical profile for the species (Ucko *et al.*, 2002). Therefore, the use of other approaches for the identification of

Mycobacterium spp. such as molecular methods or mass spectrometry analysis is being used increasingly (Kaattari *et al.*, 2004; Kurokawa *et al.*, 2013).

13.3.4.2. Mass spectrometry (MALDI-TOF)

A simple MALDI-TOF MS system called a MALDI Biotyper has been developed for rapid bacterial identification that does not require a highly trained operator (Shitikov *et al.*, 2011; Bille *et al.*, 2012). The MALDI Biotyper method has high reproducibility generating high-quality spectra, which allows the separation between species closely related to *M. marinum* and also differentiates *Mycobacterium* isolates (Kurokawa *et al.*, 2013).

MALDI-TOF MS has been demonstrated to be a rapid and accurate technique which could be an effective diagnostic tool for the identification and differentiation of clinical mycobacterial isolates (Puk *et al.*, 2018).

13.3.4.3. PCR

Several DNA-based diagnostic methods have been developed for the identification of *Mycobacterium* spp. in fish, which are particularly useful for its detection from fish tissues when the culture of *Mycobacterium* spp. fails (due to slow and poor growth of some of the species on culture media) (Toranzo *et al.*, 2005; Hashish *et al.*, 2018).

The small subunit 16S rRNA gene is commonly used for its identification due to the availability of *Mycobacterium* spp. 16S gene sequences in web repositories such as Gene Bank. A PCR has been described in 1993 by Telenti *et al.*, targeting *hsp65* sequence present in all *Mycobacterium* species. Exact PCR conditions depend on the DNA concentration in the isolate, and the type of polymerase used, so what follows is an example using GoTaq G2 Hot Start Master Mix (Promega) or similar polymerase with DNA extracted from bacterial culture using commercial kit.

Primers for *hsp65* region

| | | |
|----------------|-------------|------------------------------|
| Forward primer | Tb11 | 5'- ACCAACGATGGTGTGTCCAT -3' |
| Reverse primer | Tb12 | 5'- CTTGTCGAACCGCATACCCT -3' |

PCR mix contains the following reagents:

| Reagent | Quantity |
|---------------------------------|----------|
| Water (molecular biology grade) | 6 µl |
| Hot Start Master Mix (2x) | 10 µl |
| 10 µM primer Tb11 | 1 µl |
| 10 µM primer Tb12 | 1 µl |
| DNA sample | 2 µl |
| Total volume | 20 µl |

Thermal profile

| Initial polymerase activation | Denaturation | Annealing | Extension | Final extension |
|-------------------------------|--------------|-----------|-----------|-----------------|
| 95°C | 94°C | 60°C | 72°C | 72°C |
| 2 min | 1 min | 1 min | 1 min | 5 min |
| 45 cycles | | | | |

PCR-RFLP analysis using the 16S rRNA gene as a target has been proven a sensitive and highly specific tool for the identification of *M. marinum*, *M. fortuitum* and *M. chelonae* from fish tissues and blood samples (Talaat *et al.*, 1997). PCR-RFLP on two genes, 16S rRNA and *hsp65*, have been able to distinguish a variety of *M. marinum* isolates (Ucko *et al.*, 2002).

Due to the high homology between fish isolates, the use of a minimum of two genes for accurate identification is recommended. Other gene targets used for the molecular diagnosis are the: 1) 16S-23S internal transcribed spacer (ITS) 2) 65-kDa heat shock protein gene (*hsp65*), 3) exported repeated protein gene (*erp*), 4) RNA polymerase B subunit (*rpoB*) gene (Kaattari *et al.*, 2005).

FRET probe assay has shown to have high specificity via melting curve analysis and is able to discriminate and distinguish *M. marinum* from other *Mycobacterium* spp. The FRET assay has two steps. First, PCR with SYBR green (with a detection limit of 10² *Mycobacterium* DNA copies) and second, real-time PCR using FRET probes. The kidney is the organ with the strongest detection signal (Salati *et al.*, 2010).

Finally, two methods based on PCR coupled with reverse hybridization are currently available commercially for the rapid and accurate identification of *Mycobacterium* spp.: INNOLiPA Mycobacteria v2 (Innogenetics), based on the amplification of the ribosomal gene spacer (16-23S) (Tortoli *et al.*, 2001; Tortoli *et al.*, 2003) and GenoType Mycobacteria CM/AS (Hain Lifescience), based on the amplification of the 23S rRNA gene (Russo *et al.*, 2006).

13.3.5. Typing of the bacteria

13.3.5.1. Genotyping

A novel category of variable tandem repeats (VNTR) called mycobacterial interspersed repetitive units (MIRU) has been applied to *M. marinum* and *M. ulcerans*. The MIRU-VNTR typing is highly reproducible and can be applied directly to clinical samples and allows the intra and inter-specific differentiation among the *M. marinum*-*M. ulcerans* complex. The genotypes found, however, for *M. marinum* were not clearly related to the geographic origins of the isolates (Stragier *et al.*, 2005).

Multilocus Sequence Analysis has been shown that *M. marinum* isolates showed a higher level of intraspecific nucleotide sequence divergence than other closely related species such as *M. ulcerans* (Stinear *et al.*, 2000).

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Photos



Fig. 13.1. European seabass/granulomatous lesions of the spleen due to *Mycobacterium marinum* (a-d).

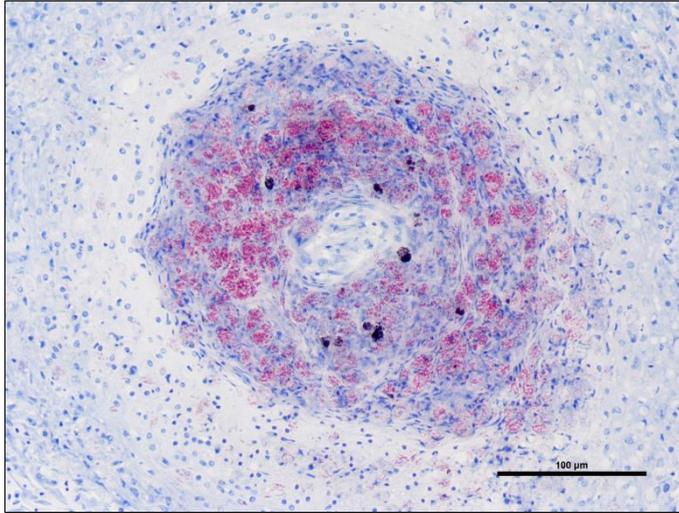


Fig. 13.2. Mycobacterial granuloma in seabass. Resin-embedded (Technovit 7100) stained with Ziehl-Neelsen (Photo courtesy of P. Katharios).