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ScienceDirect
The Veterinary Journal 180 (2009) 33–47
The Veterinary Journal
www.elsevier.com/locate/tvjl

Review

Mycobacteriosis in fishes: A review

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Accepted 13 May 2008

Abstract

Mycobacterium species have long been recognised as a significant source of morbidity and mortality in finfish aquaculture, as well as in wild finfishes. Mycobacteria infecting fishes also include zoonotic pathogens that can cause protracted illness, especially in immunocompromised individuals. Several basic aspects of mycobacterial pathobiology in aquatic animals remain poorly understood, although a number of important recent developments have been made, especially with respect to identification of novel *Mycobacterium* spp. infecting fishes and a new group of mycobacteria closely related to the human pathogen *Mycobacterium ulcerans*. This review will encompass important aspects of mycobacterial disease in fishes, discuss recent research including studies of mycobacteriosis in striped bass (*Morone saxatilis*) of Chesapeake Bay, USA, and suggest directions for future work.

Keywords: *Mycobacterium* spp.; Fish; Mycobacteriosis; Chesapeake Bay; *Morone saxatilis*

Introduction

Mycobacteriosis in fishes was first described in carp (*Cyprinus carpio*) from water potentially contaminated with *Mycobacterium tuberculosis* (Bataillon et al., 1897). The carp isolate, '*Mycobacterium piscium*,' was highly pathogenic to frogs, and infections were reportedly established experimentally in endothermic animals (reviewed by Parisot, 1958). The identity of '*M. piscium*' is presently uncertain, as type isolates are not available. Three *Mycobacterium* spp. have since dominated the literature on fish diseases: *Mycobacterium marinum*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae*.

The archaic names '*Mycobacterium platypoecilus*' (Baker and Hagan, 1942) and '*Mycobacterium anabanti*' (Besse, 1949), are considered synonymous with *M. marinum* (Van Duijn, 1981), whereas '*Mycobacterium ranae*' (Stanford and Gunthorpe, 1969) is considered to be *M. fortuitum*. '*Mycobacterium borstelense*' and '*Mycobacterium runyonii*' have been grouped under *M. chelonae* (Kubica et al., 1972). '*Mycobacterium salmoniphilum*,' originally described from salmonids by Ross (1960), has not had recognized species status since 1980 due to its high biochemical similarity to *M. fortuitum* and *M. chelonae*. Recent work, however, has indicated that '*M. salmoniphilum*' is a valid, distinct species (Whipps et al., 2007a). *Mycobacterium marinum* was first isolated from captive marine fishes in 1926 (Aronson, 1926), and has traditionally been regarded as a warm-water pathogen (Frerichs, 1993), although infections in temperate and coldwater species are known (Dalsgaard et al., 1992; dos Santos et al., 2002; Rhodes et al., 2004). *Mycobacterium fortuitum* was first isolated from the neon tetra (*Paracheirodon innesi*) in 1953 (Ross and Brancato, 1959), and is not highly host-specific with regards to temperature or salinity. *Mycobacterium chelonae* (*M. chelonei* [*sic*]), originally isolated from a turtle in 1903, was later found in chinook salmon (*Oncorhynchus tshawytscha*) and other Pacific salmonids (Ashburner, 1977; Grange, 1981).

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doi:10.1016/j.tvjl.2008.05.012

The mycobacteria

Mycobacteria, comprising the genus *Mycobacterium* (Order *Actinomycetales*, Suborder *Corynebacterineae*, Family *Mycobacteriaceae*), are pleomorphic, Gram-positive, acid-fast, aerobic, non-motile rods, 0.2–0.6 μm in diameter and 1–10 μm long. Mycobacteria have a unique cell wall that includes long-chain (60–90 carbon) 3-hydroxy mycolic acids (Draper, 1971; Gangadharam and Jenkins, 1997). As of June 2008, 130 *Mycobacterium* species and 11 subspecies were recognized by the *list of prokaryotic names with standing in nomenclature* (LPSN) (Euzéby, 1997). Mycobacteria differ greatly in their ecology, from the obligate pathogen *M. tuberculosis*, a leading cause of human mortality worldwide, to saprophytic soil residents such as *Mycobacterium terrae*.

According to the commonly used Runyon classification scheme, mycobacteria, with the exception of those not culturable in vitro (e.g., *Mycobacterium leprae*), are functionally separated by growth rate and pigmentation. Fast-growers require less than 7 days to produce colonies on solid agar, while slow-growers may require weeks to months to achieve comparable growth. With some exceptions, slow-growers have an extended helix 18 of the small subunit (16S) rRNA molecule and one rRNA operon. Fast-growers typically have a shorter helix 18 and two rRNA operons (Bercovier et al., 1986; Rogall et al., 1990; Menendez et al., 2002). Both fast- and slow-growing species may be non-pigmented, photochromogenic (form pigment in response to light), or scotochromogenic (form pigment in the absence of light). Beyond these distinctions, the nomenclature of *Mycobacterium* spp. is in a state of flux (reviewed by Gangadharam and Jenkins, 1997).

Mycobacteria from fishes

An increasingly diverse array of *Mycobacterium* spp. has been isolated from fishes in recent years, supplanting the paradigm of *M. marinum*, *M. fortuitum*, and *M. chelonae* as the only agents causing piscine mycobacteriosis (Table 1). Although many isolates from fishes have been assigned to recognized species, considerable caution must be exercised in accepting these designations.

Prior to the widespread use of molecular identification methods, mycobacteria from fishes were identified phenotypically, frequently based on a limited number of characteristics. For example, a reported isolation of *Mycobacterium simiae* and *Mycobacterium scrofulaceum* from fishes was based on only three and four biochemical tests, respectively (Lansdell et al., 1993). The danger of assigning species based on phenotype alone is demonstrated by a *Mycobacterium* spp. from snakehead fish (*Channa striata*) that was originally identified as *Mycobacterium poriferae* on the basis of biochemical characteristics and on high-performance liquid chromatography (HPLC) analysis of mycolic acids (Tortoli et al., 1996). Subsequent work using DNA hybridization indicated non-identity with a reference strain of *M. poriferae* whereas positive hybridization with *M. fortuitum*-specific probes was observed (Puttinaowarat et al., 2002).

In addition to limited power to discriminate species, phenotypic methods are subject to variability in performance and interpretation (Springer et al., 1996). Consequently, identification of *Mycobacterium* spp. from fishes made solely on the basis of phenotypic testing should be considered presumptive. In Table 1, we include several isolates that were identified phenotypically in frequently cited papers before the widespread use of molecular characterization (Backman et al., 1990; Lansdell et al., 1993). We do not, however, include other isolates identified by phenotype alone as reported in a recent study of aquarium fishes (Beran et al., 2006).

The important human pathogen *Mycobacterium ulcerans* (van der Werf et al., 2005) is not included in Table 1, despite its reported presence in *Tilapia* spp. (Kotlowski et al., 2004). Detection of *M. ulcerans* in this case was based on positive PCR results for the insertion sequence IS*2404*, which can no longer be considered specific for this mycobacterium (Ranger et al., 2006; Yip et al., 2007).

Mycobacterium abscessus was considered a subspecies of *M. chelonae* until its recent elevation to species status (Kusunoki and Ezaki, 1992). Prior to the 1990s, biochemical tests capable of differentiating between *M. chelonae* subsp. *chelonae* and *M. abscessus* were not frequently performed on human clinical isolates (Brown-Elliott and Wallace, 2002), an omission also observed in the study of fish isolates (e.g., Daoust et al., 1989), thus preventing determination of the specific etiological agent in these cases. An additional subspecies of *M. chelonae*, *M. chelonae* subsp. *piscarium*, was proposed based on biochemical characteristics (Arakawa and Fryer, 1984), but was then withdrawn because of lack of serological differences with *M. chelonae* subsp. *chelonae* (Arakawa et al., 1986). Recent work has indicated that isolates of '*M. chelonae* subsp. *piscarium*' may be '*M. salmoniphilum*' (Whipps et al., 2007a).

Mycobacterium peregrinum, a member of the *M. fortuitum* group (Brown-Elliott and Wallace, 2002; Schinsky et al., 2004) has recently been described as a fish pathogen (Kent et al., 2004). *Mycobacterium peregrinum* is differentiated from *M. fortuitum* by biochemical tests and restriction analysis of the *hsp*65 gene (Kusunoki and Ezaki, 1992; Brown-Elliott and Wallace, 2002). Adequate tests to differentiate these species have not always been performed (e.g., Frerichs, 1993; Lansdell et al., 1993).

There has been much recent interest in newly described *Mycobacterium* spp. closely related to *M. ulcerans* and *M. marinum*. These isolates are characterized by the presence of insertion sequences IS*2404* and IS*2606*, as well as by the production of the polyketide toxin, mycolactone. Both mycolactone production and the presence of IS*2404* were thought to be restricted to *M. ulcerans* prior to the discovery of an IS*2404*/IS*2606*-positive mycobacterium from *Xenopus* frogs (Trott et al., 2004). Subsequent work

Table 1
A review of *Mycobacterium* spp. identified from finfishes

Species	Host	Identification methods[a]	Reference
M. abscessus	Zebrafish (*Danio rerio*)	B/P	(Teska et al., 1997; Astrofsky et al., 2000; Chang et al., 2006)
	Medaka (*Oryzias latipes*)	B/P	
	Milkfish (*Chanos chanos*)	B/P, *hsp*65[f]	
M. avium	Dwarf Cichlid (*Apistogramma cacatuoides*)	B/P, IS	(Lescenko et al., 2003)
M. chelonae[b]	Multiple	Multiple	(Ashburner, 1977; Arakawa and Fryer, 1984; Bruno et al., 1998; Whipps et al., 2007a)
'*M. chesapeaki*'[c]	Striped bass (*Morone saxatilis*)	B/P, 16S	(Heckert et al., 2001)
M. fortuitum	Multiple	Multiple	See text
Mycobacterium gordonae	Goldfish (*Carassius auratus*)	GTM	(Lescenko et al., 2003; Pate et al., 2005; Sakai et al., 2005)
	Guppy (*Poecilia reticulata*)	B/P, GTM, 16S[d], ITS[d]	
	Angelfish (*Pterophyllum scalare*)	B/P, GTM	
	Others	B/P	
Mycobacterium haemophilum	Zebrafish (*Danio rerio*)	16S, ITS, *rpo*B, *hsp*65	(Whipps et al., 2007b)
'*Mycobacterium lentiflavum*-like'	Swordtail (*Xiphophorus hellerii*)	16S[f], ITS[f], *hsp*65[f]	(Poort et al., 2006)
M. marinum	Multiple	Multiple	See text
M. marinum[e]	Sea bass (*Dicentrarchus labrax*)	B/P, HPLC, MLS	(Ucko et al., 2002; Ucko and Colorni, 2005; Ranger et al., 2006)
	Others		
M. montefiorense	Moray eel (*Gymnothorax funebris*)	B/P, 16S, ITS, *hsp*65	(Herbst et al., 2001; Levi et al., 2003)
'*M. montefiorense*-like'	Rockfish (*Sebastes* spp.),	16S[f], ITS[f]	(Whipps et al., 2003; Gauthier et al., unpublished data)
	Rainbow trout (*Oncorynchus mykiss*)	B/P, 16S, ITS	
M. peregrinum/septicum	Zebrafish (*Danio rerio*)	B/P, 16S, ITS	(Kent et al., 2004; Pate et al., 2005)
	Cichlid (*Pseudotropheus lombardoi*)	GTM	
Mycobacterium neoaurum	Chinook salmon (*Oncorhynchus tschawytscha*)	B/P	(Backman et al., 1990)
M. pseudoshottsii	Striped bass (*Morone saxatilis*)	B/P, 16S, *hsp*65, *erp*, HPLC	(Rhodes et al., 2004; Rhodes et al., 2005)
M. scrofulaceum	Pacific staghorn sculpin (*Leptocottus armatus*)	B/P	(Lansdell et al., 1993; Perez et al., 2001)
	Silver mullet (*Mugil curema*)	B/P	
M. shottsii	Striped bass (*Morone saxatilis*)	B/P, 16S, *hsp*65, *erp*, HPLC	(Rhodes et al., 2003; Rhodes et al., 2004; Rhodes et al., 2005)
M. simiae	Black acara (*Cichlasoma bimaculatum*)	B/P	(Lansdell et al., 1993)
'*M. triplex*-like' (multiple)	Striped bass (*Morone saxatilis*)	B/P, 16S	(Rhodes et al., 2004, this work)
Mycobacterium szulgai	?	B/P, *hsp*65	(Abalain-Colloc et al., 2003)

[a] B/P, biochemical/phenotypic characterization; 16S, 16S rRNA; ITS, 16S–23S rRNA intergenic spacer region; *erp*, external repeated protein; *hsp*65, 65 kDa heat shock protein; *rpo*B, RNA polymerase B; MLS, sequenced at multiple loci (i.e., *erp*, *hsp65*, others); HPLC, mycolic acid analysis; GTM, strip hybridization (GenoType assay, Hain Lifescience); IS, PCR amplification of insertion sequences (see text).
[b] Recent work proposes revival of the name *M. salmoniphilum* for isolates of *M. chelonae* from salmonids (see text).
[c] See text.
[d] Isolates Thai-3 and-4 were highly similar to *M. gordonae* based on 16S (97.8%, 98.2% similarity, respectively) and ITS (98.5%, 98.1%) sequences.
[e] *M. marinum* as identified by phenotype and 16S rRNA gene sequence, but positive for IS *2404* and producing mycolactone F (Ranger et al., 2006).
[f] Detected *via* direct PCR on infected tissues.

on this isolate, given the provisional name of '*Mycobacterium liflandii*,' demonstrated a plasmid similar to the mycolactone-encoding pMUM in *M. ulcerans* as well as mycolactone production (Mve-Obiang et al., 2005).

A variety of IS*2404*-positive, mycolactone-producing mycobacteria (MPM) have since been described, including *Mycobacterium pseudoshottsii* from Chesapeake Bay striped bass (*Morone saxatilis*) as well as several isolates from fishes in the Red and Mediterranean Seas (Rhodes et al., 2005; Ranger et al., 2006). These isolates are postulated to have arisen from a common *M. marinum* ancestor and adapted to specific niches via acquisition of pMUM, and in some cases (e.g., *M. ulcerans*), by genomic degeneration (Stinear et al., 2000; Stinear et al., 2007; Yip et al., 2007). It is likely that the diversity of mycobacteria infecting fishes is presently under-recognised, especially with respect to mycolactone-producing and non-producing mycobacteria closely related to *M. marinum*.

Clinical signs and pathology

Piscine mycobacteriosis is predominately a chronic disease that may not produce clinical signs. All tissues of the fish may be involved, including eyes, gills, visceral organs, musculature, and fins. External clinical signs are nonspecific and include scale loss and dermal ulceration (Fig. 1), pigmentary changes, abnormal behavior, spinal defects, emaciation, and ascites (Nigrelli and Vogel, 1963; Ross, 1970; Snieszko, 1978; Wolke and Stroud, 1978; Bruno et al., 1998). Gross internal signs of infection include enlargement of the spleen, kidney and liver, and characteristic grey or white nodules in internal organs (Chinabut, 1999). Acute disease is occasionally observed in association with high bacterial loads (Wolf and Smith, 1999; Whipps et al., 2007b). Mycobacteriosis is not typically associated with large-scale mortality in wild fishes, although significant losses attributed to the disease have occurred in aquaculture (Hedrick et al., 1987; Bruno et al., 1998).

Granulomatous inflammation (Fig. 2) is the classic histopathological manifestation of piscine mycobacteriosis (Chinabut, 1999; Noga, 2000). Granulomas are composed of concentric layers of epithelioid cells forming a discrete spherical lesion. Epithelioid cells, so-called because of their morphological similarity to epithelial cells, are polygonal, eosinophilic cells with open-faced nuclei, prominent nucleoli, and faintly granular cytoplasm (Cotran et al., 1999). Mammalian epithelioid cells are derived from mononuclear phagocytes and do not possess desmosomes (Adams, 1974, 1975, 1976). Desmosomes have been found between epithelioid cells of fish granulomas, suggesting that these cells may arise from a different lineage than those in mammals (Noga et al., 1989; Gauthier et al., 2004).

Generally, piscine mycobacteriosis is a systemic disease with granulomas produced in multiple organs or tissues. Significant variation in the size and structural organization of granulomas is seen, from highly organized lesions with thick epithelioid layers to poorly organized inflammation with minimal epithelioid cell formation. Caseous necrosis and/or calcification of the core region may be observed, in a manner similar to that seen in human tuberculosis. A fibrotic and/or a leukocyte-rich capsule frequently surrounds fish granulomas. Multinucleate giant cells, characteristic of mycobacterial granulomas in mammals (Adams, 1976) have been reported in fishes, however the majority of mycobacterial lesions described in fishes lack these cells (Majeed et al., 1981; Bruno et al., 1998; Colorni et al., 1998; Gauthier et al., 2003).

Fig. 1. Ulcerative skin lesions attributed to infection with *Mycobacterium* spp. in striped bass (*Morone saxatilis*) from Chesapeake Bay, USA.

Secondary disease has been observed in striped bass infected with *M. marinum* (Gauthier et al., 2003). A granulomatous response lasting 26–32 weeks post-injection was followed by granuloma disintegration, visceral inflammation and fibrosis, and high splenic bacterial density of greater than 10^9 bacteria/g. This suggests *M. marinum* infection in poikilotherms may be useful in modeling failure of host immunity as observed in primary progressive or secondary tuberculosis.

Pathogenesis

With the exception of *M. ulcerans*, pathogenic mycobacteria in vertebrates are predominately intracellular parasites of phagocytes. Phagosomes containing mycobacteria are thought to resist the normal processes of acidification and phagolysosomal fusion (Brown et al., 1969; Armstrong and Hart, 1971; Crowle et al., 1991; Sturgill-Koszycki et al., 1994), thus promoting bacterial survival. Furthermore, pathogenic mycobacteria are thought to retard maturation of mammalian phagosomes and their progression to the phagolysosomal state by preventing fusion with components of the late, but not early, endosomal network (Clemens and Horwitz, 1995; Clemens, 1996; Russell, 2001). *Mycobacterium marinum* in mouse macrophages has also been shown to escape phagosomes and spread directly from cell to cell via actin-based motility (Stamm et al., 2003). van der Wel et al. (2007) present evidence of lysosomal fusion with *M. tuberculosis* and *M. lepraе*-infected phagosomes, and the subsequent escape of mycobacteria into the cytosol.

The behavior of mycobacteria within the macrophages of poikilotherms is less well characterized than in mammals. Using cell lines derived from carp leukocytes, El-Etr et al. (2001) demonstrated inhibited fusion of thorium dioxide-labeled lysosomes with *M. marinum*-containing phagosomes relative to phagosomes containing non-pathogenic *Mycobacterium smegmatis*. We have found, however, that *M. marinum* phagosomes in striped bass peritoneal macrophages exhibit evidence of fusion in vivo (Gauthier et al., 2004) and in vitro (Gauthier and Vogelbein, 2003) with membrane-bound electron-opaque organelles morphologically consistent with lysosomes. Similar observations have been made on frog (Bouley et al., 2001), and fish tissues (Chen et al., 1998). These observations suggest

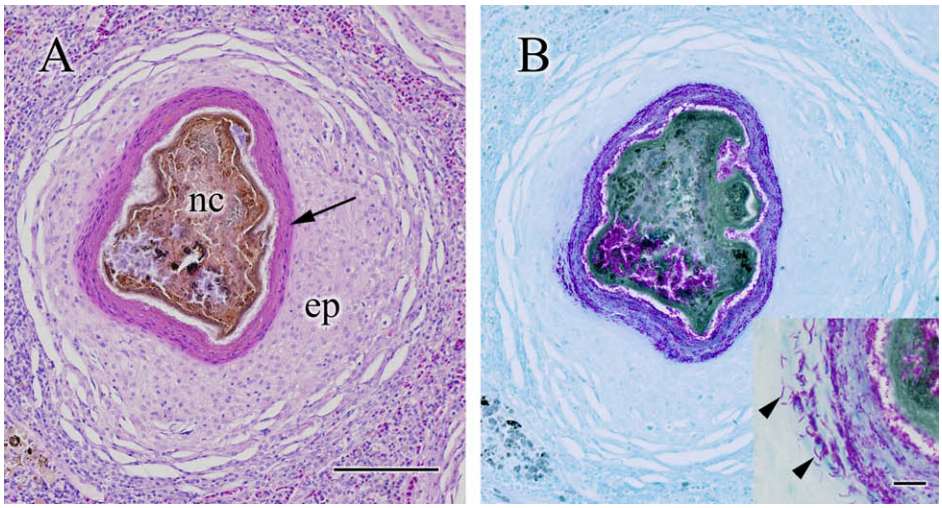

Fig. 2. *Mycobacterium marinum* granuloma induced in the spleen of a striped bass by intraperitoneal injection (Gauthier et al., 2003). (A) Photomicrograph of granuloma with necrotic core (nc), compressed epithelioid cell (spindle-cell) layer (arrow), and a epithelioid cell layer (ep). Hematoxylin and eosin stain (bar = 100 μm). (B) Photomicrograph of Ziehl–Neelsen-stained section with inset illustrating *M. marinum* bacteria (arrowheads) in the necrotic core and in the surrounding cellular layers. Ziehl–Neelsen stain (Inset bar = 10 μm).

that multiple intracellular survival strategies may be used by intracellular mycobacteria, and indicate that caution should be exercised when using pathogenic mycobacteria of poikilotherms as models of human mycobacteriosis.

Mycobacterium marinum infection of poikilotherms has received considerable interest as a model for human *M. tuberculosis* infection. *M. marinum* and *M. tuberculosis* are genetically related, and *M. marinum* produces granulomatous disease in poikilotherms that is at least superficially similar to human tuberculosis. *M. marinum*, as a biosafety level 2 organism, has considerable advantages in terms of laboratory safety procedures over *M. tuberculosis*, a biosafety level 3 organism, and *M. marinum* grows more rapidly in culture.

Although goldfish (*Carassius auratus*) and leopard frogs (*Rana pipiens*) have been proposed as poikilotherm experimental model candidates (Ramakrishnan et al., 1997; Talaat et al., 1998), zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*) appear to be emerging as the model species of choice. Zebrafish are particularly attractive in this context as they are easily propagated, their genome is sequenced, mutant and transgenic lines are available, and substantial research has been performed on the genetics and immunological function of this species (Prouty et al., 2003; Van der Sar et al., 2004; Sprague et al., 2006). The medaka is also an appealing model, with an extensive research history in toxicology and carcinogenesis, transgenic lines, and a draft genome (Kohara and Morishita, 2007). Lines of transparent zebrafish and medaka have been developed, allowing for whole-animal trafficking studies (Wakamatsu et al., 2001; Broussard and Ennis, 2007; White et al., 2008).

Mycobacterium ulcerans is unique among mycobacterial pathogens of humans in that it is predominately extracellular and secretes mycolactone (George et al., 1999; Cosma et al., 2003). Mycolactone causes apoptosis and necrosis of cultured cells in vitro (George et al., 1999; Dobos et al., 2001), inhibits pro-inflammatory cytokines and phagocytosis (Pahlevan et al., 1999; Coutanceau et al., 2005), and is responsible for the progressive necrotising skin disease seen in humans afflicted with Buruli ulcer (George et al., 2000). A number of variant mycolactones (A–D) have been identified in *M. ulcerans* from different geographical locations. Mycolactones also have been recovered from pathogens of poikilotherms, including mycolactone E from '*M. liflandii*' and mycolactone F from fish pathogens (Ranger et al., 2006). *M. ulcerans* mycolactones A and B are more cytopathic in cultured mouse cells than other forms produced in endo- and poikilothermic hosts (Mve-Obiang et al., 2003; Mve-Obiang et al., 2005; Ranger et al., 2006), however the effects of these mycolactone congeners on cultured fish cells have not been reported.

Epidemiology

Mycobacteriosis is one of the most common diseases affecting cultured and wild fishes worldwide. A review in 1963 described 151 species of fishes, representing 40 families, affected by mycobacteriosis (Nigrelli and Vogel, 1963). This list has since expanded considerably, and mycobacterial infections have been described in freshwater and marine fishes from tropical to subarctic latitudes (Lund and Abernethy, 1978; Arakawa and Fryer, 1984; MacKenzie, 1988; Daoust et al., 1989; Noga et al., 1990; Lansdell et al., 1993; Bruno et al., 1998; Diamant et al., 2000; Rhodes et al., 2004).

Transmission of mycobacteria in fishes is poorly understood. Water and associated biofilms are natural habitats for *Mycobacterium* spp. (Pedley et al., 2004)

including *M. marinum*, *M. fortuitum*, and *M. chelonae*, so waterborne transmission seems likely. Mycobacteria are known to infect a number of aquatic organisms other than fishes (Nigrelli and Vogel, 1963; Mohney et al., 1998) and survive and replicate within various protozoan hosts (Cirillo et al., 1997; Steinert et al., 1998; Skriwan et al., 2002), so vectors are potentially present throughout the food web.

Fishes are believed to be infected by ingestion of contaminated food and water (Parisot, 1958; Wood and Ordal, 1958; Ross and Brancato, 1959; Ross, 1963; Gauthier et al., 2003; Nenoff and Uhlemann, 2006). Vertical transmission of mycobacteria has been suggested (Ashburner, 1977; Chinabut, 1999), and transovarian transmission in live-bearing fishes has been reported (Conroy, 1966).

Most experimental infections have used the intraperitoneal and intramuscular routes which, although convenient, are artificial (Wolf and Smith, 1999; Gauthier et al., 2003). Zebrafish embryos have been infected with *M. marinum* via bath exposure (Davis et al., 2002), and both bath exposure and gavage have been used to infect adult zebrafish with *M. marinum* and *M. peregrinum* (Hariff et al., 2007). The latter study indicated that the gut, rather than the gills, was the primary site of infection. The use of natural routes of exposure to various *Mycobacterium* spp. and the study of the resultant pathology are clearly critical areas of future research if the pathogenesis and epidemiology of mycobacteriosis in fishes is to be further elucidated. Furthermore, effective measures for controlling mycobacteriosis in both aquacultural and natural settings will likely depend on this information.

Although mycobacteriosis is considered to be precipitated by stress, specific factors leading to disease outbreaks are seldom defined, and appear to vary between systems. Host factors likely play a role, as demonstrated by the differing susceptibility of medaka and zebrafish to *M. marinum* (Broussard and Ennis, 2007). The initial dose influences disease outcome, as evidenced by studies in which *M. marinum* produces chronic disease when a low initial inoculum is used and acute or subacute disease with higher infecting doses of greater than 10^7 CFU/g (Talaat et al., 1998; Prouty et al., 2003). Whereas *M. marinum* causes pathology in most experimental settings, consistent production of disease with other *Mycobacterium* spp. has been problematic. *Mycobacterium shottsii*, a dominant and often solitary isolate observed at high density in diseased striped bass in Chesapeake Bay (Rhodes et al., 2004), established chronic infections in experimentally exposed striped bass (Gauthier and Vogelbein, 2003). In this context, however, the disease was mild and did not resemble the severe visceral or ulcerative skin disease observed in wild fish (Gauthier et al., 2003). Similarly, isolates identified as *M. abscessus*, *M. chelonae*, and *M. peregrinum*, originally isolated from moderate to severe spontaneous disease outbreaks in zebrafish colonies, failed to produce severe disease when re-injected into zebrafish (Watral and Kent, 2007).

Diagnostics

Mycobacteria are best visualised in tissue sections with the Ziehl–Neelsen acid-fast stain, which is based on the resistance, or 'acid-fastness' of the mycobacterial cell wall to acid–alcohol decolourisation after staining with carbolfuchsin. Related actinomycetes such as *Nocardia*, *Rhodococcus*, *Tsukamurella*, *Gordonia*, and *Rhodococcus*, are partially acid-fast (Holt, 1994), and *Legionella* spp. occasionally demonstrates some acid-fastness (Bentz et al., 2000). Acid-fast bacilli are frequently visible within piscine mycobacterial granulomas, although granulomas containing no visible acid-fast bacilli have been reported in several experimentally infected species (Colorni et al., 1998; Gauthier et al., 2003; Watral and Kent, 2007). Lack of staining has been associated, in some instances, with tissue decalcification procedures (Watral and Kent, 2007). Fite's acid-fast stain or pretreatment of sections with periodic acid may improve detection (Daoust et al., 1989; Gauthier et al., 2003). Immunohistochemical detection may be useful for visualizing mycobacteria in early granulomas (Gomez et al., 1993; Sarli et al., 2005).

Mycobacteria can be effectively cultured on a variety of selective liquid and agar media (Frerichs, 1993; Teska et al., 1997; Chinabut, 1999; Rhodes et al., 2004). Plating on solid medium further allows isolation of mycobacteria in the presence of non-mycobacterial contaminants. Spread-plating of homogenates from weighed tissue and dilutions thereof permits quantification of mycobacterial densities (Talaat et al., 1999; Rhodes et al., 2004). Additionally, colony morphology may reveal mixed mycobacterial and non-mycobacterial infections (Rhodes et al., 2004). Research directed at the use of growth-enhancing additives such as mycobactin is needed as some mycobacteria grow very slowly on standard media. Non-selective media may support the growth of fast-growing mycobacteria, but are ineffective for slow-growers.

Culture at room temperature or at the environmental temperature at which fishes are collected is recommended. Some fish pathogens such as *M. marinum* will grow at 30 °C whereas others such as *M. shottsii* and *M. pseudoshottsii* grow optimally at 23 °C and poorly or not at all at 30 °C (Rhodes et al., 2003; Rhodes et al., 2005).

Diagnostic keys described for piscine mycobacterial pathogens (Frerichs, 1993; Chinabut, 1999) focus on *M. chelonae* (originally including *M. abscessus* as a subspecies), *M. fortuitum*, and *M. marinum*. Fast-growing mycobacteria are biochemically reactive and can usually be presumptively distinguished using phenotypic characterization schemes developed for clinical isolates (Kusunoki and Ezaki, 1992; Brown-Elliott and Wallace, 2002). In contrast, mycobacteria isolated from Chesapeake Bay striped bass (Rhodes et al., 2004) are dominated by slow-growers for which existing diagnostic keys do not permit species identification (Lévy-Frébault and Portaels, 1992; Tortoli, 2003).

To avoid false-negative results for slow-growers, sufficient growth must be obtained for substrate utilization

tests. Supplementary phenotypic testing such as mycolic acid analysis and antimicrobial susceptibility is also useful for the characterization of slow-growers, in addition to genotypic characterization. Tortoli (2003) presents a comprehensive review of genetic and chemotaxonomic tests used to identify mycobacteria.

The importance of a combined phenotypic and genotypic approach for identifying mycobacteria is exemplified by recognition of the moray eel pathogen, *Mycobacterium montefiorense*, as a new species (Levi et al., 2003) following initial characterization of this isolate as '*Mycobacterium triplex*-like' based on 16S rRNA gene similarities (Herbst et al., 2001). As the numbers of reported phenotypic and genotypic features of mycobacteria expand, there will no doubt be an increase in the number of recognized *Mycobacterium* spp. infecting fishes, a process likely to enhance our understanding of their taxonomic relationships.

It is important to distinguish between mycobacteria isolated from fishes, and fish-pathogenic mycobacteria. Aseptic isolation from internal organs (Rhodes et al., 2004) ensures that isolates do not represent contaminants, however efforts should be made to determine their density and any disease produced. Isolation from external sites or feces (Perez et al., 2001), or molecular identification of mycobacteria from whole-fish or whole-viscera homogenates (Prearo et al., 2004) is questionable, as isolates could be external contaminants or passively present in ingesta.

Culture-based detection of mycobacteria from external sites such as skin or gills and from environmental matrices is further complicated by the presence of background microbiota that outcompete mycobacteria on standard media. Due to their hydrophobicity, mycobacteria are highly resistant to treatment with both acidic and basic chemicals in addition to compounds such as benzalkonium chloride and hypochlorite (Brooks et al., 1984; Rhodes et al., 2004). These substances have therefore been used to aid in the isolation of mycobacteria from samples with a high microbial background, although they can also adversely affect the recovery of mycobacteria (Brooks et al., 1984; Schulze-Röbbecke et al., 1992; Rhodes et al., 2004). Consequently, use of decontamination procedures requires evaluation of minimum concentrations and exposure times, and the results of these studies should be considered semi-quantitative.

A variety of molecular diagnostic methods have been developed for detecting and identifying *Mycobacterium* spp. of human health importance, with considerable focus on *M. tuberculosis*. Molecular diagnostics of *Mycobacterium* spp. infecting fishes are less well-developed, although this is changing rapidly as the diversity of this group is recognized. The small subunit (16S) rRNA gene is a popular target for diagnostics due in part to the availability of *Mycobacterium* spp. 16S gene sequences in repositories such as GenBank and the Ribosomal Differentiation of Microorganisms database (RIDOM, 2007).

Identification of *M. marinum*, *M. fortuitum*, and *M. chelonae* via PCR-RFLP (restriction fragment length polymorphism) analysis of the 16S rRNA gene was first described by Talaat et al. (1997), and this technique or variations thereof are still in widespread use. High 16S gene sequence conservation among mycobacteria, however, limits the utility of this gene for differentiating closely related species. For example, *Mycobacterium kansasii* and *Mycobacterium gastri* share 100% 16S rRNA gene sequence homology (Rogall et al., 1990), and the striped bass isolates *M. pseudoshottsii* and *M. shottsii* differ by only 8 of 1454 nucleotides at this locus, despite differing in phenotype (Rhodes et al., 2003; Rhodes et al., 2005). Other gene targets for molecular detection of mycobacteria infecting fishes include the 16S–23S internal transcribed spacer (ITS), heat-shock protein 65 (*hsp*65), exported repeated protein (*erp*), and the RNA polymerase B subunit (*rpo*B) genes (reviewed by Kaattari et al., 2006). Unique insertion sequences, while holding great potential as species-specific gene targets, remain largely unexplored in mycobacteria infecting fishes with the exception of the MPM-associated IS*2404* and IS*2606*, and IS*901*/IS*1245* used to identify *Mycobacterium avium* in a tropical fish (Lescenko et al., 2003).

Previous studies have compared methods of detecting mycobacteria in fish tissues including histological and bacteriological examination and nucleic acid-based techniques (Knibb et al., 1993; Kaattari et al., 2005). A number of sampling and conceptual issues make interpretation of the findings of these methods problematic, given that they assess different biological phenomena. Histological examination identifies disease, and in this context, infection. However cryptic infections may exist without evidence of pathology. Bacteriological examination identifies culturable organisms, therefore indicating the presence of infection. On the other hand, molecular methods detect mycobacterial DNA, but do not indicate if the bacteria are viable or if disease is present. Different scenarios are therefore possible, including cryptic infection, disease, and a state of non-disease/non-infection but with mycobacterial DNA present. As a result, the characteristics of a 'positive' result may vary according to the goals of the particular study and the diagnostic method applied. Assignment of gold-standard status to any one diagnostic method, and thus the use of terms such as false-negative and false-positive, requires careful consideration of the context in which different tests are applied.

The standard test for previous mycobacterial exposure in humans and other mammals involves subcutaneous injection of mycobacteria-derived purified protein derivative (PPD) and subsequent observation of a delayed-type hypersensitivity response. More recently, tests directly measuring interferon production by peripheral blood leukocytes in response to stimulation with *M. tuberculosis* antigens ESAT-6 and CFP-10 have become available (QuantiFERON-TB Gold, Cellestis). This type of test has the advantage of returning less false-positive results in *Mycobacterium bovis* BCG-vaccinated individuals, as genes encoding ESAT-6 and CFP-10 are deleted from the BCG

genome (Brock et al., 2001; Mori et al., 2004). Unfortunately, similar non-lethal diagnostic tests do not exist for fishes.

In infected fishes, mycobacterial DNA can be detected in peripheral blood (Knibb et al., 1993), antibody may be present and hypersensitivity responses to mycobacterial antigens can be induced (Bartos and Sommer, 1981; Colorni et al., 1998). To date, however, these methods have not been adapted for use as routine diagnostics. Reliable, non-lethal diagnostic methods for detecting mycobacterial infection in fishes would be useful for screening broodstock and fishes imported into aquaculture facilities, as well as for the epidemiological study of infection and disease in wild fishes.

Treatment

Currently there are no widely accepted treatments for mycobacteriosis in fishes. Treatment of humans infected with *M. tuberculosis* and opportunistic mycobacteria is typically protracted, requiring up to 24 months with multiple antibiotics. Antibiotic susceptibility testing on fish isolates is rarely performed, and resistance appears to be highly dependent on the infecting species and strain. Rifampicin, streptomycin, and erythromycin have been shown to have some effectiveness against an undescribed *Mycobacterium* spp. in yellowtail (*Seriola quinqueradiata*) (Kawakami and Kusuda, 1990), and ethambutol, isoniazid, and/or rifampicin are occasionally used for treating high-value specimens in aquaria (Chinabut, 1999). Antibiotic resistance may be a significant hurdle to treatment, however, as evidenced by isolates of *M. fortuitum* that were cultured from aquaria in South Africa. These isolates were found to be resistant to standard anti-mycobacterial antibiotics including streptomycin, isoniazid, rifampicin, and ethambutol (Bragg et al., 1990). With few exceptions, the pharmacokinetics of anti-mycobacterial compounds in fishes remain unknown (e.g., Brown et al., 1990).

Unfortunately, control of mycobacteria in aquaria typically requires destruction of affected stock and disinfection of holding tanks and plumbing (Noga, 2000; Roberts, 2001). Although ethanol, benzyl-4-chlorophenol-phenylphenol (Lysol), and sodium chlorite have been found to rapidly kill *M. marinum* in water, other commonly used disinfectants such as *N*-alkyl dimethyl benzyl ammonium chloride (Roccal-D, Micronex), and potassium peroxymonosulfate (Virkon-S) are ineffective even after extended contact times. Sodium hypochlorite was an effective sterilizing agent, provided the contact time was longer than 10 min (Mainous and Smith, 2005).

Originally used in 1921, Bacille Calmette-Guérin (BCG), or attenuated *M. bovis*, remains the only accepted vaccine against tuberculosis in humans. The efficacy of this vaccine is highly variable, and it is no longer used in countries with a low incidence of the disease, due in part to fact that vaccination can result in individuals cross-reacting to the diagnostic skin test. More promising vaccines are currently in development or on clinical trial (Ginsberg, 2002; McShane et al., 2004; Hewinson, 2005; Horwitz et al., 2006).

Some progress has recently been made in development of a vaccine for *Mycobacterium* spp. in fishes. A recombinant vaccine based on the fibronectin binding protein Ag85A generated antibody and cellular immune responses, but did not elicit protection against live challenge of striped bass with *M. marinum* (Pasnik et al., 2003). Subsequent work with Ag85A DNA vaccines produced short-term protection against *M. marinum* in striped bass, although long-term protection was lacking (Pasnik and Smith, 2005; Pasnik and Smith, 2006). The production of effective vaccines against *M. tuberculosis* infection has proved daunting, and it is likely that considerable development will be necessary before economically viable vaccines are produced for use in fishes.

Zoonotic considerations

In addition to their known infectivity to fishes, aquatic mycobacteria pose significant zoonotic concerns. *M. marinum* is a well-known human pathogen, producing granulomatous lesions in skin and peripheral deep tissues (Lewis et al., 2003; Petrini, 2006). Lesions of this type were first described in 1951 (Nordén and Linell, 1951), and the causative agent was identified as '*Mycobacterium balnei*' (now known as *M. marinum*) in 1962 (Swift and Cohen, 1962). This disease has since been repeatedly described in fishermen and aquarists (Zeligman, 1972; Hay et al., 1975; Lawler, 1994) and is commonly known as fish tank- or swimming pool-granuloma, or 'fish-fancier's finger'.

Infection with *M. marinum* produces cross-reactivity to *M. tuberculosis* PPD- and *M. avium* sensitin-based skin tests. *M. marinum*-induced PPD reactions may be over 15 mm in diameter (Lewis et al., 2003), leading to false-positive results for the diagnosis of *M. tuberculosis* exposure as defined by US Centers for Disease Control criteria (CDC, 2006). Antibiotic therapy is effective for *M. marinum* infections in humans, although surgical excision is occasionally indicated (Lewis et al., 2003). In addition, *M. marinum*, *M. peregrinum* and *M. scrofulaceum* infections have been implicated in causing cutaneous granulomas in aquarists (Ishii et al., 1997). Additionally, *M. fortuitum* and *M. chelonae* are well-known opportunistic human pathogens, especially of immunocompromised persons (Pagnoux et al., 1998; Brown-Elliott and Wallace, 2002; Otsuka et al., 2005).

Considering the severity of disease caused by *M. ulcerans* infection in humans (Buruli ulcer), the recent discovery of numerous closely related mycolactone-producing mycobacteria in fishes is of some concern, although only one case of zoonosis caused by these bacteria has been reported (Chemlal et al., 2002). Although some isolates of *M. pseudoshottsii* are capable of growing at 30 °C in vitro, visible growth is not detected at 37 °C, suggesting infection of endotherms may not be possible. The closely related *M. shottsii*, which is not reported to produce mycolactone,

has similar temperature intolerance. Other *M. marinum*-like MPM, including '*M. liflandii*' have higher temperature tolerances, suggesting they could survive and replicate in peripheral sites in humans (Mve-Obiang et al., 2005; Ranger et al., 2006).

Mycobacteriosis of striped bass in Chesapeake Bay, USA

Mycobacteriosis was first described in Chesapeake Bay striped bass in 1997 (Vogelbein et al., 1998), and has since been recognized as a potential threat to the Atlantic coastal stock, which uses Chesapeake Bay as a primary spawning and nursery ground. The prevalence of visceral disease is extremely high, exceeding 70% in some samples (Cardinal, 2001; Overton et al., 2003; Rhodes et al., 2004; Gauthier et al., in press). Additionally, ulcerative skin lesions (Fig. 1) are common (Overton et al., 2003; Rhodes et al., 2004). Although the impact of this disease on the striped bass population is currently unknown, a recent tag-recapture study has indicated that natural (non-fishing) mortality of striped bass in Chesapeake Bay has increased since 1999 (Jiang et al., 2007).

Various hypotheses have been presented to explain the high prevalence of mycobacteriosis in Chesapeake Bay striped bass. It has been postulated that accelerating eutrophication within Chesapeake Bay is an important

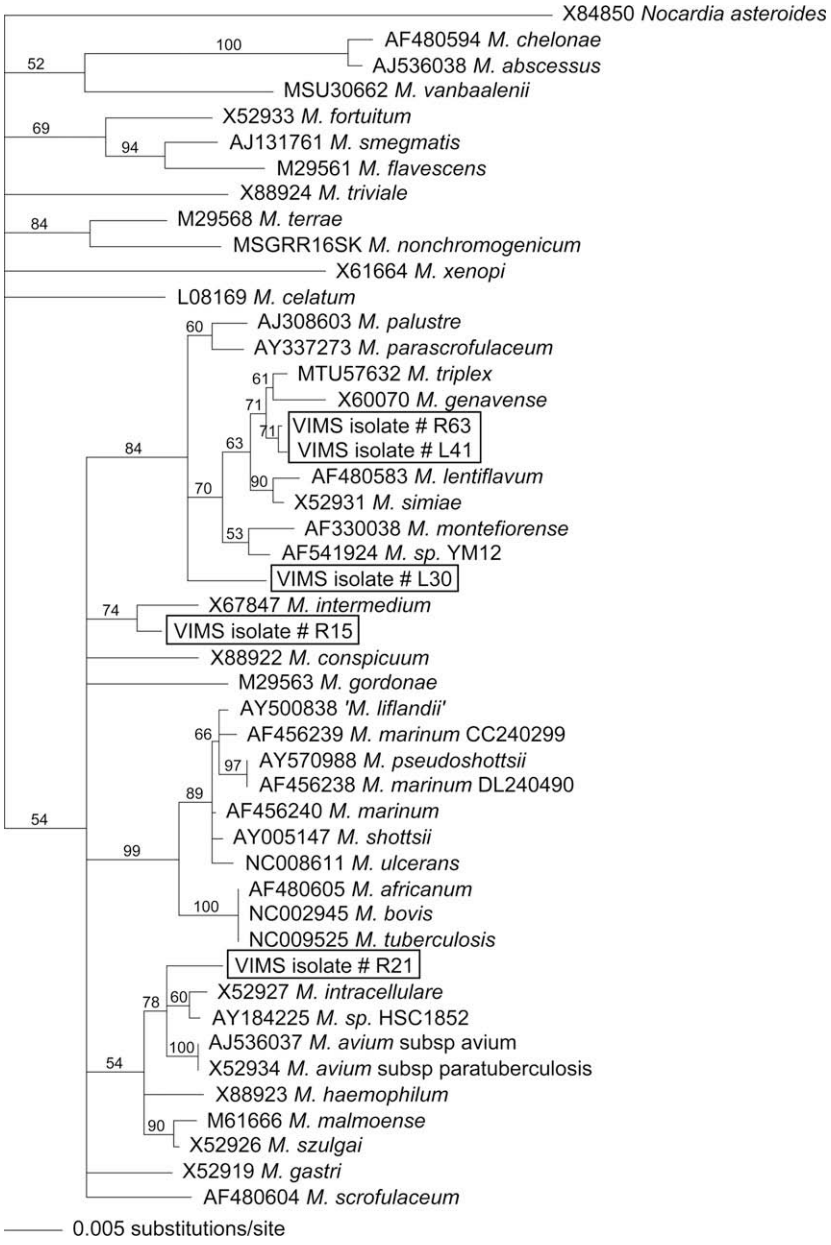

Fig. 3. Neighbour-joining analysis of *Mycobacterium* spp. 16S rRNA gene sequences, including isolates L30, L41, R63, R15, and R21 (boxed) from Chesapeake Bay striped bass. The 16S sequences have been deposited under GenBank Nos. EU541618, EU541619, EU541622, EU541620, and EU541621, respectively. One thousand boostrap replicates were performed under the '*P*' uncorrected model. Bootstrap confidence values are shown at nodes.

anthropogenic stressor that modulates disease expression (Coutant, 1985; Price et al., 1985). Adult striped bass have a maximum preferred temperature of approximately 25 °C (Coutant, 1985), which is exceeded in the surface waters of this estuary during the summer months. Although the deeper, cooler channels of the Chesapeake Bay mainstem are thought to serve as thermal refugia for resident striped bass during this time, these areas are subject to severe eutrophication and become anoxic during the summer (Boesch et al., 2001; Kemp et al., 2005). This anoxia may force fish into stressful, high-temperature water during the summer months, leading to increased disease expression (Coutant, 1985; Price et al., 1985).

A second hypothesis advanced to explain the high prevalence of disease in striped bass is nutritional stress resulting from a reduced forage base. Numbers of forage-sized Atlantic menhaden (*Brevoortia tyrannus*), which form a major portion of the striped bass diet (Hartman and Brandt, 1995; Walter and Austin, 2003), declined to near historic lows during the mid 1990's (Uphoff, 2003), while striped bass numbers have recovered to near historic highs (Field, 1997), possibly leading to a trophic imbalance. While both environmental and nutritional hypotheses have merit, neither currently has significant scientific support.

Kane et al. (2007) indicated that mycobacterial infections are increasing in Chesapeake Bay fishes, and imply that menhaden potentially serve as a vector for mycobacterial infection to striped bass. Temporal data supporting changing prevalence levels, however, have not been presented. Furthermore, given that mycobacteria were identified to genus only, and data were not presented regarding bacterial density or disease expression, it is not possible to make further assessments relating to the identity or significance of these mycobacterial isolates from Chesapeake Bay fishes. The significance of trophic transfers of mycobacteria between prey species and top predators such as striped bass also cannot be determined without knowledge of the *Mycobacterium* spp. involved.

The etiology of mycobacteriosis in Chesapeake Bay striped bass is considerably more complex than previously reported for wild fishes. Isolates obtained from diseased striped bass are dominated by two novel species, *M. pseudoshottsii* and *M. shottsii*, however, *M. marinum* and a variety of slow-growing mycobacteria are also found (Rhodes et al., 2004). The relative pathogenicity of the latter isolates remains unknown, and their taxonomic position is uncertain. Sequencing of the 16S rRNA gene, however, reveals considerable diversity within these isolates (Fig. 3). Some group closely with *M. triplex* and related mycobacteria (isolates L30, L41, R63), others with *M. intermedium* (R15), and one with *M. avium*-complex organisms (R21). The data detailed in Fig. 3 are not intended to definitively identify these isolates, but to reflect the diversity of potentially fish-pathogenic *Mycobacterium* spp. in striped bass of Chesapeake Bay.

Interestingly, fast-growing mycobacteria similar to *M. chelonae* or *M. fortuitum* have not been isolated from visceral granulomas in Chesapeake Bay striped bass (Rhodes et al., 2004), although *M. chelonae* has been reported from striped bass in Delaware Bay, which lies directly to the north of Chesapeake Bay and communicates with it through a shipping channel (Ottinger et al., 2007). '*Mycobacterium chesapeaki*', an isolate biochemically similar to *M. shottsii*, was reported by Heckert et al. (2001) from Chesapeake Bay striped bass. The 16S gene sequence for this isolate (GenBank No. AF257216) is problematic, as sequence homologies with *M. ulcerans*, *M. tuberculosis*, and *M. marinum* are less than 88%, much less than expected within this genus. Much of this variation occurs within the 3′ end of the deposited sequence, which has low homology to known *Mycobacterium* spp. We have sequenced (GenBank No. EU541624) the 16S and ITS genes for the ATCC (American Type Culture Collection) deposit of '*M. chesapeaki*' (No. BAA-422) and found both to be identical to *M. shottsii* (GenBank No. AY005147 [16S], GenBank No. EU541623 [ITS]).

Conclusions

Mycobacteriosis remains an important infectious disease of wild and aquacultured finfishes worldwide. As many mycobacteria infecting fishes are also infective for humans, the potential for zoonotic infection presents an additional challenge. Although this disease has been studied in fishes for over a century, basic questions about its pathobiology remain, including transmission and host defense mechanisms. The study of mycobacterial taxonomy is also in a major state of transition, and pitfalls still exist for accurate identification of isolates. Additionally, effective prophylaxis, control measures, and non-lethal diagnostics require development. However, with the advent of modern molecular detection methods, epidemiological techniques, and vaccinology, considerable potential exists for the improvement of our understanding and control of this disease.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors wish to thank Drs. Wolfgang Vogelbein, Howard Kator, and Ashley Haines for critical review of this manuscript. This is VIMS contribution No. 2921.

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