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Pathological and molecular studies on mycobacteriosis of milkfish *Chanos chanos* in Taiwan

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ABSTRACT: An outbreak of mycobacteriosis was investigated in milkfish *Chanos chanos*, which had a cumulative mortality of up to 66.7% over the course of 1 yr. Gross reddish- or greyish-white nodules appeared on the peritoneal surface, spleen, kidney, liver and gastrointestinal (GI) tract. Epithelioid granulomas with the formation of Langhan's type giant cells were the prominent histopathological changes. Despite large numbers of acid-fast bacilli in the granulomas, neither caseous necrosis nor dystrophic calcification were observed. Using degenerate primers that targeted the heat shock protein 65 kDa gene of *Mycobacterium* spp., a 441 bp product was amplified. When compared with published sequences, our products were identical to those of *Mycobacterium abscessus* Type II (GenBank accession number AY603554). This is the first report of *M. abscessus* infection in milkfish.

KEY WORDS: Mycobacteriosis · *Mycobacterium abscessus* · Visceral granuloma · PCR · Milkfish

INTRODUCTION

Mycobacteria are widespread in nature, particularly in the aquatic environment. Among the rapidly growing mycobacteria (RGM), *Mycobacterium marinum, M. fortuitum*, and *M. chelonae* are the most frequently isolated from infected fish, but other species including *M. neoaurum, M. simiae, M. poriferae, M. scrofulaceum, M. abscessus, M. chesapeaki, M. gordonae,* and *M. shottsii* have also been reported (Backman et al. 1990, Chinabut et al. 1990, Gomez et al. 1993, Landsdell et al. 1993, Tortoli et al. 1996, Teska 1997, Bruno et al. 1998, Heckert et al. 2001, Rhodes et al. 2001, 2003, Gauthier et al. 2003).

Within the *Mycobacterium fortuitum* complex, *M. chelonae*, and *M. abscessus* are the species of RGM most often associated with human diseases (Silcox et al. 1981, Shih et al. 1997, Brown-Elliott & Wallace 2002, Haverkort 2003). These organisms cause a variety of disseminated or localized diseases in humans, particularly pulmonary infections, as well as primary skin and soft tissue infections (Debrunner et al. 1992, Brown-Elliott & Wallace 2002, Haverkort 2003). According to a recent report of National Taiwan University Hospital (NTUH), *M. abscessus* is the most prevalent pathogen inducing atypical mycobacteriosis in Taiwan (Yang et al. 2003).

To the best of our knowledge, there are few published reports concerned with the diseases of milkfish. This study reports an outbreak of mycobacteriosis in milkfish owing to infection by *Mycobacterium abscessus*, using pathological and molecular examination.

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MATERIALS AND METHODS

Samples. In April 2001, approximately 55000 milkfish were cultured in 2 brackish-cultured ponds and subsequently became infected by *Mycobacterium abscessus*. By November, affected fish showed signs of emaciation and reddish discoloration at the base of fins and anus. Cumulative mortality reached 66.7%. Five moribund fish were submitted to the Southern Taiwan Aquatic Animal Disease Diagnostic Center (STAADDC) for necropsy in March 2002.

Pathology. Organs including brain, heart, gills, liver, spleen, kidney, gastrointestinal (GI) tract, mesentery, eyes and body wall were processed. Fish tissues were fixed in 10% neutral buffered formalin for at least 24 h prior to processing, except the gills and body wall, which were decalcified in 5% trichloroacetic acid for 24 h. The sampled tissues were dehydrated through a graded ethanol series, then embedded in paraffin, and sectioned at 5 µm. All sections were stained with haematoxylin and eosin (H&E), and selected sections were also treated with Ziehl-Neelsen acid-fast stain.

DNA extraction and PCR amplification. Bacterial DNA was extracted from formalin-fixed, paraffin-embedded tissue sections (Marchetti et al. 1998). For detection of *Mycobacterium* spp., PCR assays targeting the *hsp65* gene were used to amplify a 441 bp of expectant product as previously described (Shinnick 1987, Ringuet et al. 1999).

Cloning of PCR products. After purification (QIAquick Spin columns, Qiagen), PCR products were cloned into a T-vector using a TA cloning kit (YT&A; Yeastern Biotech), according to the manufacturer's instructions. They were then sequenced using a 373A automatic sequencer and a BigDye Terminator cycle sequencing kit (Mission Biotech).

Analysis of sequence data. Sequence similarities in percentage were calculated with the PAM250 residue weight table by using MEGALIGN/DNASTAR software (DNASTAR, Madison). Molecular phylogenetic trees were constructed from nucleotide sequences to avoid possible distortion by codon usage differences.

RESULTS

External lesions were non-specific for mycobacterial infection. Internally, reddish-white or greyish-white nodules were the prominent lesions scattered on the peritoneal surface; occasionally, several nodules coalesced into a mass with or without ulceration of the epithelium (Fig. 1). Reddish nodules were also found on the GI tract including on the oesophagus, stomach and intestine (Fig. 2). However, the most significant changes were many greyish-white nodules scattered on the spleen and kidney (Fig. 3); only a few reddish nodules appeared on the liver, which often had a yellowish discolouration.

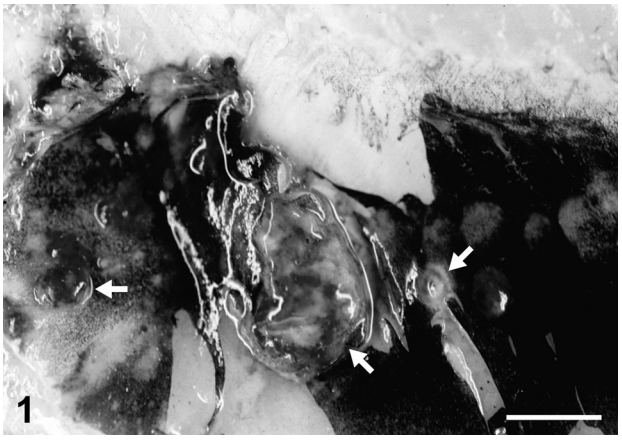

Fig. 1. *Chanos chanos*. Individual and coalescent tubercle-like lesions scattered on the peritoneal surface (arrows). Scale bar = 1 cm

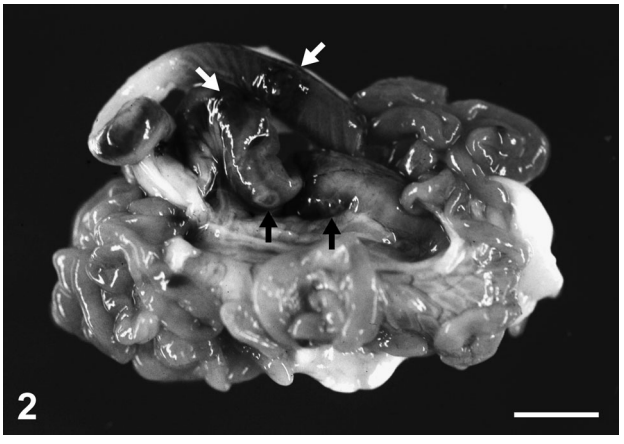

Fig. 2. *Chanos chanos*. Numerous tubercle-like lesions in the gastrointestinal wall (arrows). Scale bar = 0.5 cm

Histological features of the nodules were classified as granulomas of reticuloendothelial (RE) type. Within the granulomatous centre was a mixture of RE cells, Langhan's type giant cells, and yellowish-brown pigments, followed by well-demarcated layers of spindle and epitheloid cells (Fig. 4). The outer-most zone was encapsulated by a thin layer of fibrous tissues and/or lymphocytes. Neither caseous necrosis nor dystrophic calcification was observed in the granulomas (which sometimes contained a yellowish-brown pigment). Significant numbers of acid-fast, unbranching bacilli were recorded by Ziehl-Neelsen staining in the cytoplasm of the RE and Langhan's type giant cells.

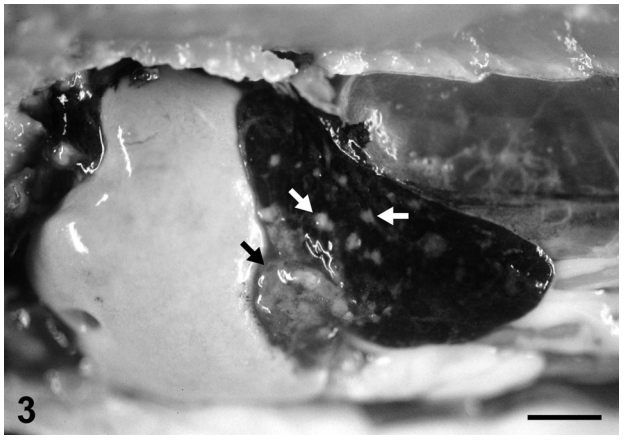

Fig. 3. *Chanos chanos*. Numerous whitish tubercle-like lesions in the spleen and swimbladder (arrows). Scale bar = 0.5 cm

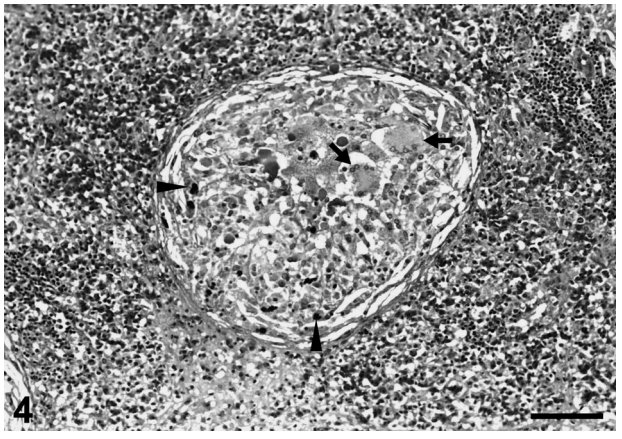

Fig. 4. *Chanos chanos*. Tuberculous granuloma composed of Langhan's type giant cells (arrows) and macrophages associated with deposition of yellowish-brown pigment (arrowheads). Scale bar = 100 µm

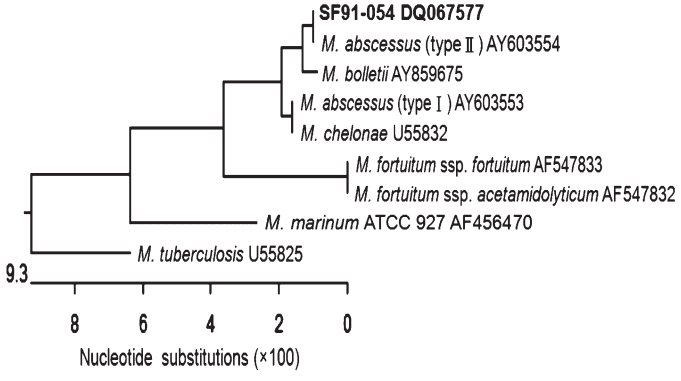

Fig. 5. Phylogenetic tree constructed on the basis of the partial nucleotide sequence of *Mycobacterium* spp. heat shock protein (*hsp65*) gene

Use of a set of primers for the *hsp65* gene resulted in amplification of a fragment of 441 bp, the expected size for mycobacteria; the similarity of our amplified products with *Mycobacterium abscessus* (GenBank accession number AY603554) was 100 % (Fig. 5).

DISCUSSION

Milkfish culture has been practiced over 400 yr in Taiwan, and this species is an important food fish in southeast Asia. Production of this fish is estimated to represent approximately 34 % of total aquaculture production.

Genotypic methods based on polymorphism of the 16S rRNA gene have been of value for the identification of slow-growing mycobacterial species. However, there is little variability within the mycobacterial 16S rRNA gene sequence in RGM, making this target a poor discriminator for closely related species such as *Mycobacterium abscessus* and *M. chelonae* (Kirschner et al. 1992, Kusunoki & Ezaki 1992). *hsp65* is highly conserved among mycobacteria. The *hsp65* sequencing method was developed for RGM species because it displays more polymorphism than does the 16S RNA gene sequence (Ringuet et al. 1999). Among the etiological agents of piscine mycobacteriosis, *M. marinum* is the most frequently isolated from fish (Decostere et al. 2004). However, based on the presence of acid-fast, unbranching rods in the RE granulomatous lesions and nucleotide sequencing of the PCR product of the *hsp65* gene, the milkfish in our study were diagnosed to be infected with *M. abscessus*.

Natural infections from the same pathogen have also been found in several freshwater tropical fish species (Gomez et al. 1993) and some pet fish including black acaras *Cichlasoma* sp., goldfish *Carassius auratus*, firemouth cichlid *Thorichthys meeki* (Lansdell et al. 1993), oscar *Astronotus ocellatus* (McCormick et al. 1995), and Japanese medaka *Oryzias latipes* (Teska 1997). The typical granuloma for piscine mycobacteriosis is often composed of a thick capsule of epitheloid cells surrounding a necrotic centre. Within necrotic lesions, large numbers of acid-fast bacilli are generally present. However, Langhan's type giant cells and dystrophic mineralization are rarely encountered together in natural and experimental cases (Grady et al. 1992, Colorni et al. 1998, Talaat et al. 1998, 1999, Astrofsky et al. 2000). Based on our observations, Langhan's type giant cells were easily identified in the granulomatous lesions induced by *Mycobacterium abscessus* infection in milkfish. Neither caseous necrosis nor dystrophic calcification was observed *in situ*.

Comparison with results of experimental studies involving sea bass infected with *Mycobacterium mar-*

inum (Colorni et al. 1998) lead us to conclude that our case might have been in the pre-granulomatous stage, because epitheloid cells were only observed in a granuloma pattern without the formation of a necrotic centre. In addition, previous research on the experimental pathogenesis of focal tuberculosis in plaice *Pleuronectes platessa* indicates that giant cells can be identified but rapidly decrease in number after 28 d (Timur et al. 1977). However, the morphological variability of tubercular lesions related to different host or different pathogens requires further study.

In humans, *Mycobacterium chelonae, M. abscessus* and *M. fortuitum* group are the major pathogens among the RGMs (Sungkanuparph et al. 2003). In its first documented report, *M. abscessus* (formerly *M. chelonae* subsp. *abscessus*) was isolated from an abscess of a human patient (Moore & Frerichs 1953). A recent report indicates that *M. abscessus* has become the most prevalent pathogen isolated from patients with atypical mycobacteriosis in Taiwan since 1992 (Yang et al. 2003). Several cases associated with *M. abcessus* infection in cystic fibrosis have been reported (Ringuet et al. 1999, Cullen et al. 2000, Sanguinetti et al. 2001, Olivier et al. 2003, Sernet-Gaudelus et al. 2003). The present study indicates a similarity of 100% between the *hsp65* gene sequence of strain SF91-054 (DQ067577) described here from milkfish *Chanos chanos* and *M. abcessus* Type II (GenBank accession number AY603554) originating from humans. Therefore, epidemiological surveys of milkfish mycobacteriosis is a priority task in relation to public health.

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