Chapter 3: Isolation and Phenotypic Characterization of Mycobacterium spp. from Freshwater Fish and Water Samples
3.1 Introduction

The vast majority of non-tuberculous mycobacteria (NTM) or environmental mycobacteria are ubiquitous and aquatic environment has been shown to be an important source of these organisms. A variety of NTM, including *M. marinum, M. fortuitum, M. chelonae, M. gordonae, M. mucogenicum, M. avium* complex, *M. malmoense, M. simiae, M. kansasii, M. terrae, M. lentiflavum* and *M. xenopi* are found in natural and artificial water resources (Covert et al., 1999; Torkko et al., 2000; Falkinham et al., 2001; Leclerc and Moreau, 2002; Le Dantec et al., 2002; Primm et al., 2004; Torvinen et al., 2004). Aquatic environments containing various living organisms form very diverse ecosystems. Fish, as part of these ecosystems, are very susceptible to mycobacteria and can act as both hosts and carriers of these bacteria (Eddyani et al., 2004).

Although primary diagnosis of fish mycobacteriosis is based on external clinical signs and granulomas in visceral organs of infected animal via necropsy (Rhodes et al., 2001), a definitive diagnosis is usually made in the laboratory based on isolation and identification of acid fast bacteria. Successful isolation and identification of the organism depend on the type of samples taken and the isolation and/or identification procedures used. The aim of this chapter was to isolate mycobacteria from fish tissues and water samples collected from the different parts of India and their tentative identification.

The traditional method for identification of mycobacteria from clinical and environmental sources includes isolation using enrichment and selective media and subsequent screening and confirmation by the phenotypic characteristics of biochemical testing, pigment production and growth characteristics, colony morphology (Kent and Kubica, 1985).
3.2 Materials and Methods

3.2.1 Fish samples

Freshwater aquarium fish (n=60) with no observable signs of disease were randomly collected from aquarium shops. Samples consisted of 6 specimens each of *Carassius auratus*, *Helostoma temminckii*, *Colisa loria*, *Danio rerio*, *Balantiocheilus melanopterus*, *Epalzeorhynchos frenatus*, *Trichogaster chuna* and 18 specimens of *Carassius auratus auratus*. In addition, 10 environmental samples (Pond water) were collected from different cities in India. All samples were processed for isolation of mycobacteria.

3.2.2 Histopathological diagnosis

Fishes were subjected to pathomorphological and microscopic examination of native preparates with a focus on lesions in the skin and in parenchymal organs (Fig. 1). Excised tissue was immersed in 70% ethanol for 5 min, drained, and the residual alcohol allowed to evaporate before mounting. The wet mounts were then stained according to Ziehl–Neelsen to identify acid-fast rods (AFR). Skin scrapings and individual organs of the body cavity (hepatopancreas, spleen, kidneys, and contents of the intestine) from each fish were examined (fig.3.1).

3.2.3 Conventional Microbiological Diagnosis.

Conventionally, the detection of aquatic *Mycobacterium* spp. is performed by culture and acid-fast staining. Before culture, decontamination of samples is necessary.
Fig. 3.1: Haemorrhagic skin ulcer in fish (B) granulomatous lesions in fish (C) Ziehl Neelsen staining of kidney showing ZN positive bacteria (D) Large numbers of ZN positive bacteria in necrotic kidney.
3.2.3.1 **Tissue homogenization and decontamination**

Samples are decontaminated because of the possible contaminating commensals present in the non-sterile clinical materials. Several methods are known for this purpose: The Petroff’s method, N-acetyl-L-cysteine-sodium hydroxide, NaOH-N-acetyl-L-cysteine, Sulphuric acid, Zephiran, Papain-Zephiran, Papain-Pentane-Zephiran, sulphuric acid, chlorhexidine and oxalic acid and the newer method: hypertonic saline with sodium hydroxide (Thoen et al., 1974; Yeboah-Manu et al., 2004). While the NaOH-N-acetyl-L-cysteine method is currently recommended for the recovery of NTM, other methods appear to be more efficient like the sulphuric acid, oxalic acid and the chlorhexidine methods (Yeboah-Manu et al., 2004; Ferroni et al., 2006). They yield a higher recovery rate and less contamination and should be considered as the method of choice. A secondary effect of decontaminating pre-treatment is the property to dissolve clinical materials which clears bacilli from intracellular containment. Therefore, decontamination of specimens is useful and should be applied to all specimens.

**3.2.3.2 Procedure for tissue samples: (Shitaye’s Method)**

1 ml aliquots of homogenate was centrifuged at 4,000×g at 4°C for 30 min and pellets were re-suspended in 1 ml of 2% HCl (method A) and 4% NaOH (method B) both containing 10μL phenolphthalein as a pH indicator. After 15 min exposure to acid or base, suspensions were neutralized by addition of 1% NaOH or HCl. Then suspension was centrifuged as above. Decant supernatant and pellets were re-suspended in 0.5 ml of 0.85 % NSS, inoculate deposit on to two slopes of L-J with antibiotics and L-J with Gruft’s supplement.
3.2.3.3 Procedure for environmental (soil and water) samples: (Modified Petroff’s Method)

To 1 ml of sample 2 ml of 4% NaOH was added, tighten cap of container and shake to digest. Let stand for 15 minutes at RT with occasional shaking and then centrifuge at 3000 g for 15 minutes. Carefully pour off the supernatant, approx. 20 ml sterile distilled water was added and re-suspend the sediment. Then centrifuge again at 3000 g for 15 minutes, decant supernatant and inoculate deposit on to two slopes of LJ with antibiotics and L-J with Gruft’s supplement.

3.2.3.4 Culture

The yield of cultivation for the detection of Mycobacterium is higher than that of acid-fast microscopy. Historically, the egg-based media, such as that of LJ, are the best-known of the solid media used for the isolation of Mycobacterium. The agar-based media, e.g. Middlebrook 7H10 and 7H11 agar, offer a better opportunity for the examination of colonial morphology and the detection of mixed cultures than do egg-based media (Ulukanligil et al., 2000). The use of a liquid medium is generally recommended for subcultures and in vitro tests (i.e. Middlebrook 7H9). Normal growth temperature for mycobacteria is 28-37°C. Most media require additives like mycobactin and OADC to increase the growth rate (containing oleic acid, albumin, dextrose, catalase and NaCl). Antibiotics are often added to inhibit the growth of contaminants: Panta, containing polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin, and/or PACT, containing polymyxin B, amphotericin B, carbenicillin, and trimethoprim (Whittier et al.,
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1993; Somoskovi and Magyar, 1999). Incubation periods for liquid media stay within 6 weeks, but cultures on lower temperatures and on solid media might require incubations up to 10 weeks (IDSA 2007).

Decontaminated homogenates were inoculated onto three media: one egg media Lowenstein–Jensen (LJ) slants (Himedia Laboratories); Middlebrook 7H11 agar (MDA) containing 0.5 % glycerol and oleic acid, albumin, dextrose, and catalases a supplement (Sigma-Aldrich); and solid Stone-brink’s medium (Himedia Laboratories). In LJ and MB media nalidixic acid used for inhibition of most of the gram-negative bacteria, lincomycin for inhibition of gram-positive bacteria and cycloheximide as a suppressor of saprophytic fungi. The concentration of these antibiotics in 1000 ml media were 0.64gm of cycloheximide, 3.2mg of lincomycin and 56.0 mg of nalidixic acid. Inoculated media were incubated at three different temperatures 25°C, 30°C and 42°C. The growth of mycobacterial strains was monitored every 1 week for a period of 2 months (fig. 3.2 and fig.3.3).
Fig. 3.2: *Mycobacterium* culture on LJ media
Fig. 3.3: (A) & (B) Subcultures of *Mycobacterium* on Middlebrook7H11 agar media
3.2.3.5 Acid-Fast Staining

Staining procedures for mycobacteria are based on the acid-fast properties of the mycobacterial cell-wall, which is a bacterial cell wall composed of a thin, inner layer of peptidoglycan and large amount of glycolipids such as mycolic acid, arabinogalactan-lipid complex, and lipoarabinomannan. During the acid-fast staining procedure, the acid-fast cell wall enables the bacterium to resist decolorization with acid alcohol and retain the original stain. Acid-fast staining is usually a Ziehl Neelsen (ZN) stain and is indicative for the presence of mycobacteria. Mycobacteria are 1-10 µm in length and 0.2-0.6 µm in diameter and visible in different colors depending on the dye (Woods and Walker, 1996). In the modified Kinyoun and the Ziehl-Neelsen (ZN) stain the dye is carbolfuchsin and is directly visible with light microscopy. The staining method can be performed either on direct material or on decontaminated material, which can therefore be more concentrated. Sensitivity rates of the ZN stain is higher than those of the Kinyoun stain (Ulukanligil et al., 2000; Somoskovi et al., 2001) and are estimated between 60%-90% compared to culture (Ulukanligil et al., 2000; Lipsky et al., 1984). Low sensitivity of fluorescence microscopy and ZN staining is also negatively influenced by formalin fixation as applied in histopathological examinations (Fukunaga et al., 2002). The acid-fast staining procedures are not fully specific for mycobacteria, since Nocardia species, some Legionella species and some Corynebacteria are also (partially) stained acid fast (Shinnick and Good, 1994). However, no speciation is possible and it is considered a pre-screening of clinical materials.

Ziehl-Neelsen: heat drives in primary stain (carbolfuchsin) then decolorize with acid-alcohol and after that counterstain with methylene blue.
Fig. 3.4: (A) procedure of Ziehl-Neelsen staining (B) Z-N Stained *Mycobacterium*
3.2.3.6 Species Identification

Species cannot be differentiated by colony morphology and further analysis subsequent to culture is necessary. The conventional identification of species consisted of biochemical tests, colony characteristics and growth temperature. Biochemical species differentiation includes reduction of nitrate, reduction of tellurite, tween 80 hydrolysis, growth on LJ with 5% NaCl, pigment production, urease activity, catalase activity at 68°C, semi-quantitative catalase activity, growth activity with arylsulfatase.

3.2.3.6.1 Nitrate Reduction

- Species of mycobacteria differ quantitatively in the ability to reduce nitrate to nitrite.
- Nitrate substrate broth heavily inoculated with organism.
- After 2 hr at 37°C sulfanilamide and N-napthylethlenediamine added.
- Nitrite forms pink-red product (no color add zinc powder to confirm negative).

Fig.3.5: Showing results of Nitrate Reduction Test
3.2.3.6.2 Pyrizinamidase

- Enzyme pyrizinamidase hydrolyzes pyrazinamide (PZA) to ammonia and pyrazinoic acid.

- Agar with Dubos broth containing PZA heavily inoculated with organism and incubated at 37°C for 4 days.

- 1% ferrous ammonium sulfate added and agar observed after 4 hr.

- Pink band forms in agar from reaction of ferrous ammonium sulfate with pyrazinoic acid in a positive reaction.

Fig.3.6: Showing results of Pyrizinamidase Test
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3.2.3.6.3 Tween 80 hydrolysis

- Lipases produced by several mycobacterial species hydrolyze the detergent polyoxyethylene sorbitan monooleate (Tween 80) into oleic acid and polyoxyethylene sorbitol.

- Tween 80 substrate solution inoculated with organism contains neutral red which is bound to Tween 80 producing an amber color.

- With hydrolysis of Tween 80 neutral red was released and produced a red color. A change in the color of Tween 80 from amber to pink or red after 24 h, 5 days, or 10 days was considered as positive result for Tween 80 hydrolysis.

3.2.3.6.4 Iron uptake

- Iron uptake test utilized to identify rapidly growing mycobacteria capable of converting ferric ammonium citrate to an iron oxide.

- LJ slant inoculated with the organism incubated until visible growth develops, aqueous ferric ammonium citrate added, and the slant incubated for up to 21 days at 37°C.

- Development of reddish brown color in the colonies indicated production of iron oxide and is a positive result.
3.2.3.6.5 Tellurite Reduction

- The ability of mycobacteria to reduce potassium tellurite to metallic tellurium within 3-4 days is determined by this test.
- Heavily inoculated Middle brooke 7H9 broth was incubated at 370°C for seven days.
- A drop of sterile Potassium tellurite was added to each test culture and control. Tubes were shaken to mix well.
- Re-incubated at 370°C for 3 days on the 3 days culture was examined for sedimentation.

Result: POSITIVE – smooth, fine, black precipitate
NEGATIVE – gray clumps (no smoke-like action).

3.2.3.6.6 5% NaCl Tolerance test

This test is used to determine the ability of an organism to grow in high concentration of NaCl. It is used to distinguish rapid growers from slow growers [except for M. flavescens].

Result: POSITIVE – substantial growth (rapid growers)
NEGATIVE – little or no growth (slow growers)
3.2.3.6.7 Urease

- Urease production useful in identification of scotochromogens and nonchromogens.
- Urea broth with phenol red pH indicator inoculated with organisms visually read after 1, 3, and 7 days of incubation.
- Urease hydrolyzes urea to ammonia and CO2 increasing broth pH to alkaline values.
- Positive reaction is a pink to red color of phenol red at an alkaline pH.

Fig. 3.7: Showing results of Urease Test
3.2.3.6.8 Arylsulfatase

- Arylsulfatase hydrolyzes the sulfur ester bond linking the aromatic rings of tripotassium phenolphthalein disulfate, releasing free phenolphthalein.
- Although arylsulfatase activity can be detected with all mycobacteria with prolonged incubation, a 3-day incubation identifies several species.
- Dubos liquid medium containing tripotassium phenolphthalein disulfate is inoculated, incubated 3 days, and Na2CO3 added to alkalinize the medium.
- Free phenolphthalein turns a pink color at alkaline pH, and development of a pink color with addition of Na2CO3 is a positive reaction.

![Fig.3.8: Showing results of Arylsulfatase Test](image)
3.2.3.6.9 Catalase (Semi quantitative and Heat stable) test

Most of mycobacteria, except *M. tuberculosis* complex (INH resistant strain) and *M. gastri* (non photochromogen) produce intracellular catalase enzyme which converts \( \text{H}_2\text{O}_2 \) into water and oxygen.

(1) Semi quantitative catalase test:
- Production of bubbles
  - More than 45 mm elevation (+)
  - Less than 45 mm elevation (-)

(2) Ability of the enzyme to remain its activity after heating at 68 °C for 20 mins.

![Fig.3.9: Showing results of catalase test](image-url)
3.3 Results and Discussion:

Multiple colony types appeared on plates inoculated with tissue and water samples and cultured at 3 different incubation temperatures. Bacterial colonies which grew up overnight were excluded from further analysis. However, colonies which appeared on the second day of incubation onwards were ZN stained, according to the standard protocol. In total I got 18 positive samples with acid fast rods out of which 15 from fish and 3 from water samples. Pure colonies of acid-fast bacteria were identified according to their morphological appearance and using biochemical methods and growth tests (Wayne et al., 1993). In 18 positive samples I got 11 rapid growers, 5 slow growers and two strains of the same species which grew rapidly at 30°C and slowly at 37°C. Results of biochemical tests were shown in the table 3.1.

Due to long generation time and overgrowth by other micro-organisms this a difficult task to isolate mycobacteria from fish as well as environmental samples. Hence, non-mycobacteria have to be eliminated before culturing the samples. Paradoxically, a harsh decontamination has adverse effects on the recovery of mycobacteria while non-stringent treatments could result in overgrowth of other microbial flora (Kamala et al., 1994). Parashar et al. (2004) could also not isolate any mycobacteria from water due to contamination with other organisms. In turn, they developed a decontamination procedure by which treatment with 3% SDS plus 4% NaOH (15 and 30 min for rapid- and slow-growers, respectively) was followed by incubation with 2% cetrimide (5 and 15 min for rapidly- and slowly-growing mycobacteria, respectively); this procedure was found to
completely eliminate contamination with other organisms in water and wet soil samples and resulted in the isolation of only mycobacteria.

Apart from overgrowth of cultures by other organisms, the number of mycobacteria in the water samples may have been lower than the limit of sensitivity of the isolation method (Le Dantec et al., 2002). In this regard, the observation of few AFB in samples stained with ZN may prove this hypothesis. Another possibility for this finding is the lack of live mycobacterial cells in the samples. However, taking into consideration that NTM have an astonishing ability to survive under starvation conditions (Smeulders et al., 1999) and samples in this study, were carefully transported to lab, these factors are less likely for poor recovery of NTM. In this study, the clinical examination of infected ornamental fish indicated the presence of various clinical signs including ulcerations, detachment of the scales, exophthalmia, body deformity and external hemorrhages. These observations supported those reported by (Rychlik and Pavlik, 1997).

During necropsy there is significant potential for introduction of external mycobacterial contaminants whose presence can complicate isolation of the true pathogen (Rhodes et al., 2004). Before culture, clinical specimens from non-sterile body sites must be subjected to a pretreatment involving homogenization, decontamination, and concentration. This procedure will eradicate more rapidly growing contaminants such as normal flora (other bacteria and fungi) but not seriously affecting the viability of the mycobacteria (George and William, 1967). The isolation of mycobacteria takes a few days in rapidly growing mycobacterial species and several weeks or months in slow-growing species (Wayne et al., 1993). Sample decontamination is required to reduce background
overgrowth, to inactivate other bacteria that might be present in the sample, to avoid their faster growth and media nutrients exhaustion. It is noteworthy, however, that the efficacy of these procedures is highly influenced by the time of exposure to the reagent used for decontamination (George and William, 1967).

Determination of mycobacterial isolates by plating provides information regarding both intensity of infection and the presence of poly infections. It is clear that, although acid-fast microscopy and direct microscopic examination are important adjuncts to the detection of mycobacterial infections, they are not an adequate criterion alone and must be followed by growth detection. Thus, culture for the presence of mycobacteria is still indispensable for the following reasons: (1) culture is more sensitive for the detection of mycobacteria than acid-fast microscopy, (2) growth of those NTM that are not covered by the presently available NAA assays is necessary for precise identification, (3) drug susceptibility tests require viable organisms, and (4) genotyping of particular cultured NTM (Mangione et al., 2001). However, identification methods based on growth characteristics and biochemical tests are still considered much reliable. Conventional detection / identification of fish mycobacteria are based on histopathology, culture and biochemical characteristics. Culture examination revealed more mycobacteria than AFR detection by microscopy. In this study complementary molecular approach was used to assist in mycobacterium identification.
References


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LJ: Lowenstein–Jenner medium, RG: Rapid grower, SG: Slow grower, MG: Moderate grower, S: Scotochromogenic mycobacteria