[4] Diseases Caused By Bacterial Pathogens in Saltwater

seriolae in injected yellowtail. Unfortunately, the isolates provided minimal protection to a challenge with virulent *N. seriolae*.

2.7. Conclusion

In Japanese marculture, nocardiosis is a disease most difficult to control. No effective drug or a vaccine has so far been developed. Therefore, the only prophylaxis is to remove the infected fish, and not to give stress to fish. In the future, development of effective vaccine against this disease is much anticipated.

Glossary

BHIA: Brain heart infusion agar,

PCR: Polymerase chain reaction,

LAMP: Loop-mediated isothermal amplification,

3. MYCOBACTERIAL DISEASE

Kim D. Thompson and Alexandra Adams

3.1. Synopsis

Fish mycobacteriosis (or fish tuberculosis), caused by *Mycobacterium* spp., is a progressive disease affecting a wide range of wild and cultured marine and freshwater fish species. The economical losses experienced by the aquaculture industry due to mycobacteriosis, the lack of effective treatment regimes and the zoonotic nature of the bacteria involved, highlight the need for rapid methods to detect and identify the bacterial species associated with disease.

Detection and identification of the mycobacteria is traditionally based on histopathology, culture, and biochemical properties, although these do not offer the sensitivity or specificity of nucleic acid-based amplification methods. As with other bacterial pathogens, DNA-based techniques have revolutionised the identification and classification of the mycobacteria. *M. marinum*, *M. fortuitum* and *M. chelonae* are the species most predominantly associated with mycobacteriosis. However, the recent use of genotyping-based techniques has resulted in an increase in the identification of a number of new *Mycobacterium* spp. associated with mycobacteriosis, and highlighted problems with polymerase chain reaction (PCR) amplification due to cross-reactions of species-specific primers with closely related mycobacteria spp.

3.2. Introduction

Mycobacteriosis, caused by non-tuberculosis mycobacteria (or atypical mycobacteria), has been reported in a wide range of freshwater and marine fish species, and can result in significant economic losses to the aquaculture industry. Three species of *Mycobacterium*, *M. marinum*, *M. fortuitum* and *M. chelonae*, have been cited as the main species involved in these infections, although various other non-tuberculous mycobacteria, including a

number of new species, have also been associated with disease outbreaks in fish (Gauthier and Rhodes, 2009).

The occurrence of clinical outbreaks is associated with water temperature, over-crowding, poor nutrition and poor environmental conditions, and infection is believed to occur by both vertical and horizontal transmission.

Many of the mycobacteria spp. that infect fish also have the potential to infect humans, especially immunocompetent or immunosuppressed individuals. Phylogenetic studies have shown *M. marinum* to be closely related to *M. ulcerans* and *M. tuberculosis*, which shares many of their virulence factors and pathological traits. It is therefore used as a model organism to study the pathogenesis of *M. tuberculosis* in poikilotherm model species such as zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*).

Recent research has focused on more accurate methods of identifying and speciating the mycobacteria spp. involved in mycobacteriosis and the development of vaccines.

3.3. Disease Agent (Characteristics, Genome Size, Serological Classification, Molecular Classification, Pathogenesis)

Mycobacterium spp. belong to the family *Mycobacteriaceae* of the actinomycetes (Pitulle *et al.*, 1992). They are aerobic, non-motile, acid fast, Gram-positive bacteria (although this can be difficult to confirm), with a curved or rod shaped morphology (0.2-0.6×1.0-10 μm). Classification of species within the *Mycobacterium* genus is complex, and current classification is based on a polyphasic approach using phenotyping, chemo-taxonomy and molecular analysis (Adékambi and Drancourt 2004; Wallace *et al.*, 2005). Mycobacteria have a diverse range of phenotypic characteristics (Smole *et al.*, 2002), and are divided into two groups based on their pathogenicity i.e. tuberculosis and non-tuberculosis (or atypical) mycobacteria (Eisenstadt and Hall, 1995). Non-tuberculosis mycobacteria are characterised based on their growth (slow or rapid grow), their colony colour (white/cream to yellow/orange) and the biochemical composition of their cell walls. *Mycobacteria* have a chemotype IV cell wall composition containing mycolic acid. Phenotypic methods can have limited ability to discriminate between mycobacteria spp. due to interspecies homogeneity, intraspecies variability, and the existence of unclassified species (Springer *et al.*, 1996; Tortoli *et al.*, 2001). On the other hand, molecular taxonomy has had a significant impact on the classification of the genus *Mycobacterium* (Tortoli, 2003). The 16S ribosomal RNA (rRNA) sequence can differentiate between slow and rapidly growing species; the majority of slowly growing mycobacteria contain a long helix 18 at position 430 - 500 (Tortoli, 2003), and the majority of rapidly growing mycobacteria contain two copies of the 16S rRNA gene (Helguera-Repetto *et al.*, 2004).

Although PCR has been used for the rapid identification of *Mycobacterium* spp, there are problems with closely related species within the genus cross reacting with species-specific primers and commercially available probes. Additional methods are therefore required to identify mycobacteria to species level, such as PCR-restriction enzyme pattern analysis (PRA) and sequence analysis. Nucleic acid sequencing is frequently used to identify mycobacteria to species level, i.e. fragments or an entire gene,

amplified by PCR, are sequenced and compared to known sequences within available databases e.g. GenBank. Regions in the *Mycobacterium* genome used for this include the 16S rRNA gene (Kirschner *et al.*, 1993), the heat shock protein 65 gene (*hsp65*) (Ringuet *et al.*, 1999), the internal transcribed spacer 1 (ITS1) (Roth *et al.*, 1998), *dnaJ* (Yamada-Noda *et al.*, 2007), and the ß subunit of RNA polymerase gene (*rpoB*) (Kim *et al.*, 1999). The presence of identical/highly similar 16S rRNA sequences between species has limits its use as a target for species differentiation (Clarridge, 2004). As a result of the sequence variability within the 16S-23S spacer region, several ITS1 sequence based assays have been developed as an alternative region for the identification of mycobacteria (Pourahmad, 2007).

The *M. marinum* genome is composed of a single circular chromosome of 6,636,827 bp, with 5424 protein-coding sequences (CDS), 65 pseudogenes, 46 tRNA genes, a single rRNA operon and a 23 kb mercury-resistance plasmid (pMM23) (Stinear *et al* 2008). It also contains an ESX-1 secretion system essential for mycobacterial pathogenesis (Lewis & Chinabut, 2011).

As well as *M. marinum*, *M. fortuitum* and *M. chelonae*, several other mycobacteria spp. have been associated with mycobacteriosis in fish. A selection of these include *M. abscessus, M. avium, M. barombii, M. chesapeaki, M. gordonae, M. haemophilum M. lentiflavum-like, M. montefiorense; M. montefiorense*-like', *M. neoaurum, M. pregrinum/septicum, M. pseudoshottsii, M. salmoniphilum, M. scrofulaceum, M. shottsii, M. simiae, M. triplex-like* and *M. szulgai* (Gauthier & Rhodes, 2009; Pourahmad *et al* 2009; Zerihun *et al* 2011). The susceptibility of fish to *M. ulcerans* is still unclear (Gauthier & Rhodes, 2009; Mosi *et al*, 2012).

Mycobacteria are facultative intracellular bacterial pathogens (Pasnik *et al.*, 2003), able to survive and replicate in host macrophages by preventing phagosome maturation (Rybniker *et al.*, 2003; Solomon *et al.*, 2003). They are also zoonotic, and have been associated with tuberculoid infections in people using public swimming pools and are a hazard for people involved with aquaculture and aquarium-related industries and hobbies. The pathogen enters through cuts or broken skin, resulting in the formation of skin lesions on hands or extremities 3 weeks to 9 months after contact with the pathogen.

3.4. Diagnostic Methods (Clinical Signs, Gross Pathology, Histopathology, Diagnosis: PCR, Antibody)

Mycobacteriosis is a chronic disease, sometimes taking months or years for clinical signs to appear (Hedrick *et al.*, 1987; Knibb *et al.*, 1993). The severity of the disease can vary dramatically, with only a few fish dying from chronic infections or high levels of mortality during severe outbreaks (Whipps *et al.*, 2003). Clinical signs of mycobacteriosis tend to be non-specific, but may include emaciation, anorexia, exophthalmia, ascites, pigmentation changes and dermal ulcerations. On post-mortem examination, grey-to-white granulomas are evident (e.g. especially in anterior kidney, spleen, mesenteries and liver). Identification of acid fast bacteria in lesions by Ziehl-Neelsen (Figure 3.1) is, however, both non-specific and not especially sensitive.

Culture is traditionally considered the gold standard for definitive diagnosis of *Mycobacterium*, using specialised media such as Lowenstein-Jensen, Petragnani, Sautons, Middlebrook 7H10, and Dorset egg media at 20 to 30^{o}C. It can take between 2 to 30 days before colonies appear and often cultures become overgrown with contaminating bacteria. Initial characterisation of bacteria is based on growth characteristics (e.g. pigment production, growth rate, and gross and microscopic colony morphologies) and biochemical activity.

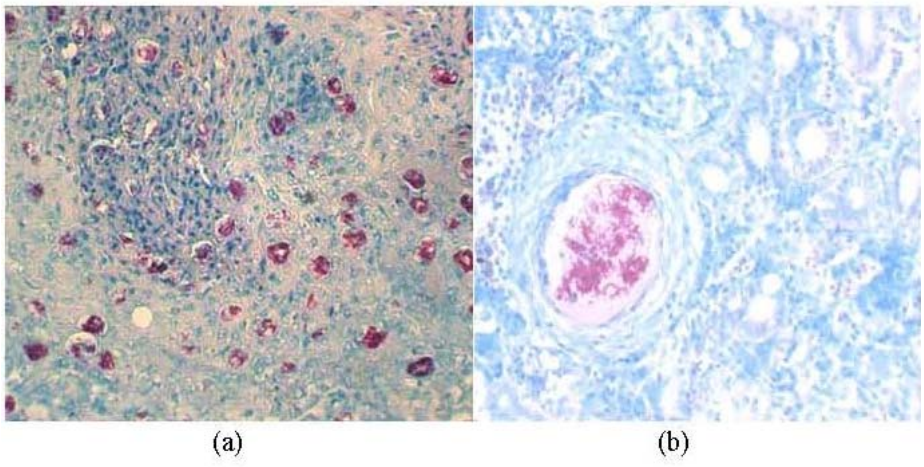

Figure 3.1. Representative Ziehl-Neelsen-stained sections of fish tissues showing granumatous reactions. (a) Spleen tissue of a cichlid following natural infection with *M. stomatepiae* (x200) (b) Kidney tissue of a rosy barb naturally infected with *Mycobacterium* sp. (x·400) (modified from Pourahmad *et al*, 2009)

Molecular detection methods based on nucleic acid amplification have great potential for rapid diagnosis of mycobacteria (i.e. PCR amplification of species-specific sequences (Devallois *et al.*, 1996), amplification and restriction enzyme analysis (Telenti *et al.*, 1993; Talaat *et al.*, 1997), hybridization with species-specific DNA probes (Alcaide *et al.*, 2000), PCR followed by reverse cross blot hybridization (Puttinaowarat *et al.*, 2002) and DNA sequencing (Kirschner *et al.*, 1993; Swanson *et al.*, 1996).

(b)

Monoclonal antibodies have been produced against various mycobacteria for use in immunohistochemistry, ELISA and Western blotting (Verstijnen *et al.*, 1991, Adams *et al.*, 1995 and 1996; Blackwell *et al.*, 2001). However, false-positive results from cross-reaction with environmental mycobacteria can be an issue (Ramachandran and Paramasivan, 2003).

3.5. Control (Prevention, Chemotherapy, Vaccine)

Mycobacterium infections in fish can be very difficult to treat, especially since no commercial vaccines are available for mycobacteriosis. Treatment with antibiotics can be time-consuming, expensive, and the bacterium is often resistant to the antibiotics commonly used in aquaculture. Also, there are no authorized antibiotics currently available to treat *Mycobacterium* infections in fish (Stoffregen *et al.*, 1996). It is also unclear if the use of antibiotics eliminates the bacterium or if fish become asymptomatic

carriers as a result. In light of this, the best approach for controlling mycobacteriosis is through good bio-security. The most effective course is to destroy infected stock, disinfect the system, and restock with *Mycobacterium*-free fish.

Mycobacteria are more resistant to disinfection than other bacteria; their waxy cell wall protects them from many of the commonly used disinfectants. Ethyl alcohol (50% and 70%), benzyl-4-chlorophenol/phenylphenol (1%), and sodium chlorite (mixed as 1:5:1 or 1:18:1[base: water: activator]) have been shown to be good mycobacteriocidal agents, killing all detectable *M. marinum* within 1 min of contact (Mainous and Smith, 2005). Lysol®, Roccal® and Virkon® are three commercially available mycobacteriocidal agent used in aquaculture.

Stringent quarantine procedures are required prior to adding new fish to a system. Quarantine times of 30 days are required to allow clinical signs of mycobacteriosis to develop, and additional confirmation that fish are *Mycobacterium*-free may be necessary, and the sensitivity of molecular methods may be necessary to confirm the fish's *Mycobacterium* status. It may not be possible to kill expensive ornamental fish to screen for mycobacteria however, and there are currently no non-lethal sampling methods available for screening fish for mycobacteria.

3.6. Recent Topics

There has been increased interest in the development of improved methods to detect and differentiate the *Mycobacterium* spp infecting fish. This, in part, is due to the increased incidence of mycobacteriosis in aquaculture systems, not only for food and ornamental fish, but also for zebra fish used in clinical research to develop specific pathogen-free colonies. Recent developments for detection include FRET assays (Salati *et al.*, 2009) and sensitive real time PCR methods (Lloyd *et al.* 2009, Parikka *et al.* 2012), and for differentiating *Mycobacterium* spp., polygenic sequencing (Pourahmad, 2007; Kurokawa *et al*. 2012) and a MALDI Biotyping system (Kurokawa *et al*. 2012). Attempts to develop an effective vaccine for mycobacteriosis also continues (Kato *et al.* 2010, 2011).

Glossary

PCR: Polymerase chain reaction,

FRET assays: Fluorescence/förster resonance energy transfer

4. PASTEURELLOSIS

Tae-Sung Jung and Takashi Aoki

4.1. Synopsis

Pasteurellosis, caused by *Photobacterium damselae* subspecies *piscicida*, appeared in 1963 and has since been causing problems to the aquaculture industry. It is especially known to infect various fish species including yellowtail in Japan and sea bass and sea bream in the Mediterranean region. Pasteurellosis can be controlled using antibiotics at the initial stages of infection but have also led to the emergence of antibiotic resistant