Detection and characterisation of aquatic *Mycobacterium* spp.

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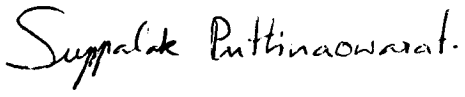
To my parents,
sisters and brothers

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been previously submitted for any other degree or qualification.

The work of which it is a record has been carried out myself. The nature and extent of any work carried out by, or in conjunction with, others has been specifically acknowledged by reference.



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Abstract

Mycobacterium spp, the etiological agent of mycobacteriosis, has recently been responsible for serious infections in two economically important fish species cultured in Thailand; snakehead (*Channa striata*) and Siamese fighting fish (*Betta splendens*). Attempts to detect and identify the pathogens to species level, in fish tissue and the environment, have so far been unsuccessful, mainly due to the poor levels of sensitivity and specificity of the detection methods used, based on conventional bacteriology and histology. In this study, a variety of novel techniques were developed and used for more effective identification of *Mycobacterium* spp., including monoclonal antibody-based assays, DNA-based techniques and mass spectrometry.

A monoclonal antibody (Mab 8F7) probe was developed against *M. marinum*, which was successfully used to identify *M. marinum* in infected fish tissue by immunohistochemistry (IHC), and from pure bacterial cultures by enzyme-liked immunosorbent assay (ELISA). The molecular-based techniques employed to detect the pathogen included *in situ* hybridisation (ISH), polymerase chain reaction (PCR) and reverse cross blot hybridisation. The PCR was developed using primers available from the literature which amplified mycobacterial 16S rDNA. The products of the reaction were identified to species level by PCR-reverse cross blot hybridisation. *M. marinum*, *M. fortuitum* and *M. chelonae* were identified using this method. The same primers as those used in the PCR, were used as probes in ISH to identify *Mycobacterium* spp to genus level in infected fish tissues.

A range of *Mycobacterium* spp. isolated from fish located in different geographical regions were identified and characterised using Mab 8F7, pyrolysis mass spectrometry (PyMS) and PCR-reverse cross blot hybridisation and PyMS analysis showed that three distinct groups of mycobacteria were involved in mycobacteriosis in Thailand and Israel. The groups were clustered around either type strains *M. fortuitum-M. chelonae* or *M. marinum*, or around an unspeciated *Mycobacterium* spp. The unspeciated isolates were identified as *M. marinum* by PCR analysis and were mainly isolated from fish cultured in Israel. *M. marinum* from Israel and Thailand appeared to be different from each other since the isolates from Thailand reacted positively with Mab 8F7, whereas isolates obtained from fish in Israel were negative.

PCR-reverse cross blot hybridisation was used to establish the identity of *Mycobacterium* spp involved in mycobacteriosis outbreaks affecting Siamese fighting fish and snakehead fish in Thailand. PCR was also utilised to analyse environmental samples taken from these farm sites. Siamese fighting fish farmers are known to suffer from skin lesions caused by *Mycobacterium* spp and therefore biopsies were taken from the farmers for analysis by PCR-reverse cross blot hybridisation. Analysis revealed that two species, *M. fortuitum* and *M. marinum*, were involved in the mycobacterial infections observed in both fish species. *M. fortuitum* and *M. marinum* were also both found in environmental samples including water, sediment and fish food. However, *M. fortuitum* was the isolate most frequently found. Skin lesions were only observed amongst the Siamese fighting fish farmers, while the snakehead fish farmers did not seem to be effected. Analysis of the biopsies from the skin lesions by PCR reverse cross blot hybridisation revealed that *M. fortuitum* was the main etiological agent associated with these lesions.

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Abbreviations

µl	Microlitre
16S rDNA	16 small subunit ribosomal deoxyribonucleic acid
16S rRNA	16 small subunit ribosomal ribonucleic acid
A	Absorbance
ABC	Avidin-biotinylated horseradish peroxidase complex
AHL	*Arachis hypogaea*
APES	3-aminopropyltriethoxysilane
ASP	Ammonium persulphate
ATCC	American Tissue Culture Center
BCG	Bacilli Calmette-Guerin
BCIP	5-bromo-1-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
CARD	Catalyzed reporter deposition
Con A	Concanavalin A (*Canavalia ensiformis*) lectin
CVA	Canonical Variates Analysis
DAB	3, 3'-diaminobenzidine tetrahydrochloride
dATP	2'-Deoxyadenosine 5'-Triphosphate
dCTP	2'-Deoxycytidine 5'-Triphosphate
dGTP	2'-Deoxyguanosine 5'-Triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DR	Direct repeat
dTTP	2'-Deoxythymidine 5'-Triphosphate
dUTP	2'-Deoxyuridine 5'-Triphosphate
ECP	Extracellular product
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
fg	Fantogram
GML	*Glycine max* soybean
h	Hour
HCA	Hierarchical cluster analysis
HGL	*Dolichos biflorus* horse gram
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSW	High salt wash buffer
IFAT	Immunofluorescence test

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IOA	Institute of Aquaculture, Stirling University
ip	Intra-peritoneal
ISH	*In situ* hybridisation
kDa	kilodalton
KIT	The Royal Tropical Institute, Amsterdam, The Natherlands
LSW	Low salt wash buffer
M	Molar
m/z	Mass:charge ratio
Mab	Monoclonal antibody
Mabs	Monoclonal antibodies
Mc	*M. chelonae*
Mf, F	*M. fortuitum*
min	Minute
MIRU	Mycobacterial interspersed repetitive unit
ml	Millilitre
mM	Millimolar
Mm	*M. marinum*
mRNA	Messenger ribonucleic acid
MSA	Modified Sauton's agar
MSB	Modified Sauton's broth
Myc	*Mycobacterium* spp.
NBT	Nitro blue tetrazolium
NCIMB	National Collections of Industrial and Marine Bacteria
nd	No data
ng	nanogram
Pab	Polyclonal antibody
Pabs	Polyclonal antibodies
PBS	Phosphate buffer saline
PCA	Principal Components Analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulse-field gel electrophoresis
pg	Picogram
PIPES	Piperazine-N, N,-bis-(2-ethanesulfonic acid)
PyMS	Pyrolysis mass spectrometry
RAPD	Random amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

SAPU	Scottish Antibody Product Unit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Saline-sodium citrate buffer
SSCP	Single-stranded conformational polymorphism analysis
TBE	Tris borate EDTA buffer
TBS	Tris buffered saline
TdT	Terminal Transferase
TE	Tris-EDTA buffer
TEMED	N, N, N`, N`-Tetramethylethylenediamine
TMS	3, 3', 5, 5'-tetramethylbenidine dihydrochloride
TTBS	Tween 20-tris buffered saline
TVL	*Triticum vulgaris*
U	Unit
UDG	Uracil DNA glycosylase
UEA-1	*Ulex europaeus* agglutinin
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Voltage
WB	Western Blot
ZN	Ziehl-Neelsen stain

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General introduction

Background of this study

Siamese fighting fish (*Betta splendens*) is an important ornamental species in Thailand, which is exported worldwide. The occurrence of mycobacteriosis in Siamese fighting fish in Thailand was first described in 1989 by the National Inland Fisheries Institute, Bangkok (Pungkachonboon *et al*, 1992). A few years later, Pungkachonboon *et al* (1992 & 1994) isolated *Mycobacterium* spp. from Siamese fighting fish. The disease was confined not only to fish, but was also transferred to fish farmers. This became evident in the early 1990s, when it was reported that *M. marinum* was frequently isolated from chronic lesions on the skin of patients who were involved with fish culture (Kullavanijaya *et al*, 1993).

Snakehead (*Channa striata*), one of the most important freshwater food fish in Thailand, has also been reported as susceptible to mycobacteriosis (Limsuwan *et al*, 1983; Chinabut *et al*, 1990). It is a common species farmed in Suphanburi Province, Thailand. The disease normally occurs during the warm season when there is a shortage of water in the river for water exchange in the fish ponds (S. Areerat, pers. comm.). Mortality levels as high as 20% have been reported (Adams *et al*, 1996). However, there are no reports of snakehead farmers with granulomas caused by *M. marinum*, or other *Mycobacterium* species.

Sea bass (*Dicentrarchus labrax*), an important marine food fish in Israel, have had severe outbreaks of mycobacteriosis in recent years (Colorni, 1992; Knibb *et al*, 1993). During outbreaks of the disease, entire stocks were found to be infected. The

pathogen was identified as *M. marinum* by PCR and direct sequencing of 16S rDNA (Knibb *et al*, 1993).

Attempts to characterise *Mycobacterium* spp isolates from Siamese fighting fish and snakehead have been difficult since the criteria used to do so have been based on biological characteristics such as growth rate, pigmentation, optimal growth temperature and analysis by biochemistry (Pungkachonboon *et al* 1992; Pungkachonboon, 1994). Such traits are not always clear cut between the different *Mycobacterium* spp. which infect fish. Adams *et al* (1995; 1996) introduced immunological-based techniques to characterise and identify the different isolates obtained during outbreaks of mycobacteriosis in fish, however the results remained inconclusive with reference to species identification. Puttinaowarat (1995) subsequently examined some of the same isolates by ELISA and PCR. Restriction enzyme analysis of the PCR products enabled identification of *M. marinum*. The results also indicated that some of the *Mycobacterium* spp. previously identified to species level on the basis of biochemistry may need to be re-evaluated.

It is clear that identification of the pathogens involved in mycobacteriosis and their characterisation to species level would provide necessary information for effective treatment of fish. Diagnosis of the disease in the early stages of infection may allow the farmer to manage the disease more effectively. It is also important to establish the source of infection for both fish and man. Understanding the disease with respect to pathogenesis, epidemiology, and mode of transmission to both fish and man may in turn help to alleviate the problems experienced by mycobacteriosis.

Objectives of this study

The aims of this study were firstly to develop specific and sensitive methods for the detection and identification of *Mycobacterium* spp isolated from fish in Thailand, Israel and other geographical regions. This information will establish which *Mycobacterium* species are involved in mycobacteriosis, particularly in Siamese fighting fish and snakehead in Thailand, and sea bass in Israel. The presence of mycobacteria in environment samples from Siamese fighting fish and snakehead fish farms will also be analysed.

More specifically the aims of the thesis were to develop Mabs which could be used to characterise mycobacterial isolates to species level both in pure bacterial culture (ELISA) and infected fish tissue by IHC. A comparison will be made between *Mycobacterium* isolates identified with the Mabs to those identified by PyMS among strains collected from different geographical regions. This will establish the main etiological agent involved the disease in the different geographical regions.

DNA-based methods such as ISH, PCR, and reverse cross blot hybridisation assay will be established by using oligonucleotide sequences (primers), that have already been used in the study of human tuberculosis. These primers will be used in PCR to amplify mycobacterial 16S rDNA, and the product will be analysed to species level by reverse cross blot hybridisation. The same nucleotides sequences used as primers in PCR will be used as a genus-specific probes to detect *Mycobacterium* spp in infected fish tissue by ISH.

Finally, the PCR-reverse cross blot hybridisation will be used as a tool to examine the etiological agent involved mycobacteriosis in snakehead and Siamese fighting fish farms in Thailand and to investigate the link between the transfer of the disease in fish to man. Environmental samples including water, sediment and fish food will be analysed to determine the source of infection. Environmental and fish samples obtained for routine bacterial diagnosis from different regions and also Siamese fish samples from the exporting aquaria around Bangkok will also be analysed to examine the general epidemiology of the disease and any potential risk to public health.

Chapter 1

Literature Review

1.1 Mycobacteriosis

Mycobacteriosis is usually a chronic disease of fish where the etiologic agent is an acid-fast bacillus in the genus *Mycobacterium.* Two terms are used to describe the disease, either "piscine tuberculosis" or "mycobacteriosis". The term "tuberculosis" was previously used to describe diseases of fish, which involved any acid-fast bacilli. Since the typical tubercular inflammatory response observed in mammals was absent in fish, Parisot & Wood (1960) suggested that "mycobacteriosis" was a more appropriate expression for the disease.

Mycobacteria are responsible for a number of diseases in a wide range of hosts, including mammals and aquatic animals. There are 54 official mycobacteria species at present and it seems likely that some still remain to be identified (Collins *et al,* 1984).

1.2 Aetiology

Many bacteria in the genus *Mycobacterium* have been reported to cause mycobacteriosis in fish. Dulin (1979) reported numerous mycobacteria pathogenic to fish species including *M. piscium*, *M. platypoecilus*, *M. fortuitum*, *M. ranae*, *M. marinum*, *M. anabanti* and *M. salmoniphilum.* Other species associated with the disease have also been reported such as *M. simiae* and *M. scrofulaceum* (Lansdell *et al*, 1993). However, most of these species were later reclassified as outlined in Table 1.1. The most common species infecting fish are *M. marinum*, *M. fortuitum* and *M.*

chelonae (Giavenni & Finazzi, 1980; Arakawa & Fryer, 1984; Dalsgaard *et al*, 1992; Lansdell *et al*, 1993).

Table 1.1 *Mycobacterium* spp. isolated from fish (Collins *et al*, 1984; Dalsgarrd *et al*, 1992; Austin & Austin, 1993)

Described species	Synonym	Accepted name
M. piscium (Bataillon *et al*, 1902)	*M. marinum*	-
M. ranae (Küster, 1905)[a]	*M. fortuitum*	*M. fortuitum*
M. marinum (Aronson, 1926)	*M. marinum*	*M. marinum*
M. fortuitum (Nigrelli, 1953)	*M. fortuitum*	*M. fortuitum*
M. platypoecilus (Baker & Hagan, 1942)	*M. marinum*	-
M. anabanti (Besse, 1949)	*M. marinum*	-
M. salmoniphilum (Ross, 1960)	*M. fortuitum*	-
M. borstelense (Bernstad, 1974)	*M. chelonae*	*M. chelonae*
M. chelonae subsp. *piscarium* (Arakawa & Fryer, 1984)	*M. chelonae*	-
M. scrofulaceum (Lansdell *et al*, 1993)	-	-
M. gordonae (Bozzeta *et al*, 1995)	-	-

[a] It was first isolated from frog liver.

Mycobacterium are aerobic, non-motile slow-growing rod-shape bacteria. Due to the waxy material of the cell walls, these bacteria are able to resist decolorisation with acidified alcohol as well as with strong mineral acids (Wayne & Kubica, 1986). This property of acid-fastness is an important feature for the identification of mycobacteria.

By Bergey's system of classification, *Mycobacterium* belong to the family Mycobacteriaceae and the genus *Mycobacterium*, which consists of 54 species in total. *M. tuberculosis* complex, *M. leprae* and some atypical mycobacteria species

have been reported to cause mycobacterial disease in man (Wayne & Sramek, 1992). In fish, three species, *M. marinum*, *M. fortuitum* and *M. chelonae* have often been reported as causing disease (Nigrelli & Vogel, 1963; Giavenni & Finazzi, 1980; Daoust *et al*, 1989; Colorni, 1992; Lansdell *et al*, 1993)

M. marinum are described as moderate to long rods, with frequent cross-barring. Colonies are smooth to rough and non-pigmented when grown in the dark. However, when they are grown in the light or exposed to light when they are young, they become brilliant yellow (photochromogenic). *M. marinum* is a slow growing, acid-fast bacterium, taking 7 or more days to grow at 30°C. The optimum temperature range is from 25°C to 35°C, however it may adapt to growth at 37°C (Williams & Riordan, 1973; Belas *et al*, 1995).

M. fortuitum can be either coccoid, or short to long rods. The cells are acid-fast after a 5 day incubation at 28°C. The colonies are usually off-white or cream in colour, but they may absorb the green dye when grown on medium containing malachite green. Their optimum temperature range for growth is reported as 30°C to 37°C (Wayne & Kubica, 1986; Belas *et al*, 1995).

M. chelonae is a pleomorphic organism ranging from long narrow to short thick rods (0.2-0.5 × 1-6 μm), but coccoid forms are also reported. Colonies may be smooth, moist and shiny or rough, and they are usually nonchromogenic to creamy buff in colour. The optimum growing temperature ranges from 22°C to 40°C (Wayne & Kubica, 1986). *M. chelonae* was more commonly isolated from cold water fish species such as salmonid fish (Frerichs, 1993).

1.3 Fish Infections

1.3.1 History

Mycobacteriosis in cold-blooded animals was first reported by Bataillon *et al* in 1897 in carp, from a pond believed to be contaminated with the organism by a person suffering from tuberculosis. The organism was named *Mycobacterium piscium* by Bataillon *et al* in 1902. In 1903, *M. chelonae* was first isolated from turtles by Friedman (Belas *et al*, 1995). Later in 1913, the first report of the infection in a wild marine fish was made in cod (*Gadus morhua*) (Alexander, 1913; Johnstone, 1913). Aronson isolated *M. marinum* from a moribund aquarium fish in 1926, while in 1950, *M. fortuitum* was first described in giant frogs in Brazil by Darzins (Belas *et al*, 1995). A different strain of acid-fast bacilli, *M. fortuitum,* was isolated from Neon Tetra (*Hyphessobrycon innesi*) by Ross & Brancato (1959). Since its initial discovery, Nigrelli & Vogel (1963) reported mycobacteriosis to occur worldwide in more than 40 families and 150 species of marine and freshwater fishes as outline in Table 1.2. Ashburner (1977) isolated *M. chelonae* from chinook salmon, which was later classified as *M. chelonae* subspecies *piscarium* (Arakawa & Fryer, 1984). However, Arakawa *et al* (1986) suggested that the name *M. chelonae* subspecies *piscarium* be withdrawn and be referred to as *M. chelonae.* Fish are not the only animals susceptible to these acid-fast bacteria. Other susceptible animals include oysters, shrimps, crayfish, frogs, lizards, turtles, as well as mammals, including man (Lightner & Redman, 1986; Alderman *et al*, 1986; Beecham *et al*, 1991; Ramakrishnan *et al*, 1997).

Table 1.2 Fish species from which *Mycobacterium* spp have been isolated (Nigrelli & Vogel, 1963)

Family	Species	Common name
Salmonidae	*Oncorhynchus gorbuscha*	Humpback salmon
	O. keta	Dog salmon
	O. kisutch	Silver salmon
	O. nerka	Blueback salmon
	O. tschawytscha	Chinook/ king salmon
	Salmo gairdneri	Steelhead, rainbow trout
Osmeridae	*Osmerus mordax*	American smelt
Umbridae	*Umbra pygmaea*	Mud-minnow
Characidae	*Aphyochorax rubripinnis*	Bloodfin
	Gymnocorymbus ternetzi	Black tetra
	Hemigrammus rhodostomus	Rummy-nosed characin
	H. unilineatus	Feather fin
	H. erythrosona	Glowlight tetra
	H. ocellifer	Head-and-tail light
	Hyphessobrycon bifasciatus	Yellow tetra
	H. flammeus	Flame characin
	H. cardinalis	Cardinal tetra
	H. innersi	Neon tetra
	H. pulcher	-
	H. rosaeus	Rosy barb
	H. callistus	Serpa tetra
	Moenkhausia pittieri	Pittier's Moenkhausia
	Pyrrhulina rachoviana	Rachow's pyrrhulian
	Pristella riddlei	Riddle's pristella
Cyprinidae	*Notemigonus crysoleucas*	Dace or roach
	Barbus fluviatilis	European barb
	Brachydanio albolineatus	Pear danio
	B. analipunctatus	Tail-spot danio
	B. nigrofasciatus	Spotted danio
	B. rerio	Zebra danio
	Danio malabaricus	Giant danio
	Carassius auratus	Goldfish
	C. carassius	Cruscian carp
	Cyprinus carpio	Common carp, golden carp
	Puntius conchonius	Rosy barb
	P. lineatus	Lined barb
	P. nigrofasciatus	Black ruby barb
	P. phutunio	Dwarf/ Pygmy barb
	P. semifasciolatus	Chinese barb
	P. tetrazona	Sumatra barb
	P. partipentazona	Five-banded barb
	P. ticto	Ticto barb
	Rasbora einthoveni	Brilliant rasbora
	R. heteromorpha	Red rasbora
	R. lateristriata	Stripped rasbora

Family	Species	Common name
	R. leptosoma	Slender-bodied rasbora
	Tanichthys albonubes	White-cloud mountain fish
Cyprinidae	*Idus melanotus*	Golden orf
	Tinca tinca	Tench
Siluridae	*Silurus glanis*	Wels/ European catfish
Bagridae	*Rhamdia sapo*	-
Clariidae	*Clarias dumerili*	-
Loricariidae	*Plectostomus punctatus*	-
Gadidae	*Gadus collarias*	Atlantic codfish
Cyprinodontidae	*Aphyosemion australe*	Lyretail
	Oryziae latipes	Medaka
	Aplocheilus panchax	Panchax
	Rivulus cylindraceus	Cuban rivulus
	Cynolebias wolterstorffi	-
	C. adeoffi	-
	C. elongatus	-
Poeciliidae	*Lebistes reticulatus*	Guppy
	Xiphophorus helleri	Swordtail
	Platypoecilus maculatus	Platyfish
	Molliensia sphenops	Mollie
Belonidae	*Belone belone*	European garfish
Holocentridae	*Holocentrus ascensionis*	Squirrelfish
Atherinidae	*Melanotaenis nigrans*	Australian rainbow fish
Serranidae	*Centropristis furvus*	-
	C. striatus	Black sea bass
	Epinephelus adscenionis	Rock hind
	E. guttatus	Red hind
	E. morio	Red grouper
	Epinephelus sp.	Gray grouper
	Epinephelus sp.	Queen grouper
	E. striatus	Nassau grouper
	Morone americana	White perch
	M. labrax	European bass
	Mycteroperca bonaci	Black grouper
	M. falcata	Scamp
	Roccus saxatilis	Striped bass
Centrarchidae	*Lepomis gibbosus*	Pumpkinseed
	Micropterus dolomieu	Smallmouth bass
Percidae	*Lucioperca lucioperca*	European pike-perch
	Perca flavescens	Yellow perch
Carangidae	*Trachinotus carolinus*	Common pompano
	Vomer setapinnis	Moonfish
Lutianidae	*Ocyurus chrysurus*	Yellow-tailed snapper
	Lutianus apodus	Schoolmaster
	L. griseus	Gray snapper
	L. jocu	Dog snapper
	L. synagris	Lane (spot) snapper
Sciaenidae	*Cynoscion regalis*	Weakfish, gray squeteague
	Leiostomus xanthurus	Spot

Family	Species	Common name
	Micropogon undulatus	Atlantic croaker
	Pogonias cromis	Black sea drum
Pomadasyidae	*Bathystoma* sp.	Red-mouth grunt
	Plectorhynchus sp.	Sweet lip
	Anisotremus surinamensis	Black margate
	A. virginicus	Porkfish
Toxotidae	*Toxotes jaculatus*	Archerfish
Kyphosidae	*Kyphosus secatrix*	Bermuda chub
Sparidae	*Archosargus probatocephalus*	Sheephead
	Sargus sargus	Sargo
	Cantharus lineatus	Oldwife, black sea bream
Maenidae	*Spicara argus*	Picarel martin pècheur
Scatophagidae	*Scatophagus argus*	Scat
	Monodactylus argentus	Silver angelfish
Cichlidae	*Apistogramma ramirezi*	Ramirez's dwarf cichlid
	Cichlasoma facetum	Chanchita
	C. biocellatum	Jack dempsey
	C. festivum	Festive cichlid
	C. meeki	Firemouth
	Aequidens portalegrensis	Port
	A. curviceps	Flag cichlid
	Haplochromis multicolor	Egyptian mouth-breeder
	Hemichromis bimaculatus	Jewelfish
	Nannacara anomala	Golden-eyed dwarf cichlid
	Pteropyllu scalare	Angelfish, scalare
	Symphysodon discus	Disc cichlid
Pomacentridae	*Abudefduf saxatilis*	Sergeant major
	Amphiprion percula	Clownfish
	A. laticlavius	White-saddled clownfish
	A. xanthurus	Chocolate clownfish
	A. akallaopsis	Skunkfish
	Dascyllus auranus	White-tailed puller
	Pomacentrus leucostictus	Beau Gregory
	Premnas biaculeatus	Spiny clownfish
Chaetodontidae	*Pomacanthus arcuatus*	Black angelfish
	Angelichthys isabelita	Blue angelfish
Labridae	*Lachnolaimus maximus*	Hogfish
	Tautog onitis	Tautog
Acanthuridae	*Acanthurus coeruleus*	Blue Tang
Anabantidae	*Anabas testudineus*	Climbing perch
	Betta splendens	Siamese fighting fish
	Colisa lalia	Dwarf gourami
	Macropodus opercularis	Paradisefish
	Trichogaster trichopterus	Three-spot (blue) gourami
	T. leeri	Pearl gourami
	Trichopsis vittatus	Croaking gourami
Triglidae	*Prionotus carolinus*	Common sea robin
Pleuronectidae	*Hippoglossus hippoglossus*	Atlantic halibut
Bothidae	*Lophopsetta maculata*	Windowpane

Family	Species	Common name
	Paralichthys dentatus	Fluke, summer flounder
Monacanthidae	*Alutera monacanthus*	Filefish
Balistidae	*Balistes vetula*	Queen triggerfish
	B. carolinensis	Common triggerfish
Diodontidae	*Chilomycterus schoepfi*	Spiny boxfish, burrfish
	Diodon hystrix	Porcupine fish
	Sphaeroides maculatus	Northern puffer
Batrachoididae	*Opsanus tau*	Northern toadfish

1.3.2 Clinical signs

The clinical signs of mycobacteriosis vary between fish species and the species of pathogen can also influence the clinical symptoms observed. Various external signs reported include emaciation, exophthalmia, anorexia, dyspnoea, listlessness and skin lesions (Austin & Austin, 1993; Frerichs, 1993) whereas the characteristic internal symptoms are granulomata formation in various organs, including spleen, kidney, liver, heart, mesenteries, pancreas, gonad, gills, skin, muscle, brain, and eyes (Majeed *et al*, 1981; Colorni, 1992; Gómes *et al*, 1996; Talaat *et al*, 1998). In some host species there is skin discoloration. In tropical fish, the skin usually becomes paler while in temperate fish, such as salmonids, it becomes brighter (silvery) (Wood & Ordal, 1958; Ross, 1970). Although, granulomata formation in visceral organs is commonly found in most fish species with mycobacteriosis, Majeed & Gopinath (1983) reported a case where clinical internal signs were absent in carp (*Cyprinus carpio*) that were infected with acid-fast bacilli.

Histological examination of diseased fish generally reveals typical granulomata structures with necrotic-calcified centres, rich in mycobacteria, surrounded by a dense zone of epithelioid macrophages (Giavenni & Finazzi, 1980). The lesions observed varied between host species. Lund & Abernethy (1978) reported a lack of

encapsulation in infected whitefish (*Prosopium williamsoni*), while in tropical fish, the lesions were often encapsulated with central necrosis and mineralization. Moreover, there was a fibroma-like area of repair in the hepatic lesions, which was not found in other piscine species. It should be noted, however, that the presence of granulomas can also occur with other agents such as *Flavobacterium* sp. (Majeed *et al*, 1981), *Ichthyophonus hoferi*, *Nocardia* sp., protozoa, migrating helminths (Wolke, 1975; Snieszko *et al*, 1964), and viruses causing pseudo-tumours (Beckwith & Malsberger, 1980).

1.3.3 Transmission

The most probable transmission route of the disease is orally. Nigrelli & Vogel (1963) suggested that the natural mode of infection was by the ingestion of contaminated feed or by ingesting the organism directly from the water. Ross (1970) reported that guppies, fed with infected chinook salmon viscera, developed lesions within two and a half months. Transmission is also thought to be possible by other means such as transovarian transmission (Ashburner 1977), and this has been observed in embryonic platyfish and guppies (Nigrelli & Vogel, 1963). However, Ross (1970) was unable to transmit the disease via ova and milt in steelhead trout, and Parisot (1970) failed to transfer the pathogen to Pacific salmon by introducing it into the milt at spawning. Transmission of infections from lesions in skin and gills has also been considered as a mode of horizontal transmission.

Wedemeyer (1970) and Roberts (1978) shared the view that environmental stress can cause immunosuppression in fish. Although there is no firm evidence to confirm that environmental stress can cause mycobacteriosis infection, Giavenni & Finazzi (1980)

believed that an unnatural environment, such as an aquarium, may actually promote the disease. Mycobacteriosis in yellow perch (*Perca flavescens*) held in an aquarium, was thought to occur due to a depressed immune response in these fish (Daoust *et al*, 1989).

1.3.4 Economic loss

Parisot & Wood (1960) reported that infections in some wild fish populations might be as high as 15%, whereas Smith (1996) reported that infections in fish populations in intensive culture may reach 100%. Giavenni & Finazzi (1980) examined approximately 300 marine and freshwater tropical fish for mycobacteriosis, and found that the mortality rate amongst these fish was between 5-10%. In Thailand, snakehead (*Channa striata*), a valuable fresh water farmed fish, has a mortality rate for this disease which can be as high as 20% (Somsiri, pers. comm.). Fish infected with mycobacteriosis, particularly food fish, are of no market value as they are unfit for human consumption. Smith (1996) reported that companies raising striped bass in the USA have condemned several million dollars worth of fish over the past few years due to mycobacterial infections. Colorni (1992) also described mycobacteriosis outbreaks in cultured sea bass (*Dicentrarchus labrax*) in Israel, and reported a 100% prevalence of *Mycobacterium* infection with mortality over 50%.

The ornamental fish industry has also experienced problems due to mycobacteriosis, with a number of ornamental fish species reported as being susceptible to the disease (Nigrelli & Vogel, 1963). Siamese fighting fish (*Betta splendens*), an economically important fish for export from Thailand, is one species particularly susceptible to

Mycobacterium infections (Adams *et al*, 1995). In central Thailand, up to 45% of fish are thought to be infected on some fish farms (S. Kanchanakhan, pers. comm.).

As well as Siamese fighting fish, other ornamental fish including Guppy (*Poecilia reticulatus*), Goldfish (*Carassius auratus*), Oscar (*Astronotus acellatus*), Angel fish (*Pterophyllum scalar*), and Disc Cichid (*Symphysodon discus*) have also been traded. Although there are no published reports of mycobacteriosis associated with these species *Mycobacterium* spp. have occasionally been isolated from them (S. Kanchanakhan & T. Somsiri, pers. comm.).

1.3.5 Treatment and control

Mycobacterium are sensitive to many kinds of antibiotics, for example rifampin, penicillin, terramycin, and kanamycin sulphate. Treating infected pearl gouramies (*Trichogaster* leeri) by applying penicillin to the skin of the fish (Reichenebach-Klinke, 1955), and treating black widows (*Gymnocorymbus ternetzi*) with terramycin by a continuous bath have both proven successful means of treatment (van Duijn, 1961). *Mycobacterium* infections in Siamese fighting fish were successfully controlled by the addition of kanamycin sulphate (100 ppm) to aquarium water so as to maintain a permanent bath of the antibiotic over a 5-day period (Conroy, 1966). This antibiotic has also been succcessfully used to control mycobacteriosis outbreaks in three-spot gouramies (*Trichogaster trichopterus* Pallas) using the same treatment protocol (Santacana *et al*, 1982). Hedrick *et al* (1987) on the other hand found that feeding rifampin to striped bass (6mg/100g of food for 60 days) was not effective as a treatment. Colorni (1992) confirmed that mycobacteria are resistant to rifampin, whereas cycloserine reduced mortalities caused by the bacterium. Isoniazid and

rifampin have, however, been recommended by Dulin (1976) and Kingsford (1975) as treatments for valuable exotic marine fish.

Antibiotics are generally not a hundred percent effective for treating fish against mycobacteria infections. Continuous use of antibiotic drugs can also introduce drug resistant strains of the bacterium. Thus, ideal management is one of prevention. Treating trash feed before use is recommended to minimise the spread of disease. The feeding of Pacific salmon with pasteurised carcasses may reduce the incidence of infection (Hublou *et al*, 1959). Dulin (1979) suggested that fish, known to be infected, should be burnt or buried with quicklime at a site far from water drainage. Moreover, new stocks of fish should be quarantined and screened before introduction into the hatchery or aquarium.

1.4 Infections in man

1.4.1 History

M. tuberculosis complex and *M. leprae* have been known as human pathogens for many years. This review high-lights other species of *Mycobacterium* known to infect man, which are associated with an aquatic environment. This group of mycobacteria are often referred to as atypical or non-tuberculous mycobacteria.

In 1938, da Costa Cruz isolated *M. fortuitum* from a human abscess (Wayne & Sramek, 1992). MacCallum *et al* (1948) reported a new *Mycobacterium* species isolated from diseased skin which was later named *M. ulcerans* (Fenner, 1950). Although *M. marinum* was first isolated from fish in 1926 (Aronson, 1926), it was not recognised as a human pathogen until 1951 (Norden & Linell, 1951). *M. chelonae*

was first described as potential pathogen in 1953 (Moore & Frerichs, 1953; Guay, 1996). In 1953, Buhler & Pollak reported a new acid-fast bacterial species, later named as *M. kansasii*, that is responsible for pulmonary disease in man. Prissich & Masson (1956) reported cervical lymphadenitis in children caused by *M. scrofulaceum.*

1.4.2 Clinical symptoms

Popp & Reichenbach-Klinke (1982) reported that two mycobacteria pathogenic to fish, *M. marinum* and *M. fortuitum* were pathogens for man. *M. marinum* caused granulomatous skin lesions called "swimming pool granuloma" (Philpott *et al*, 1963; Popp & Reichenbach-Klinke, 1982; Paul & Gulick, 1993). The pathogen of the disease (*M. marinum*) was generally spread by infected fish and contaminated water (Collins *et al*, 1984; Huminer *et al*, 1986; Suh & Hoffman-Steeger, 1992; Kullavanijaya *et al*, 1993; Phillips *et al*, 1995; Feddersen *et al*, 1996; Tong, 1996; Ryan & Bryant, 1997; Ramakrishnan, 1997; Brady *et al*, 1997; Boyce, 1997). The source of *M. fortuitum* and *M. chelonae* infection is more likely from else where. *M. fortuitum* is more commonly associated with post-traumatic and surgical infections, whereas *M. chelonae* is more often associated with pulmonary or disseminated infections (Wallace *et al*, 1983; Griffith *et al*, 1993; Carey *et al*, 1994; Guay, 1996). *M. fortuitum* and *M. chelonae* appear to infect deeper structures such as bones, joints, and lung (Griffith *et al*, 1993; Butt & Janney, 1998; Hadjiliadis *et al*, 1999), however skin infections due to these organisms have also been reported. Zahid *et al* (1994) reported a case of *M. chelonae* infection of the skin, whereas Escalonilla *et al* (1998) reported over ten cases of cutaneous and soft tissue infections caused by non-tuberculous mycobacteria including *M. fortuitum*, *M. chelonae* and *M. marinum.*

Although there are only a few published reports of *M. fortuitum* infections in man, none of those implicates fish as the source of infection (Carey *et al*, 1994; Hong *et al*, 1995; Escalonilla *et al*, 1998; Levett *et al*, 1999; Kullavanijaya, 1999), although transfer from water was suggested.

The incubation period of atypical mycobacterial infection can range from one week to two months (Engbaek *et al*., 1980; MacLellan & Moon, 1982). *M. marinum* infections on the skin appear as brown-red papules or as granulomatous nodules with central ulceration, whereas the cutaneous infection with *M. fortuitum* or *M. chelonae* may be presented as cellulitis, abscess, nodules, sporotrichoid-like lesion, sinuses and ulcers with serosanguinous or purulent discharge (Ingram *et al*, 1993; Tong, 1996; Kullavanijaya, 1999). The cutaneous infection is common on extremities such as arms, hands, fingers, legs, knees, ankles and feet (Escalonilla *et al*, 1998).

1.4.3 Epidemiology

There are many reports of man being infected with atypical *Mycobacterium* and a selection of these are reported here. One report is a woman who scratched herself with a shrimp spine, after which a small lump developed on her left finger. She underwent surgery several times to remove the lump, which was being treated as an arthritic nodule. *M. marinum* infection was finally diagnosed and she was put on a regimen of ethabutol and rifampin (McLain, 1989).

In another case, a 33-year-old man who worked in a yoghurt factory, had a painful swelling above his right elbow for over 6 months. The swelling was tender but without local inflammation. Ziehl-Neelsen staining of pus, aspirated from the lesion,

revealed polymorphonuclear leukocytes together with scant, acid-fast bacilli. After culturing the pus on Lowenstein-Jensen, *M. fortuitum* was identified within 3 days (Westmoreland *et al,* 1990). However, the source of infection in this case was unknown.

Paul and Gulick (1993) also reported two cases of patients infected by *M. marinum.* One was a 46-year-old man who injured his finger while handling a sick catfish. Within 3 weeks his finger had become swollen and painful. *M. marinum* was later identified. In the other case, a 46-year-old woman accidentally stabbed her thumb with a fork while cleaning a fresh-water fish tank. One week later, erythema, swelling, and lymphangitis developed. The biopsy showed granulomatous inflammation and finally *M. marinum* was identified.

A 66-year-old man, who harvested oysters, developed a swollen left hand with non-draining nodular lesions along the ulnar palm. The biopsy revealed acid-fast bacilli which were identified as *M. marinum* (Beecham *et al*, 1991).

There are many reports in the literature which document the transmission of atypical mycobacteria from fish to man. There are, however, no reports revealing their transmission from man to man.

The cutaneous infection, frequently reported, was often due to exposure to the pathogen through an open wound. In some cases the severity of the infection was actually increased because of an immunodeficient state of the host. Lacaille *et al* (1990) reported the *M. marinum* infection in a 5-year-old boy who was sick with an

immune defect. He had had pimple-like lesions on his face 4 years earlier, which had progressively generalised. Most of his body, except the trunk, was affected by crusted ulcerative granuloma, some of which had drained spontaneously and caused pain.

1.4.4 Treatment

Successful chemotherapy has been reported using the following treaments; rifampin and ethambutol, tetracyclines, or sulfamethoxazole and trimethoprim (Brown & Sanders, 1987). The combination of ethambutol and rifampin has been especially effective in the treatment of *M. marinum* infections and has minimal toxicity. Paul and Gulick (1993) found amikacin and kanamycin to be effective, while Suh & Hoffman-Steeger (1992) reported that mycobacteria were susceptible to tetracycline but resistant to isoniazid and streptomycin.

McLain (1989) and Donta *et al* (1986) reported that treatment of mycobacteriosis patients with steroids could worsen the infection. Treatment with steroids is not effective against mycobacteria, and the steroid may immunosuppress the patient (McLain, 1989). Antibiotic treatments seemed to be effective in some cases, however, surgery may also sometimes be required (Williams & Riordan, 1973; Huminer *et al*, 1986).

1.5 Environment

Attention has focused on the environment in which the fish live, as a means of controlling mycobacteriosis because of the belief that diseased fish discharge the pathogen into the water via biological secretion or directly from wounds.

1.5.1 Mycobacteria species occurring in the environment

A variety of *Mycobacterium* species have been isolated from the environment including freshwater, marine water and soil samples. *M. chelonae*, *M. fortuitum*, *M. avium-intracellulare*, *M. gordonae*, *M. kansasii* have all been isolated from water (Pattyn *et al*, 1971; Goslee & Wolinsky, 1976; Landsdell *et al*, 1993). *M. marinum* has also been isolated from different aquatic habitats including fish tanks, swimming pools and hot tubs (Linell & Norden, 1954; Swift & Cohen, 1962; Barrow & Hewitt, 1971).

1.5.2 Water

There are few reports in which mycobacteria have been isolated from water in which diseased fish live (Goslee & Wolinsky, 1976; Dailloux *et al*, 1992). This probably reflects the investigators' interest with the pathology, rather than the epidemiology of the disease. Consequently, few environmental studies have been performed.

Mycobacterium has been detected in both freshwater and the marine environment. Gruft *et al* (1975) and Jamieson *et al* (1976) revealed that mycobacteria can survive in seawater. Falkinham *et al* (1978 & 1981) recovered acid-fast bacilli from coastal water of south-eastern USA, while Viallier (1967) isolated mycobateria from water samples from the coast of France.

Viallier & Viallier (1973) detected many different strains of *Mycobacterium* from freshwater including *M. gordonae*, *M. terrae M. phlei*, *M. vaccae*, *M. fortuitum*, *M. chelonae*, and *M. scrofulaceum*. In addition, *M. kansasii* and *M. marinum* have also been isolated from stream water (Beerwerth, 1973). Joynson (1979) demonstrated

that *M. kansasii* could survive up to 12 months in water without any change in culture characteristics. The author suggested that water is the natural habitat of *M. kansasii*. A number of reports in the literature state that *M. simiae* is also a common species present in environmental water. Howard *et al* (1987) isolated *M. simiae* from tap water in Arizona, while Wayne & Sramek (1992) isolated *M. simiae* from the water supply of a hospital in Gaza.

Besides routine monitoring of diseased fish, examining water for the presence of the pathogen may also be useful for farm management, thus allowing managers to make more informed decisions about their stock (Ford, 1994). A technique frequently used to recover acid fast bacilli from water is filtration, combined with bacteriological characterisation (Engelbrecht *et al,* 1974; Engelbrecht & Haas, 1977; Haas *et al,* 1983). Recently polymerase chain reaction (PCR) has been utilised to detect and identify fish pathogens in water samples including *Aeromonas salmonicida* (Gustafson *et al*, 1992; O'Brien *et al*, 1994) and *Vibrio* spp.(Arias *et al*, 1995).

1.5.3 Soil (Sediment)

Soil or sediment is another habitat from which *Mycobacterium* spp. has been isolated. Kubica *et al* (1963) successfully isolated *M. fortuitum*, *M. smegmatis* and *M. phlei* from soil samples. The presence of a variety of mycobacteria in the soil of an agricultural farm was reported by Donoghue *et al* (1997), and Katila *et al* (1995) identified potential human pathogenic mycobacterial species in water and soil samples. Unfortunately, there appear to be no documented reports of sediment analysis from fish ponds.

1.5.4 Fish food

As *Mycobacterium* infects a wide range of fish, feeding healthy stock with trash fish is of concern (Nigrelli & Vogel, 1963; Raa *et al*, 1992; Colorni, 1992; Knibb *et al*, 1993). Winsor (1946) has also identified *Mycobacterium* spp in market fish used as feed for aquaria fish. Moreover, Hublou *et al* (1959) reported that feeding Pacific salmon with pasteurised offal reduced the incidence of the disease. Ross (1970) reported that the occurrence of mycobacteriosis among Pacific salmonid fishes disappeared after feeding the fish with pasteurised viscera and fish flesh. In addition, Whipple & Rohovec (1994) stated that the prevalence of mycobacteriosis in cultured fish was due to the feeding of infected raw fish. There are no reports of other types of fish food such as zooplanktons being infected with the bacterium.

1.6 Detection and identification techniques

In the past, methods for detecting the etiologic agent of mycobacteriosis, relied on conventional bacteriology and histological examination. Bacteriology can be time consuming due to the slow growth of the organism and the isolates were subsequently identified by biochemical tests. The stains used in histology, carried out on fixed tissue samples, are not specific for *Mycobacterium* spp. Thus, rapid, specific, and practical methods for detection of the pathogen are required for effective control of the disease.

The clinical signs of mycobacteriosis vary according to both the host infected and the strain of the pathogen. Giavenni & Finazzi (1980) suggested that various *Mycobacterium* species or strains can influence the pathological pattern of the disease. In some cases, there are no external signs, while in other cases there are both external

and internal signs. The internal signs appeared as typical granulomata, however, there are other agents which may also cause granulomas.

Modern approaches to identify mycobacteria include immunological techniques (serological tests), applied genetic methods (DNA probes), and physicochemical methods. The first approach includes enzyme-linked immunosorbent assays (ELISA) (Adams & Thompson, 1990; Duffield, 1990; Adams *et al*, 1995), Western blot (WB) analysis (Adams *et al*, 1996) and immunohistochemistry (IHC) (Gómez *et al*, 1996; Navarro *et al*, 1991), while the second approach involves PCR (Knibb *et al*, 1993, Talaat, 1997), hybridisation (Kox *et al*, 1995b; 1996; 1997) and *in situ* hybridisation (ISH) (Arnoldi *et al*, 1992; McNicol & Farquharson, 1997). Physicochemical methods have also been used. These employ an instrument-based approach, with which no prior knowledge of the organisms' identity is needed. Pyrolysis mass spectrometry (PyMS), one such approach, is a versatile method for mycobacterial identification and characterisation (Wieten *et al*, 1981a; Sisson *et al*, 1992; Freeman *et al*, 1993). It is a rapid and accurate method that requires little sample for analysis and many samples can be performed at the same time (Weiten *et al*, 1983).

1.6.1 Direct microscopy

Microscopy is the conventional way of identifying *Mycobacterium* spp, in combination with Ziehl-Neelsen (ZN) staining. ZN staining, although a simple and cheap method for identification, is however not specific. The sensitivity of the method has been reported to be within the detection limits of 5×10^3 to 10^4 bacteria ml^{-1} (Verbon, 1992). The staining technique can be performed either on fresh smears or on formalin-fixed tissue (Lansdell *et al*, 1993;Chinabut *et al*, 1990; Colorni, 1992).

Due to the low sensitivity of the stain, early infection by the pathogen is not normally detected. Moreover, ZN stains a wide range of acid fast mycobacterial organisms such as *Mycobacterium* spp. and *Nocardia* spp. (Smith, 1996; Olson *et al*, 1998). In addition, bacteria cannot be identified to species level.

1.6.2 Culture and biochemical tests

Attempts to isolate *Mycobacterium* spp often fail, since they may be slow growing requiring 1 to 4 weeks for visible colonies to appear (Jacobs *et al*, 1991; Colorni, 1992). Often they are out-competed by other bacteria. It has been reported that successful media for primary isolation of mycobacteria are Dorset's media, Petroff's egg agar (Nigrelli & Vogel, 1963), Lowenstein-Jensen, Petragnani, Middlebrook 7H10 (Dulin, 1979; Shotts & Teska, 1989; Colorni, 1992; Austin & Austin, 1993), Dubos agar (Ashberner, 1977), Sauton's agar (Stanford & Beck, 1968), Ogawa egg (Ashburner, 1977; Arakawa & Fryer, 1984), and modified Sauton's agar (Chen *et al*, 1997). Shotts & Teska (1989) reported that MacConkey agar may be used successfully for the isolation of some rapidly growing strains.

Attempts to identify *Mycobacterium* to species level have focused on the growth rate of the bacterium on different medium, their ability to produce pigments, and their biochemical characteristics (Kubica, 1973; Kubica *et al*, 1973; Marks, 1976; Wayne *et al*, 1974; Wayne *et al*, 1976). After isolation, the profiles of biochemical growth characteristics are normally analysed for the cultures (Kent & Kubica, 1985; Pungkachonboon *et al*, 1992; Lansdell *et al*, 1993). Some of these biochemical characteristics are summarised in Table 1.3.

Table 1.3 Typical biochemical characteristics of *Mycobacterium* spp. isolated from fish (Lansdell *et al*, 1993; Frerichs, 1993)

Charactertistics	*M. marinum*	*M. fortuitum*	*M. chelonae*
Growth rate	slow	rapid	rapid
Growth at 25°C	+	+	+
Growth at 37°C	-	+	-
Growth on MacConkey	-	+	+
Pigmentation	photochromogen	-	-
Nitrate reduction	+/- [(a)]	+	-
Iron uptake	not known	+	-
Sucrose utilisation	not known	+	-

(a) Lansdell *et al*, 1993 reported that *M. marinum* was positive to nitrate reduction whereas Frerichs, 1993 reported that *M. marinum* was negative.

1.6.3 Immunological based tests

Monoclonal antibodies (Mabs) and polyclonal antibodies (Pabs) against *Mycobacterium* spp. have been developed as immunological probes for identification of the pathogen. Compared with culture and conventional biochemical identification, Mabs can provide a more rapid means of identification of pathogens and often to species level. Studies using Mabs to examine human pathogens such as *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*) have been extensively reported by different groups (Kolk *et al*, 1984, 1985, 1988 & 1989; Verstijnen *et al*, 1989; Havelková *et al*, 1989; Verbon *et al*, 1993). Verstijnen *et al* (1989) reported that the Mabs developed against species-specific epitopes of *M. tuberculosis* complex and *M. avium* complex (*M. avium*, *M. intracellulare* and *M. scrofulaceum*) successfully identified the relevant bacteria in WB analysis and immunofluorescene test (IFAT). Mabs have been used in a variety

of techniques requiring high specificity such as ELISA, WB, IFAT and IHC. These techniques have been applied to examine many aspects including diagnosis, epidemiology and taxonomy.

Detection of the atypical mycobacteria in fish was reported by Gómez *et al* (1996) who used commercial Pabs (anti-*M. paratuberculosis* and anti-*M. bovis* BCG) and Mab (anti-*M. avium*) to diagnose mycobacteriosis in swordtail (*Xiphophorus helleri*). These authors reported that the Pabs were more sensitive than Mabs. In fact, the former could detect a wider range of *Mycobacterium* species. However, it was not possible to identify the pathogen to species level. Attempts to classify fish *Mycobacterium* species was reported by Adams *et al* (1995 & 1996). The authors successfully developed Mabs against *Mycobacterium* spp isolated from fish, which they used in ELISA for diagnosis of mycobacteriosis. However, these Mab were not functional in IHC. Recently, Blackwell (1998) also reported on the use of Mab against *M. marinum* to identify the pathogen by ELISA and WB.

Serology is not only useful for diagnosis, but is also used in taxonomy. Tsang *et al* (1984) analysed *M. fortuitum-M. chelonae* complex by ELISA and agglutination. The authors classified *M. peregrinum* as a distinct species from *M. fortuitum*, and they found *M. chelonae* subsp. *abscessus* to be identical to *M. chelonae* subsp. *chelonae*. Arakawa *et al* (1986), using serology, established that *M. chelonae* isolated from salmonid fish, was a single species *M. chelonae* and withdrew the name *M. chelonae* subsp. *piscarium* proposed by Arakawa & Fryer in 1984.

Detection of *Mycobacterium* spp during infections can also be confirmed from the level of anti-mycobacteria antibodies present in blood serum. More than a decade ago, ELISA was first used to detect IgG antibodies in human against *Mycobacterium tuberculosis*, with favourable results (Raheman, 1990). Many studies have focused on optimisation of the assay to enhance it sensitivity and specificity. Yokomizo *et al* (1983) reported that pre-absorbing serum to remove cross-reactive antibodies before use in the ELISA appeared to improve its specificity. An ELISA set up to detect antibody to mycobacteria requires a specific probe for the technique to be functionable. Many are available for studies in man and many other mammals but few are available for fish. Chen *et al* (1997) studied the immune response of rainbow trout to extracellular products of *Mycobacterium* spp by using Mab anti-rainbow trout immunogoblulin as a tool.

1.6.4 Physico-chemical methods

Pyrolysis mass spectrometer (PyMS) is a physico-chemical technique that can be employed to obtain biochemical fingerprints of whole micro-organism (Magee, 1993). In general, a mass pyrogram is produced when an organic sample is thermally degraded, or pyrolyzed, *in vacuo*. Curie point PyMS was used in this study to heat a ferro-magnetic foil holding the samples within a high-frequency alternating magnetic field. The vapour from the pyrolysed sample was bombarded with low energy electrons to produce a variety of molecular fragments, which were then separated by a quadrudpole mass spectrometer on the basis of their mass:charge ratio (m/z). A biochemical fingerprint was obtained for each bacterium analysed in the form of a quantitative mass spectrum.

PyMS was firstly used to analyse biological materials by Zemany in 1952 (Berkeley *et al*, 1990). Meuzelaar & Kistemaker (1973) introduced a curie-point pyrolysis mass spectrometer for fingerprinting complex samples. Later, it was developed into a fully automatic integrated instrument for analysing bacteria (Meuzelaar *et al*, 1976). PyMS has been successfully applied to identify a wide range of bacterial genera, fungi and yeast including *Mycobacterium* (Wieten *et al*, 1981a, 1981b & 1983; Sisson *et al*, 1991; Freeman *et al*, 1993), *Bacillus* (Boon *et al*, 1981; Shute *et al*, 1984; 1988), *Corynebacterium* (Hindmarch *et al*, 1990), *Listeria* (Freeman *et al*, 1991c), *Fusobacterium* (Magee *et al*, 1989a), *Neisseria* (Beuvery *et al*, 1983), *Streptococcus* (Magee *et al*, 1989b; Magee *et al*, 1991; Winstanley *et al*, 1992), *Rhizobium* (Goodacre *et al*, 1991), *Candida* (Magee *et al*, 1988; White *et al*, 1994), *Actinomycete* (Sanglier *et al*, 1992) and *Aphanomyces* (Lilley, 1997).

1.6.5 Biomolecular tests

Biomolecular techniques have been extensively used to study human mycobacterial diseases such as tuberculosis and leprosy (Kolk *et al*, 1992; Van der Vliet *et al*, 1993; Beige *et al*, 1995). Recently, many groups have reported a gene coding for 16S rRNA (16S rDNA) to be highly conserved among the genus *Mycobacterium*. Edwards *et al* (1989) characterised 16S rRNA of *M. kansasii*. Rogall *et al* (1990a) reported the 16S rDNA sequence from 20 species of *Mycobacterium* to be highly conserved among the genus. The analysis of nucleic acid sequences coding for 16S rRNA is useful for the characterisation of an organism, and can be used as a target site for species-specific probes for identification purposes. After the report of mycobacterial 16S rRNA sequences, amplification of this sequence by PCR has shown to be useful for the identification and detection of *Mycobacterium* spp.

The most extensively employed technique based on nucleic acid composition is the polymerase chain reaction (PCR). With this technique, the target sequences are exponentially amplified by specific oligonucleotide primers in a thermal cycling machine. The target DNA and primers are crucial in the assay since they are the main factors determining the specificity and sensitivity of the reaction (Böddinghaus *et al*, 1990; Ross *et al*, 1997b).

Among these target sequences, 16S rDNA appears to be the most promising for the identification of *Mycobacterium* spp in fish, since the 16S rDNA is highly conserved among this genus and offers more alternatives for primers to identify the species (Knibb *et al*, 1993; Kox *et al*, 1994 & 1995b).

The amplified DNA is usually detected by agarose gel electrophoresis or hybridisation (Kox *et al*, 1994; 1996; 1997). Amplification can be further exploited by more specific methods such as restriction fragment length polymorphism (RFLP) (Plikaytis *et al*, 1992), restriction enzyme digestion, random amplified polymorphic DNA analysis (RAPD) (Ross & Dwyer, 1993), single-stranded conformational polymorphism analysis (SSCP) (Telenti *et al*, 1993), pulsed-field gel electrophoresis (PFGE) (Hector *et al*, 1992; Wallace *et al*, 1993b) and spoligotyping (Goyal *et al*, 1997; Sola *et al*, 1998).

Knibb *et al* (1993) pioneered PCR to identify fish mycobacteria and have used it to detect *M. marinum* in European sea bass (*Dicentrarchus labrax*) in Israel. The authors actually succeeded in identifying the presence of the bacteria before the onset of clinical symptoms of the disease.

The PCR technique is a sensitive taxon-specific detection system for mycobacteria without the need for prior cultivation of the pathogen (Böddinghaus *et al*, 1990; Rogall *et al*, 1990a; 1990b). The application of PCR is extensively used for the identification of a variety of bacteria (Selander *et al*, 1986; Bej *et al*, 1991; Lampel *et al*, 1990; Knibb *et al*, 1993; Varela *et al*, 1994; Zhang *et al*, 1995; Steingrube *et al*, 1997; Sechi *et al*, 1999). Moreover, the taxonomy of *Mycobacterium* is still controversial, and PCR has been introduced to aid in the classification to species level.

Pao *et al* (1990) reported that the analysis of bacterial cultures and clinical specimens by PCR was highly sensitive and specific in detecting mycobacteria. The PCR technique has been reported to be more sensitive than serological techniques such as ELISA (Puttinaowarat, 1995).

In situ Hybridisation (ISH)

In situ hybridisation (ISH) was firstly described in 1969 (Gall & Purdue, 1969; John *et al*, 1969). The technique is based on complementary nucleic acid sequences of target DNA or RNA specific to the pathogen located within individual cells on tissue sections. The target sequences can be in bacterial, viral or parasitic DNA (Schoone *et al*, 1991). The specific nucleic acid sequences (probes) are labelled with reporter molecules, which can be isotopic molecules or non-isotopic molecules. Visualisation of the reporter molecule gives the precise location of the pathogen within the infected tissue or cells. ISH has been used in a variety of applications including, diagnosis of infection (Arnoldi *et al*, 1992), identification of gene expression by detection of mRNA, studies on the cell cycle (Vermura *et al*, 1992), and studies of interphase

cytogenetics such as the investigation of tumours (Taylor *et al*, 1993; Beck *et al*, 1995). However, there are no published reports on the use of ISH for detection or identification of *Mycobacterium* spp in fish.

Chapter 2

Development of a monoclonal antibody (Mab) against *Mycobacterium marinum*

2.1 Introduction

Mycobacterium spp are slow growing bacteria and, therefore, attempts to isolate such pathogens from fish infected with mycobacteriosis is both difficult and time consuming. Ziehl-Neelsen (ZN) staining is subsequently used to identify the bacterium. Although ZN is a rapid and inexpensive method for diagnosing the disease, it is non-specific since it only identifies acid-fast bacteria. Acid-fast bacteria can be detected easily in heavily infected tissue with this stain. However, in the early stages of infection bacteria are frequently overlooked as ZN staining is normally negative (Fridman 1995; Talaat *et al*, 1998). This leads to misdiagnosis of mycobacteriosis. Therefore, although ZN staining may be useful as a pre-screening technique to establish the presence of the bacteria, further analysis using more sensitive and specific probes is still required for both ZN negative and positive samples.

Gómez *et al* (1996) used a polyclonal antibody to diagnose mycobacteriosis in swordtail (*Xiphophorus helleri*). However, they were unable to identify the species of the *Mycobacterium* pathogen. Monoclonal antibodies (Mabs) are ideal probes for identifying these pathogens. Mabs are frequently used to diagnose tuberculosis in man (Kolk *et al*, 1984 & 1989; Minden *et al*, 1984; Andersen, *et al* 1986; Havelková *et al*, 1989; Verbon *et al*, 1990), and they have also been used in the diagnosis of a variety of fish diseases caused by pathogens such as *Aeromonas* spp (Adams & Thompson, 1990; Cartwright *et al*, 1994; Neelam *et al*, 1995), *Vibrio* spp (Espelid *et al*, 1988; Chen *et al*, 1992; Miyamoto & Eguchi, 1997), *Photobacterium damselae* subs. *piscicida*

(Bakopoulos *et al*, 1997), PKX (Adams *et al*, 1992; Saulinier & De Kinkelin, 1996; Morris *et al*, 1997) and *Mycobacterium* spp (Adams *et al*, 1995 & 1996). Mabs raised against *Mycobacterium* spp in fish were produced by Adams *et al* (1996). These were used successfully to identify the bacteria by ELISA, however, they did not react using immunohistochemistry (IHC). In addition, the Mabs were only able to characterise the bacteria to genus level and differentiation between *Mycobacterium marinum, M. fortuitum* and *M. chelonae* was not possible. The reason why it is difficult to produce a species-specific probe for mycobacteria is possibly due to the cell wall of the bacterium, which is constructed of three layers comprising peptidoglycan, arabinogalactan and a lipopolysaccharide lining (Brennan & Draper, 1994). The thick cell wall may mask species-specific antigens so that the mouse is unable to respond to them upon immunisation. This may also explain why the Mabs produced by Adams *et al* (1996) do not work in IHC. Preparation of bacteria for immunisation by either heat killing or sonication may destroy the outer lipid layer of the bacteria and remove species-specific antigens. In the present Chapter the antigen was prepared in such a way as to preserve the integrity of the antigenic determinants on the surface of the cell. Bacteria were killed by UV-treatment.

The purpose of this study was to produce Mabs against *M. marinum* for use in the detection and identification of the organism in fish and their environment. It has been reported that *M. marinum* is transferable to man, and causes a chronic disease called "swimming pool granuloma" (Gray *et al*, 1990). People at greatest risk are those who are in direct contact with infected animals or infected water.

Mycobacterium sp (S267) isolated from snakehead fish (*Channa striata*) during a disease outbreak in Thailand was chosen as a representative *M. marinum* isolate to immunise mice for the production of Mabs. This isolate was previously identified as *M. marinum* using polymerase chain reaction (PCR) (Puttinaowarat, 1995). The Mabs produced here were then used in a variety of immunological assays to identify *M. marinum* in fish or in pure bacterial cultures isolated from infected fish.

2.2 Materials and Methods

2.2.1 Monoclonal antibody production

2.2.1.1 Bacterial preparation

M. marinum (S267) isolated from snakehead fish was cultured on modified Sauton's agar (MSA) at 30°C for 7 days (Stanford & Beck, 1968). The bacteria were gently scraped off the agar and washed twice with sterile phosphate buffered saline (PBS: 0.02 M Phosphate, 0.15 M NaCl, pH 7.2) by centrifugation at 3000 xg (Mistral 3000i, MSE). The absorbance of the bacterial suspension was adjusted to an OD of 0.8 at 610 nm (Ultrospec 2000, Pharmacia Biotech) and exposed to UV light (Gelaire, BSB48) at an activity of 60 Joule sec^{-1} for 4 h. The viability of the bacteria was checked by plating a sample onto MSA and incubating the plate at 30°C for 3 weeks.

2.2.1.2 Immunisation of mice

The bacteria (10^7 cells ml^{-1}) were mixed 1:1 (v:v) with TiterMax adjuvant (Vaxcel^{TM}, Inc.) to form an emulsion. The mixture was injected intraperitoneally into four 7-week-old balb/c mice at 100 µl $mouse^{-1}$. The mice received a second injection three weeks later, which contained the same amount of antigen. The antibody response was monitored and four weeks later, the mouse with the highest antibody response was

injected intra-venously with 100 µl of S267 (10^7 cells ml^{-1} in PBS). After, three days the mouse was bled out, killed and its spleen removed.

2.2.1.3 Determination of antibody response

Prior to immunisation, the mice were bled to collect normal non-immune serum. Fourteen days after the first injection, a blood sample was taken from the tail vein, kept overnight at 4°C, centrifuged at 3000 xg for 5 min and the serum collected. A blood sample was taken again 18 days after the booster injection.

The level of antibody in the serum was analysed by an enzyme-linked immunosorbent assay (ELISA). The ELISA plates were coated with 0.1% poly-L-lysine (Sigma) in carbonate-bicarbonate buffer, pH 9.6 (Sigma) for 1 h, then washed twice with low salt wash buffer (LSW, 0.02 M Trisma base, 0.4 M NaCl, 0.05% Tween 20). Bacteria, cultured and prepared as described above, were adjusted to 10^7 cells ml^{-1} (v:v) in PBS. This suspension was added to the wells (100 µl $well^{-1}$) and the plate was incubated overnight at 4°C. The following day 50 µl $well^{-1}$ of 0.05% (v:v) gluteraldehyde in PBS was added to the wells and the plate incubated for a further 20 min. The plate was then washed three times with LSW. The plate was post-coated with 1% bovine serum albumin (BSA) (250 µl $well^{-1}$) for 1 h and washed three times with LSW. Anti-sera samples were serially diluted ten fold in PBS and 100 µl $well^{-1}$ was applied to the plate and incubated for 1 h at 20°C. Pre-immune sera and PBS were added to the plate as negative controls. The plate was then washed 5 times with a 5 min soak on the last wash using high salt buffer (HSW: 0.02 M Trisma base, 0.5 M NaCl, 0.1% Tween 20, pH 7.7). The plates were incubated with an anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) [Scottish Antibody Product Unit (SAPU), Lanarkshire,

Scotland] (a 1/1000 dilution in PBS) at 100 µl $well^{-1}$ for 1 h, after which they were washed with HSW as described above. Finally, chromogen (42 mM TMS, 3, 3', 5, 5'-tetramethylbenidine dihydrochloride) (Sigma) in substrate buffer (0.1 M citric acid, 0.06 M sodium acetate, 1% H_2O_2, pH 5.4) was applied to the wells at 100 µl $well^{-1}$. After 10 min the reaction was stopped using 50 µl $well^{-1}$ 2 M H_2SO_4, and read with an ELISA reader (Dynatech, MR 5000) at 450 nm.

2.2.1.4 Myeloma cell culture

Myeloma cell line, SP2/0-Ag14, (Imperial Laboratories) was cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma) with 20% foetal calf serum (FCS), 200 mM L-glutamine, 5000i.u. penicillin/streptomycin, 100 mM sodium pyruvate at 37°C in a 5% CO_2 atmosphere. The cells were checked daily and fresh medium added as required. A week before the fusion, the cells were expanded daily to maintain growth in the log phase.

2.2.1.5 Cell fusion

Cell fusion was carried out by the method of Campbell (1984) with modification. Basically, the mouse with the highest antibody titre was killed by exposure to CO_2 and a blood sample collected by cardiac puncture. The mouse was swabbed with 70% ethanol and the spleen carefully removed and placed into 20 ml of serum-free DMEM with additives. The spleen was washed 3 times with serum-free DMEM warmed to 37°C. Both ends of the spleen were removed and 5 ml of warm DMEM was passed through it using a needle and syringe. The cell suspension was placed in a Universal and allowed to stand for 1 min. The supernatant was collected and made up to 50 ml with serum-free DMEM at 37°C. The suspension was washed twice by centrifugation

at 150 xg for 10 min. The cells pellet was gently re-suspended in 10 ml of the warmed serum-free DMEM.

The myeloma cells were collected by centrifuging at 150 xg for 10 min, washed as described above for the spleen cells and then re-suspended in 10 ml of serum free DMEM at 37°C. Both sets of cells were counted, then mixed together at a ratio of one myeloma cell to ten spleen cells (8×10^6: 8×10^7). The cells were centrifuged at 100 xg for 10 min and the supernatant carefully removed. Polyethylene glycol (PEG 1500, Boehringer Mannheim) [1.5 ml 50% (w:v) in warmed DMEM] was gently added to the cells over 15 sec and allowed to stand for 1 min 45 sec. Warmed serum-free DMEM (50 ml) was very carefully added to the cells, which were then centrifuged at 100 xg for 5 min. The supernatant was removed and the pellet was re-suspended in 10 ml of DMEM with additives (0.1 mM hypoxathine, 0.016 mM thymidine, 2 mM glutamine, 0.5 mM sodium pyruvate, 50 i.u. ml^{-1} penicillin/streptomycin and 20% FSC) and incubated for 3 h at 37°C in a CO_2 atmosphere. The cell suspension was centrifuged at 100 xg for 5 min and the pellet gently re-suspended in 200 ml of DMEM with the above additives as well as 0.4 µM aminopterin and mouse red blood cells (3×10^7 cells ml^{-1}). The cell suspension was placed into 96 well tissue culture plates at 180 µl $well^{-1}$. Positive and negative controls of spleen cells and myeloma cells were set up in parallel with the fused cells. The plates were incubated in a CO_2 incubator (0.5% CO_2) at 37°C for 10 days, after which the hybridoma clones were screened by ELISA as described Adams *et al* (1995) to establish which were positive. Positive clones were expanded and re-cloned three times by limiting dilution as described by Campbell (1984).

2.2.1.6 Isotyping

The monoclonal antibodies were isotyped using a commercial isotyping kit (BioRad), following the protocol outlined by the manufacturer.

2.2.2 Characterisation of the monoclonal antibody

The Mab was characterised with respect to specificity and sensitivity by ELISA, and the antigenic determinants were analysed with different strains of *Mycobacterium* spp. by Western blot (WB) analysis. The antigenic determinants were subsequently characterised by a variety of methods including proteinase K treatment, glycoprotein test, lectin staining, and immunogold staining.

2.2.2.1 Specificity in ELISA

The ELISA plates were coated with 0.01% poly-L-lysine in coating buffer for 1 h at 20°C followed by two washings with LSW. Sixteen strains of non-mycobacteria and 10 mycobacterial isolates including 3 reference strains (described in Section 2.3), were added to the well at 10^8 cells ml^{-1}. The remainder of the assay was as described in Section 2.2.1.3.

2.2.2.2 Sensitivity in ELISA

Ninety-six-well-plates were coated with 10 µg ml^{-1} acid-treated rabbit serum anti-*Mycobacterium* sp (S267) (courtesy of S-C Chen) in carbonate-bicarbonate buffer, pH 9.6 (Sigma) and incubated overnight as described by Adams (1992). The plates were washed three times with LSW before blocking non-specific sites with 1% (w:v) BSA, 250 µl $well^{-1}$ for 1 h at 20°C. The plates were again washed with LSW before added 100 µl $well^{-1}$ of either UV-treated whole cell or sonicated *M. marinum* (S267), serial

diluted to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 cells ml^{-1}, and PBS as a negative control. The plates were incubated for 1 h at 20°C. The plates were washed 5 times with HSW with a 5-min incubation on the last washing. Mab supernatant was applied to the plates at 100 µl $well^{-1}$ and incubated for 1 h at 20°C. They were again washed with HSW with a 5 min soak on the last wash. The plates were incubated with an antimouse IgG antibody conjugated to HRP (at 1/1000 dilution in PBS) at 100 µl $well^{-1}$ for 1 h after which the assay proceeded as described in Section 2.2.1.3. Values above three times the negative control mean value were considered positive.

2.2.2.3 Reaction of monoclonal antibody (8F7) with *Mycobacterium* lysates by Western blot analysis

Ten mycobacteria strains including 3 reference strains *M. marinum*, *M. fortuitum*, and *M. chelonae* (described in Section 2.3) were sonicated at an amplitude of 18 microns for 2 min. The protein concentration of the lysate was estimated using the relationship, an OD of 1.4 at 280 nm is equivalent to 1 mg ml^{-1}, and it was then adjusted to 1 mg ml^{-1} with PBS. The lysates were mixed with sample buffer in a ratio of 3:1 ratio and boiled for 5 min. Polyacrylamide gel electrophoresis-sodium dodecyl sulfate gel (SDS-PAGE) was performed as described by Laemmli (1970) using a 12% separating gel and a 4% stacking gel (see Appendix 1). Samples were loaded onto the gel at 20 µl $well^{-1}$ containing 15 µg protein ml^{-1} per sample, while prestained molecular weight standards (BioRad) were loaded onto the gel at 10 µl $well^{-1}$. The gels were subjected to 150 V for 1.5 h, then transblotted onto nitrocellulose membranes using a wet system (Electrophoresis unit, Hoefer) by applying 55 V for 1.5 h. The buffer used for the transblotting was 25 mM Tris-hydrochloride, 192 mM glycine and 20% (v:v) methanol, pH 8.3. The membrane was subsequently incubated in 1% BSA for 2 h to block non-

specific binding sites and washed three times with Tris buffered saline (TBS: 0.05 M Tris, 0.15 M NaCl, pH 7.6) containing 0.1% (v:v) Tween 20 with soaking for 5 min during the last washing. The membranes were incubated with Mab supernatant (8F7) overnight at 4°C, after which the membranes were washed as above. They were then incubated with 1% anti-mouse IgG-biotin (SAPU) in TBS for 1 h at 20°C and again washed as above. The membranes were incubated in 1% streptavidin-HRP (SAPU) in TBS for 1 h following with washing as described above. The reaction between antibody and antigen was developed using 2 ml of 4-chloronaphthol solution (0.018 M in methanol) (Sigma), 10 ml of PBS with 10 µl H_2O_2 until bands of colour appeared. The membrane was washed with distilled water to stop the reaction.

2.2.2.4 Reaction of monoclonal antibody (8F7) with *Mycobacterium* extracellular products (ECP) by Western blot analysis

Five ml of *M. marinum* (S267) (1 x 10^7 CFUs) was used to inoculate 500 ml of MSB and incubated at 28°C for 11 days, and then shifted from 28°C to 37°C for a further 4 days incubation (Chen *et al* 1997). The bacteria were heat killed by placing the bacterial suspension at 75°C for 15 min. Killed bacteria were collected by centrifugation at 3000 xg, 4°C for 1 h. The supernatant was filtered through filter paper (Whatman No.1) and then filtered through 0.2 µm Millipore filters. The filtrate was placed into a dialysis tube (12-14 kDa molecular weight cut-off, Medicell), covered with PEG (Sigma) and incubated overnight at 4°C. The concentrated ECP in the dialysis tubes was then dialysed in PBS (pH 7.2) for 24 h changing the buffer every 6 h. The protein concentration of the ECP was determined at 280 nm, adjusted to 1 mg ml^{-1} and diluted 1:1 (v:v) with sample buffer. SDS-PAGE and Western blotting were carried out as previously described in Section 2.2.2.3.

2.2.2.5 Proteinase K treatment

M. marinum (S267), *M. marinum* (NCIMB1297) and the ECP of S267 were digested with proteinase K (25% w/w on the basis of protein), by incubating at 60°C for 1 h (Neelam *et al.*, 1995) to remove protein components. The digested samples were analysed by SDS-PAGE and Western blotting as described in Section 2.2.2.3 and the profiles of whole cells were compared with the ECP.

2.2.2.6 Glycoprotein detection

M. marinum type strain (NCIMB1297) and *M. marinum* (S267) were sonicated as described above and samples were prepared in duplicate. One of the samples was treated with proteinase K and another remained untreated. ECP of S267, treated or untreated with proteinase K, were also included. Samples at a concentration of 1 mg ml^{-1}, were diluted with sample buffer (1:1) and loaded onto a 12% gel. The gel was run at 180 V until the samples nearly reached the bottom of the gel. The gel was blotted onto nitro-cellulose paper as described in Section 2.2.2.3 and the membrane was then analysed using a glycoprotein detection kit (BioRad), following the manufacturer's instructions. Briefly, the membrane was incubated with 10 ml PBS at 20°C for 10 min with agitation. The membrane was then incubated in 10 ml of 10 mM sodium periodate in sodium acetate/EDTA buffer at 20°C for 20 min. The membrane was washed three times with 10 ml of PBS with 10 min soaking in the wash. Biotinylation solution was prepared freshly before use by adding 2 µl hydrazide solution to 10 ml sodium acetate/EDTA buffer. The membrane was incubated in this solution at 20°C for 1 h. The membrane was washed three times with 10 ml of TBS at 20°C for 10 min and then incubated in 10 ml of blocking solution at 4°C overnight. The membrane was again washed three times with 10 ml of TBS at 20°C for 10 min. Conjugate solution was

prepared by adding 5 µl streptavidin-alkaline phosphatase to 10 ml of TBS. The membrane was incubated in this solution at 20°C for 1 h and then washed three times with 10 ml of TBS for 10 min. The colour development solution was freshly prepared before use by adding 50 µl NBT (nitro blue tetrazolium) and 37.5 µl BCIP (5-bromo-1-chloro-3-indolyl phosphate) to 10 ml of colour development buffer and mix well. The membrane was incubated in this solution for 10 min and the reaction was stopped by rinsing in distilled water.

2.2.2.7 Lectin staining

Lysates of *M. marinum* (S267) and the type strain *M. marinum* (NCIMB 1297) were prepared as described in Section 2.2.2.3. Samples were loaded at 200 µl $well^{-1}$ onto the gel and were subjected to 150 V. The gels were blotted onto nitrocellulose paper as described in Section 2.2.2.3. Each membrane was divided into two, one half was stained with Mab 8F7 as described in Section 2.2.2.3, while the another half was stained with a variety of lectins (Table 2.1) to examine carbohydrate binding sites. A multiscreen apparatus (BioRad) was used for the Western blot analysis. The lectins used included Concanavalin A (Con A, Sigma), *Glycine max* soybean (GML, Sigma), *Dolichos biflorus* Horse gram (HGL, Sigma), *Ulex europaeus* agglutinin (UEA-1, Sigma), *Triticum vulgaris* (wheat germ) (TVL, Sigma) and *Arachis hypogaea* (peanut) (AHL, Sigma). The membranes were incubated with the lectins at 0.25 mg ml^{-1} in PBS for 1 h. The remained of the protocol was as described in Section 2.2.2.3.

Table 2.1 Lectins used to characterise carbohydrate present in *Mycobacterium* spp.

Lectins	Abbreviation	Carbohydrate specificity
Concanavalin A (*Canavalia ensiformis*)	Con A	α-D-mannosyl and α-D-glucosyl residues
Soybean (*Glycine max*)	GML	N-acetyl-D-galctosamine
Horse gram (*Dolichos biflorus*)	HGL	N-acetyl-α-D-galactosaminyl residues
Gorse seed (*Ulex europaeus*)	UEA-1	L-frucose
Wheat germ (*Triticum vulgaris*)	TVL	N-acetyl-β-D-glucosaminyl residues and N-acetyl-β-D-glucosamine oligomers
Peanut (*Arachis hypogaea*)	AHL	D-galactose

2.2.2.8 Immunogold staining of mycobacteria with Mab 8F7

M. marinum type strain (NCIMB 1297) and *M. marinum* (S267) were cultured on MSA for 7 days at 30°C. The bacteria were scraped off the agar and resuspended in PBS. The suspension was then centrifuged at 3000 xg for 5 min and washed twice with PBS. The bacteria were fixed with 5% gluteraldehyde/0.2 M PIPES, Piperazine-N, N,-bis-(2-ethanesulfonic acid) (Sigma), pH 5.5 overnight, rinsed three time with 0.2 M PIPES, pH 5.5, then three times with PBS. The bacteria were dehydrated through a series of ethanol concentrations (30%-70%) at 40 min intervals, and then infiltrated overnight with 70% ethanol: LR White resin (London Resin Co.) (1:1). The solution was replaced twice with 100% LR White, by incubating for 8 h after the first change and then overnight after the second change. The bacteria were filled in pointed capsules with fresh LR White and polymerised at 50°C for 24 h. The samples were removed from the capsules and cut into ultra-thin sections mounted on nickel grids. To block the non-specific sites on the sections, they were placed face down onto drops of washing buffer containing blocking reagents (0.2 M TBS, pH 7.5, 1% Tween20, 1% BSA and 10% FCS) which had been spotted onto a piece of a parafilm placed in a moist container. The sections were incubated overnight at 4°C. The grids were transferred to neat supernatant containing Mab 8F7 and incubated at 4°C for 24 h. The grids were

then washed with washing buffer for 90 min by floating them in 24-well-plate with occasional agitation. The buffer was changed and the sections were washed for another 90 min. The samples were incubated overnight at 4°C in 1/40 anti-mouse IgG gold conjugate, 10 nm (Sigma) in the blocking buffer. The grids were held under distilled water for a few seconds and then counter-stained as described by Epstein & Holt (1963). Briefly the grids were stained with 50% uranyl acetate in ethanol for 1 min and wash three times with 50% ethanol, once with 25% ethanol and then with water. The grids were stained with Reynolds lead citrate for 7 mins and washed three times with water. Finally, the samples were viewed by transmission electron microscopy under 80 kv (Philips 301).

2.2.3 Use of monoclonal antibody 8F7 to identify *M. marinum* using ELISA, Western blot, and IHC

Once the Mab had been characterised, it was used as a tool to identify *M. marinum* in ELISA, Western blot and IHC.

2.2.3.1 Examination of culture strains by ELISA

The Mab was tested against a variety of *Mycobacterium* spp isolated from both farmed and ornamental fish from different geographical regions including Thailand, Israel, Denmark, Germany, Greece, USA, Chile and UK (described in Section 2.3.3). They were cultured at 30°C from 14 to 30 days depending on the growth time of the strain. The bacteria were prepared for ELISA as described in Section 2.2.1.3. These were then used to coat the ELISA plates and tested with the Mab as described in Section 2.2.1.3.

2.2.3.2 Western blot analysis

Twenty-nine mycobacteria strains from Thailand, nine from Israel, two from Greece, two from Germany, two from Denmark, and one from USA as well as three reference strains, *M. marinum*, *M. fortuitum*, and *M. chelonae* were sonicated at an amplitude of 18 microns for 2 min and the protein concentration of the lysate adjusted to 1 mg ml^{-1} with PBS. The samples were analysed as described in Section 2.2.2.3.

2.2.3.3 Immunohistochemistry (IHC)

Spleens and livers, infected with mycobacteria, were collected from Siamese fighting fish (*Betta splendens* Regan) and fixed in 10% formalin buffer overnight. The samples were then embedded in wax and cut into 5 μm thick sections. These were dewaxed in xylene (2 x 5 min), 100% ethanol (5 min) and 70% ethanol (3 min). Sections were stained with ZN staining prior to examining them for the presence of mycobacteria. Briefly, the sections were stained in carbol fuchsin for 1 h at room temperature. The sections were then blotted on filter paper and rinsed in 1% acid alcohol for 1 min. The sections were again rinsed in tap water and then counterstained in 1% acidified methylene blue for 1 min. Excess stain was removed by rinsing in tap water and then dehydrated in alcohol dilutions. The slides were mounted with Pertex (Cellpath, UK).

The remaining sections were carried on by IHC as described by Adams & Marin de Mateo (1994). Briefly, the sections were incubated in 10% hydrogen peroxide in methanol for 10 min at 20°C, and then washed three times with TBS. Non-specific binding sites were blocked with 10% goat serum diluted in TBS. Neat supernatant of Mab 8F7 was applied to the sections and incubated for 3 h, and a negative control of PBS was also included. The sections were washed with TBS, then incubated for 1 h

with 1% anti-mouse IgG-biotin (SAPU) diluted in TBS. The sections were washed and incubated for a further 60 min with streptavidin-HRP (SAPU) (1% in TBS), after which they were again washed with TBS. The sections were visualised by incubating them in 3'3'-diaminobenzidine tetrahyrochloride (DAB) for 10 min. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted with pertex.

2.3 Results

2.3.1 Monoclonal antibody production

Three hybridoma cell lines were produced in this study against *M marinum* (S267). However, two of the clones exhibited poor growth and had a low response in both ELISA and Western blot. The remaining clone (8F7) grew well and had a good response to isolate S267. This was, therefore, the only Mab, which was characterised from the fusion and used for diagnostic purposes. The Mabs from this clone were of a IgG_{2a} subclass with a κ light chain.

2.3.2 Characterisation of the monoclonal antibody

2.3.2.1 Detection of specificity and sensitivity by ELISA

The sandwich ELISA test using rabbit polyclonal antisera as the capture antibody and Mab 8F7 as the detection antibody had a sensitivity threshold of 10^5 cells ml^{-1} with whole cells, and 10^4 cells ml^{-1} with sonicated cells. The Mab recognised both the type strain *M. marinum* (NCIMB1297) and *M. marinum* isolate S267 as determined by PCR (Puttinaowarat, 1995). It did not cross-react with any other *Mycobacterium* species (Table 2.2) nor the non-mycobacteria (Table 2.3).

Table 2.2 Cross-reaction of monoclonal antibody cell line (8F7) with the human pathogenic mycobacteria and other mycobacteria strains determined by an enzyme-liked immunosorbent assay

Bacteria	Origin	Absorbance [a]	Reaction[b]
Mycobacterium avium	KIT	0.091	-
Mycobacterium kansasii	KIT	0.085	-
Mycobacterium tuberculosis	KIT	0.081	-
Mycobacterium gordonae	KIT	0.085	-
Mycobacterium vaccae	KIT	0.099	-
Mycobacterium intracellulare	KIT	0.127	-
Mycobacterium poriferae	NCIMB12538	0.138	-
Mycobacterium fortuitum	NCIMB1294	0.116	-
Mycobacterium chelonae	NICMB1474	0.170	-
Mycobacterium marinum	NICBM1297	0.771	+
Mycobacterium marinum (S267)	Snakehead fish, Thailand	0.848	+

[a] The mean value of duplicate samples at 450 nm; the negative control was 0.077
[b] The cut-off point was three times of the average negative value
KIT: N.H. Swellengrebel laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands.
NCIMB: National Collection of Industrial and Marine Bacteria, Scotland.

Table 2.3 Cross-reaction of monoclonal antibody cell (8F7) line with a variety of non-mycobacteria determined by an enzyme-liked immunosorbent assay

Bacteria	Origin	Absorbance [a]	Reaction[b]
Aeromonas hydrophila	NCIMB 1134	0.060	-
Aeromonas salminicida	NCIMB 1109	0.068	-
Arthrobacter oxydans	NCIMB 9333	0.070	-
Bacillus subtilis	ATCC 6633	0.075	-
Bacillus cereus	ATCC 11778	0.068	-
Edwardsiella ictaruli	IOA	0.064	-
Edwardsiella tarda	IOA	0.070	-
Escherichia coli	NCTC 10418	0.059	-
Micrococcus luteus	NCIMB 8553	0.069	-
Nocardia asteroides	NCIMB 1890	0.053	-
Pseudomonas aerufinosa	ATCC 27853	0.095	-
Pseudomonas fluorescens	NCIMB 1953	0.071	-
Pasteurella piscicida	IOA	0.080	-
Streptococcus faecalis	IOA	0.101	-
Vibrio ordalli	NCIMB 2167	0.055	-
Yersinia ruckeri	NCIMB 1316	0.059	-

NCIMB: National Collection of Industrial and Marine Bacteria, Scotland
ATCC: America Tissue Culture Centre
NCTC: National Culture Collection
IOA: Institute of Aquaculture, University of Stirling, Scotland
[a] The mean value of duplicate samples at 450 nm; the negative control was 0.065.
[b] The cut-off point was three times of the average negative value. Postive reactions were reported for results greater than three times the average negative value.

2.3.2.2 Examination of culture strains by Western blot analysis

Mab 8F7 recognised *M. marinum* isolate S267 from snakehead fish, *M marinum* (KIT) isolated from human and the type strain *M. marinum* (NCIMB 1297) by Western blot analysis. Bands were observed at 4-17 and 71 kDa with lysates of the isolates, whereas the Mab recognised a band about 18-22 kDa in the ECP of S267 (Fig 2.1). There was no reaction with other mycobacteria type strains (ie *M. fortuitum* and *M. chelonae*).

When the samples were treated with proteinase K, the 71 kDa band disappeared in the profile of both the type strain *M. marinum* (NCIMB 1297) and *M. marinum* isolate S267, but the broad 4-17 kDa band remained intact (Fig 2.2). The 18-22 kDa band was not digested by the proteinase K when added to the ECP sample.

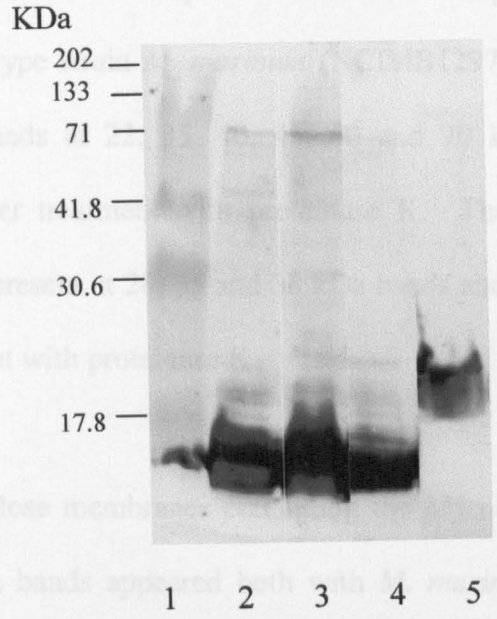

Fig 2.1 The response of Mab 8F7 to *M. marinum* and its ECP by Western blot analysis: lanes: (1) standard marker, (2) S267; (3) *M. marinum* (KIT); (4) *M. marinum* (NCIBM 1297); (5) ECP of S267

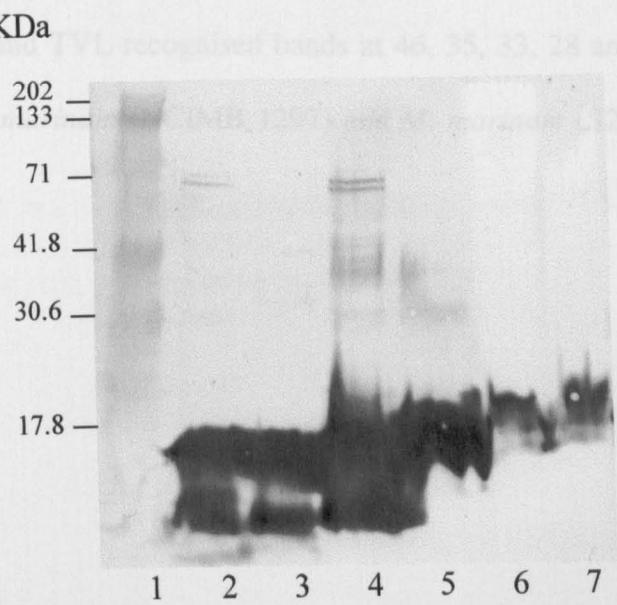

Fig 2.2 The response of Mab 8F7 to proteinase K-treated *M.marinum* and its ECP by Western blot analysis: lanes: (1) standard marker; (2) *M. marinum* (NCIMB1297); (3) *M. marinum* (NCIMB1297) digested with proteinase K; (4) S267; (5) S267 digested with proteinase K; (6) ECP of S267; (7) ECP of S267 digested with proteinase K

Bacteria untreated or treated with proteinase K were compared using the glycoprotein kit in Fig 2.3. The type strain *M. marinum* (NCIMB1297), *M. marinum* isolate S267 had glycoprotein bands at 22, 35, 40, 45, 60 and 70 kDa bands and these bands appeared fainter after treatment with proteinase K. The ECP of isolate S267 had glycoprotein bands present at 28, 35 and 38 kDa bands and again these bands appeared fainter after treatment with proteinase K.

When the nitrocellulose membranes containing the *Mycobacterium* preparations were stained with lectins, bands appeared both with *M. marinum* (NCIMB 1297) and *M. marinum* (S267). Upon staining with GML, Con A, HGL, UEA-1, TVL and AHL, different bands were stained to those recognised by the Mab (Fig 2.4 and 2.5). Bands at 132, 71 and 64 kDa in both *M. marinum* (NCIMB1297) and *M. marinum* (S267) were recognised by all lectins. Con A also recognised bands at 34, 22, 20 and 5 kDa, HGL recognised a number of bands between 56 and 12 kDa, UEA-1 recognised bands at 48, 36, 32, 16 and 12 kDa, and TVL recognised bands at 46, 35, 33, 28 and 2 kDa. Mab 8F7 recognised both *M. marinum* (NCIMB 1297) and *M. marinum* (S267) at 132, 71, 64 and 10 kDa bands.

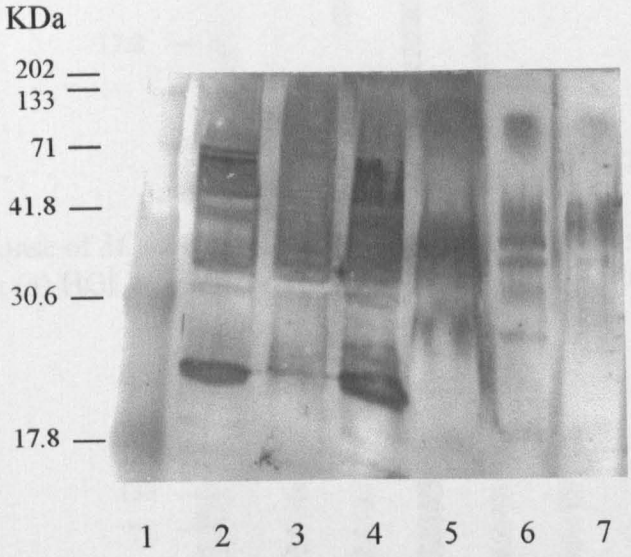

Fig 2.3 The response of *M. marinum* to glycoprotein staining: lanes: (1) standard marker, (2) *M. marinum* (1297); (3) *M. marinum* (1297) digested with proteinase K; (4) S267; (5) S267 digested with proteinase K; (6) ECP of S267; (7) ECP of S267 digested with proteinase K

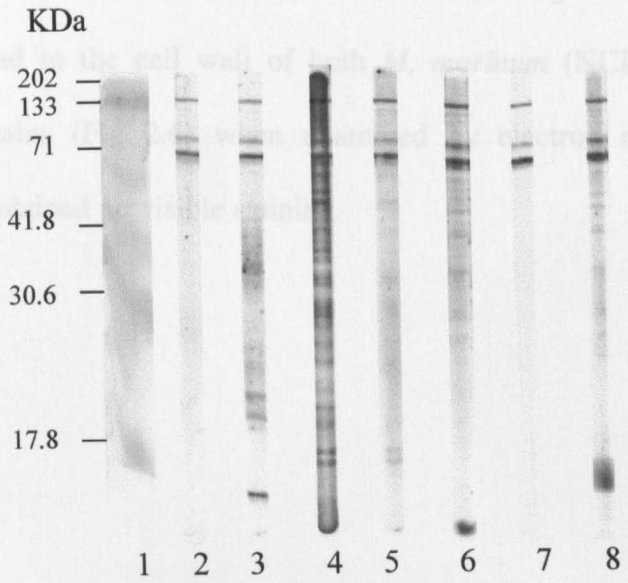

Fig 2.4 The response of *M. marinum* (S267) to lectins: lanes: (1) standard marker; (2) GML; (3) ConA; (4) HGL; (5) VEA-1; (6) TVL; (7) AHL; (8) Mab (8F7)

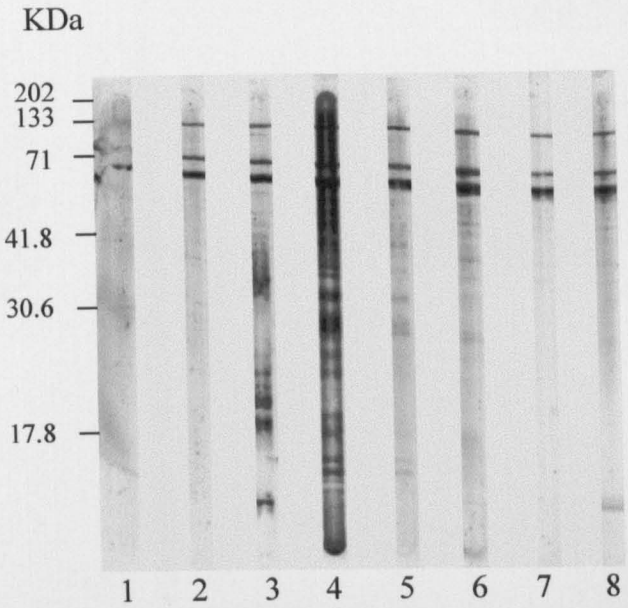

Fig 2.5 The response of *M. marinum* (NCIMB 1297) to lectins: lanes: (1) standard marker; (2) GML; (3) ConA; (4) HGL; (5) VEA-1; (6) TVL; (7) AHL; (8) Mab (8F7)

2.3.2.3 Immunogold staining analysis

Immunogold staining of *M. marinum* isolates using Mab 8F7 was performed to further characterise the antibody and establish the location of binding. The probes labelled with gold were found in the cell wall of both *M. marinum* (NCIMB1297) and *M. marinum* (S267) strains (Fig 2.6) when examined by electron microscopy. The untreated bacteria contained no visible staining.

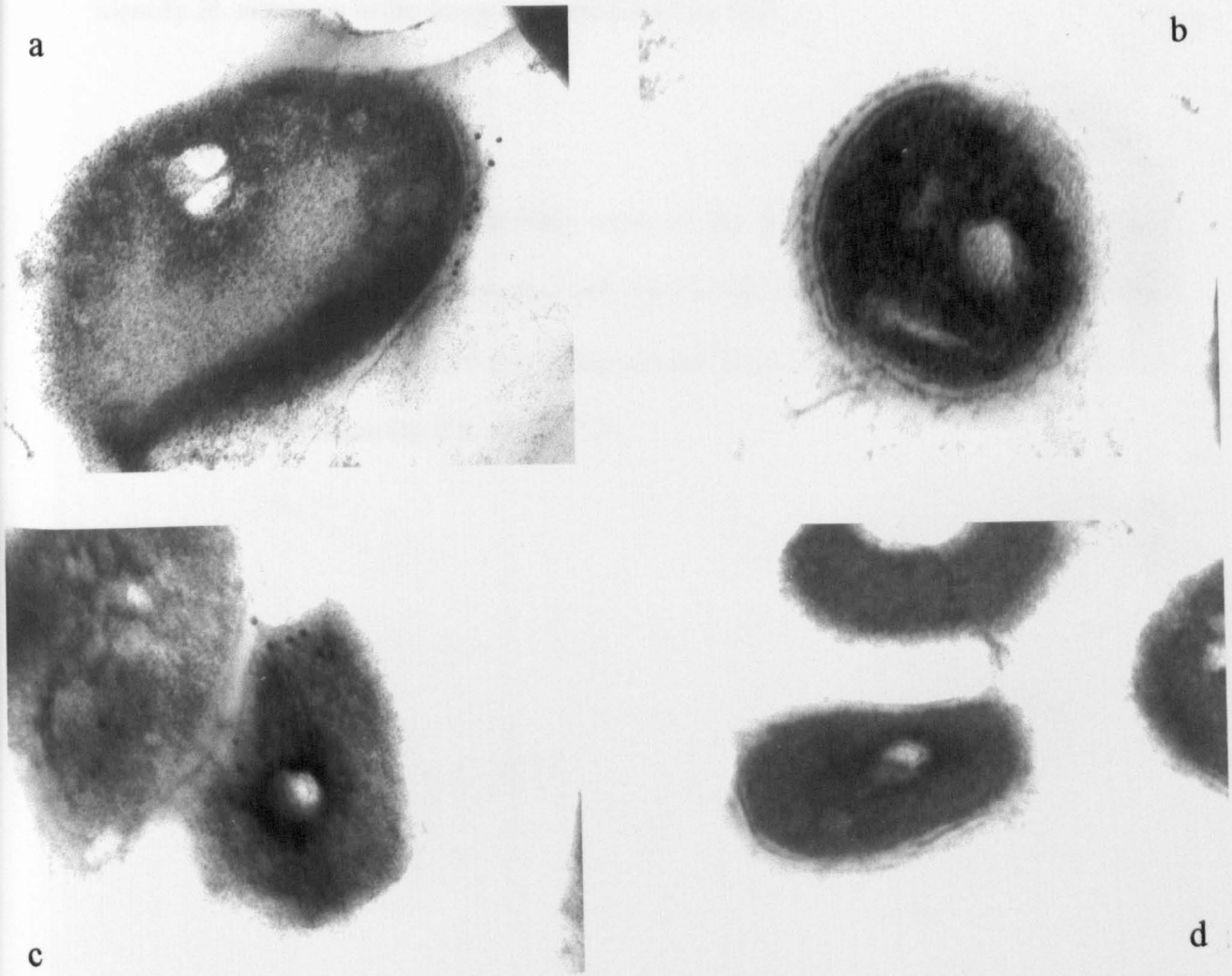

Fig 2.6 Immunogold labelling of *M. marinum* with Mab 8F7 examined by electron microscopy: (a) immunogold labelling of *M. marinum* (S267) with Mab 8F7 (x 98,000); (b) immunogold labelling of *M. marinum* (S267) with PBS (x 98,000); (c) immunogold labelling of *M. marinum* (NCIMB1297) with Mab 8F7 (x 130,000); (d) immunogod labelling of *M. marinum* (NCIMB1297) with PBS (x 98,000).

2.3.3 Detection of *M. marinum* using Mab 8F7

The Mab 8F7 was used as a tool to identify *Mycobacterium* spp. isolated from fish from different geographical regions by ELISA and WB analysis. The Mab was also used to identify *M. marinum* in the formalin fixed tissue by IHC.

2.3.3.1 By ELISA

Mab 8F7 reacted positively with only some of the *Mycobacterium* isolates obtained from Thailand (Table 2.4). It was also only positive for some of *Mycobacterium* isolates isolated from around the world, including isolates from USA, Germany, Denmark and one of the isolates from the UK (Table 2.5).

Table 2.4 Reaction of Mab 8F7 with a variety of *Mycobacterium* spp. from Thailand determined by an enzyme-linked immunosorbent assay

Isolates	Fish	Absorbance[a]	Reaction[b]
S1	Snakehead fish	0.938	+
S2	Snakehead fish	0.860	+
S3	Snakehead fish	0.692	+
S4	Snakehead fish	0.552	+
S5	Snakehead fish	0.128	-
S6	Snakehead fish	0.521	+
S7	Snakehead fish	0.123	-
S8	Snakehead fish	0.076	-
S9	Snakehead fish	0.428	+
S10	Snakehead fish	0.542	+
S11	Snakehead fish	0.145	-
S12	Snakehead fish	0.088	-
S13	Snakehead fish	0.089	-
S14	Snakehead fish	0.112	-
S15	Snakehead fish	0.457	+
S18	Snakehead fish	0.124	-
S267	Snakehead fish	0.848	+
S268	Snakehead fish	0.580	+
S269	Snakehead fish	0.113	-
TB1	Siamese fighting fish	0.060	-
TB7	Siamese fighting fish	0.853	+
TB38	Siamese fighting fish	0.082	-
TB40	Siamese fighting fish	0.184	-
TB42	Siamese fighting fish	0.772	+
TB43	Siamese fighting fish	0.100	-
TB44	Siamese fighting fish	0.116	-
TB45	Siamese fighting fish	0.787	+
TB62	Siamese fighting fish	0.806	+
TB73	Siamese fighting fish	0.129	-

a. The mean value of duplicate samples at 450 nm; the negative control was 0.077
b. The cut-off value was set at three time of the mean negative value.

Table 2.5 Reaction of Mab 8F7 with a variety of *Mycobacterium* spp. isolated from different geographical regions determined by an enzyme-linked immunosorbent assay

Isolates	Fish	Origin	Absorbance[a]	Reaction[b]
E1	Sheepshead (*Puntazzo puntazzo*)	Eilat, Israel	0.088	-
E2	Sea bass (*Dicentrarchus labrax*)	Eilat, Israel	0.083	-
E3	Thalassa (*Dicentrarchus labrax*)	Greece	0.160	-
E4	unknown	USA	0.614	+
E5	unknown	Germany	0.070	-
E6	Red Drum (*Sciaenops ocellatus*)	Eilat, Israel	0.093	-
E7	Butterfly fish (*Chaetodon fasciatus*)	Eilat, Israel	0.110	-
E8	Thalassa (*Dicentrarchus labrax*)	Greece	0.069	-
E9	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	0.082	-
E10	unknown	Hernandez, Germany	0.615	+
E11	Sea bass (*Dicentrarchus labrax*)	Atlit, Israel	0.070	-
E12	Sea bass (*Dicentrarchus labrax*)	Denmark	0.582	+
E14	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	0.070	-
E15	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	0.078	-
E16	Sea bass (*Dicentrarchus labrax*)	Ein Yahav, Israel	0.085	-
MB1	Coho salmon (*Onchorhyncus kisutch*)	Chile	0.072	-
MB2	Frog (*Rana rugulosa*)	Thailand	0.579	+
MB3	Rossy barb (*Barbus conchonius*)	UK	0.480	+
MB4	Gold fish (*Carassius auratus*)	UK	0.079	-

[a] The mean value of duplicate samples at 450 nm; the negative control was 0.075.

b The cut-off point was three times of average negative value

2.3.3.2 Western blot analysis

Mab 8F7 reacted with isolates S1, S2, S3, S4, S6, S9, S10, S15, S267, S268, TB7, TB42, TB45, TB62, E4, E10 and E12 by Western blot analysis with the broad band between 4-17 kDa recognised in all isolates (Fig 2.7a-g). Among these, isolates S4, S6, S9, S10, S15, S267, and S268 were also recognised by the Mab 8F7 with bands identified at 71 and 82 kDa, while isolates S1, TB7, TB42, TB45 and TB62 had a high molecular band at 90-200 kDa which reacted with the Mab. Isolates S1, S2 and S3 also had a reaction band at 42-34 kDa (Fig 2.7 a). Mab 8F7 did not react with the isolates S5, S7, S8, S14, TB1, TB40, TB38, TB43, TB44, TB73, E6, E7, E14, E15 and E16, whereas it recognised at least one of the bands at 71 and 82 kDa in the isolates S11, S12, S13, S18, S269, E1, E2, E3, E5, E8, E9 and E11 (Fig 2.7 a-g).

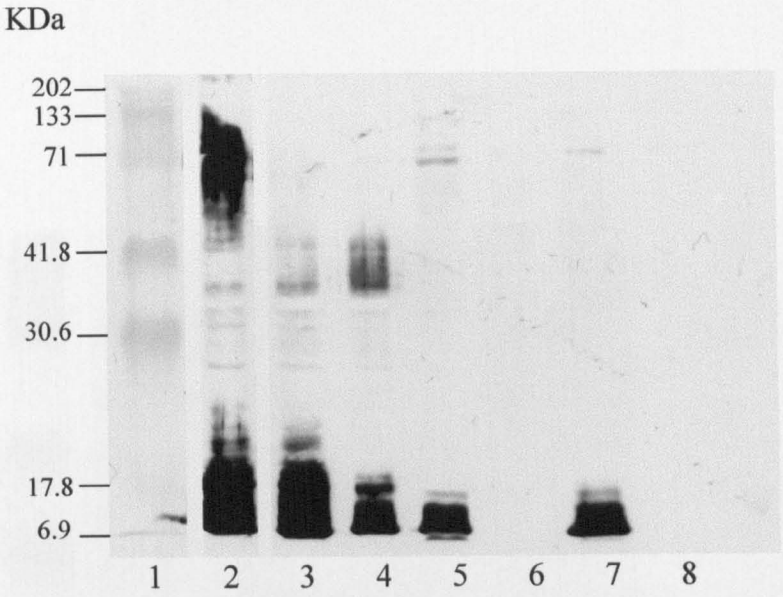

Fig 2.7a The response of Mab 8F7 to *Mycobacterium* spp isolated from snakehead by Western blot analysis: lanes: (1) Standard marker; (2) S1; (3) S2; (4) S3; (5) S4; (6) S5; (7) S6; (8) S7

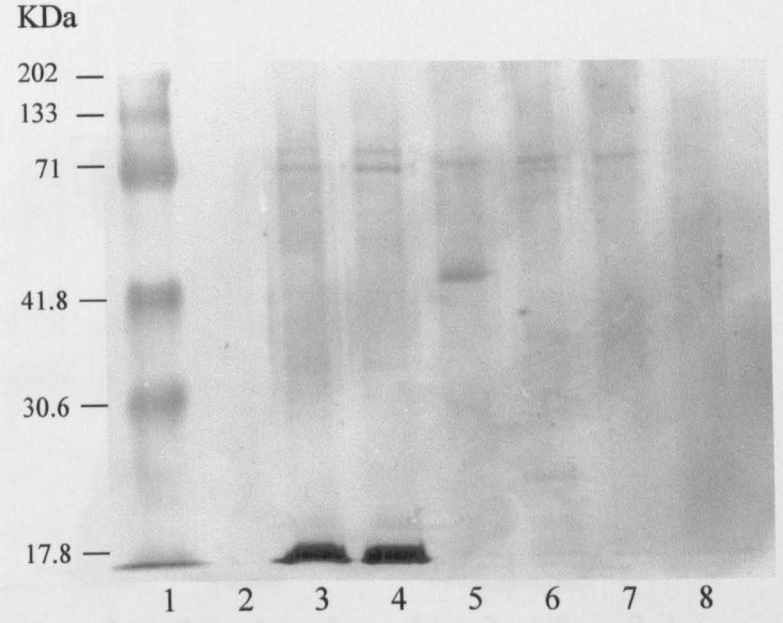

Fig 2.7 b The response of Mab 8F7 to *Mycobacterium* spp isolated from snakehead by Western blot analysis: lanes: (1) standard marker; (2) S8; (3) S9; (4) S10; (5) S11; (6) S12; (7) S13; (8) S14

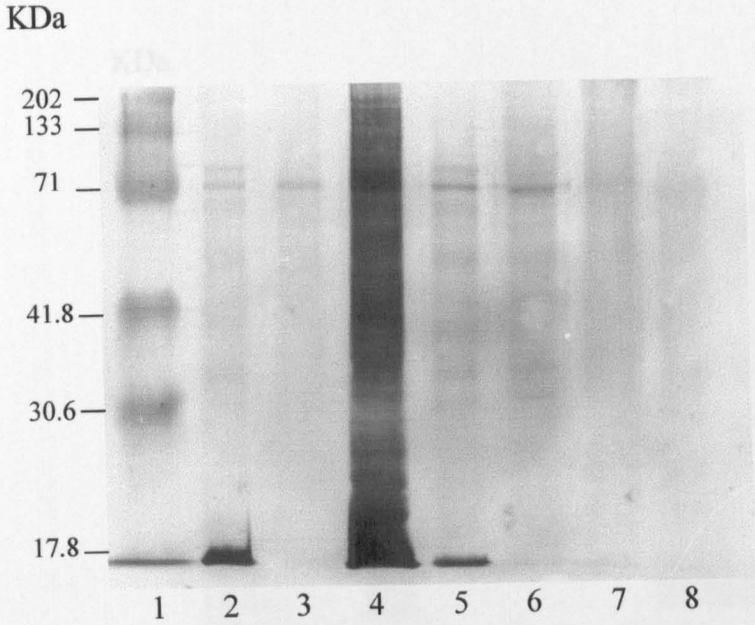

Fig 2.7c The response of Mab 8F7 to *Mycobacterium* spp isolated from snakehead and Siamese fighting fish by Western blot analysis: lanes: (1) standard marker; (2) S15; (3) S18; (4) S267; (5) S268; (6) S269; (7) TB1; (8) TB40

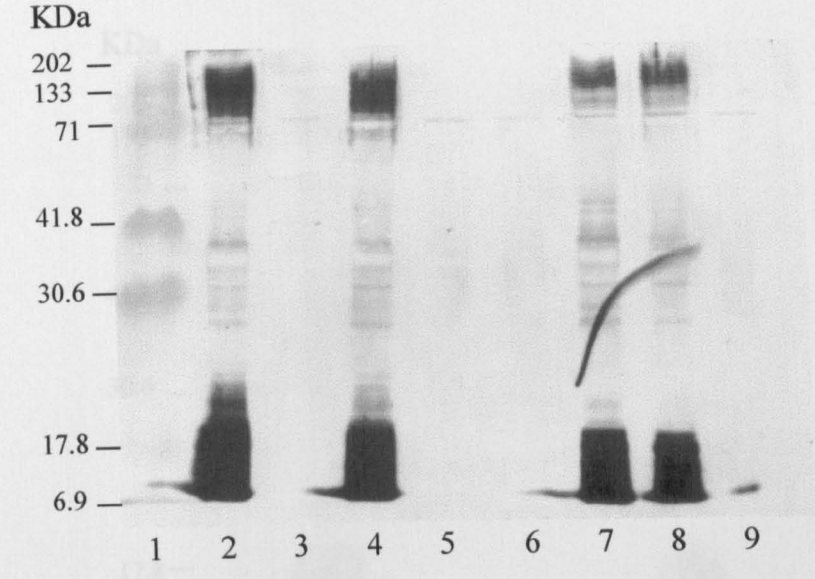

Fig 2.7d The response of Mab 8F7 to *Mycobacterium* spp isolated from Siamese fighting fish by Western Blot analysis: lanes: (1) standard marker; (2) TB7; (3) TB38; (4) TB42; (5) TB43; (6) TB44; (7) TB45; (8) TB62; (9) TB73

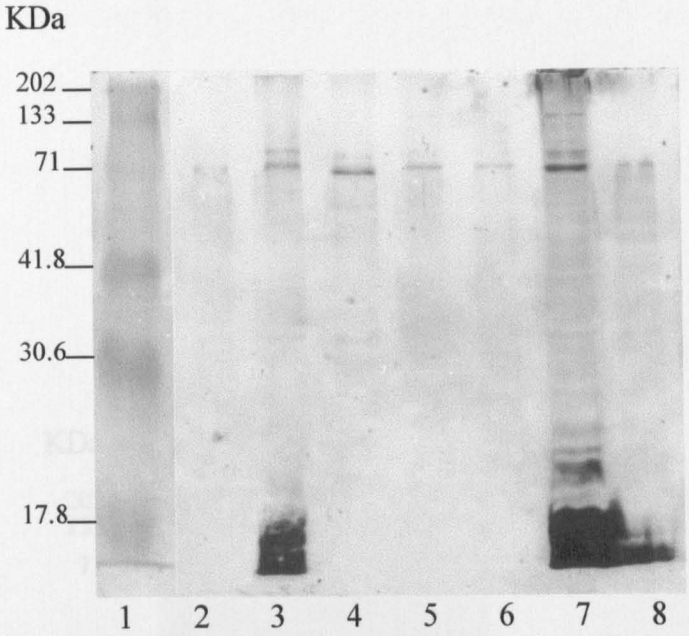

Fig 2.7e The response of Mab 8F7 to *Mycobacterium* spp isolated from variety places by Western blot analysis: lanes: (1) standard marker; (2) E5; (3) E4; (4) E3; (5) E2; (6) E1; (7) S267; (8) *M. marinum* (NCIMB1297)

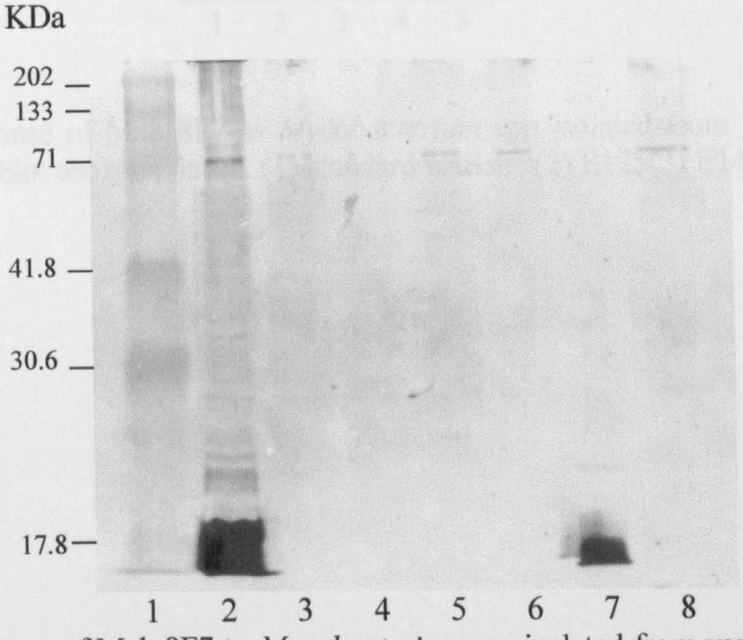

Fig 2.7f The response of Mab 8F7 to *Mycobacterium* spp isolated from variety places by Western blot analysis: lanes: (1) standard marker; (2) S267; (3) E6; (4) E7; (5) E8; (6) E9; (7) E10, (8) E11

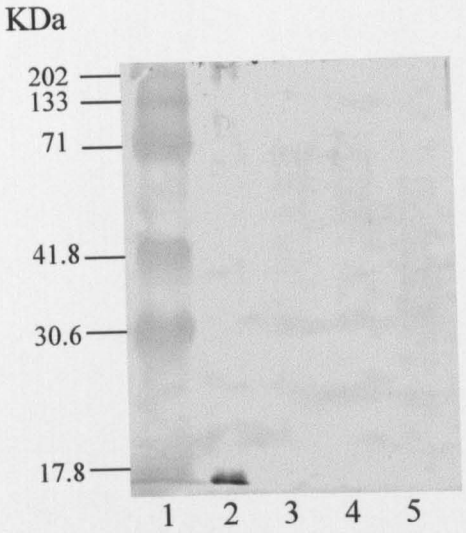

Fig 2.7g The response of Mab 8F7 to *Mycobacterium* spp isolated from variety of places by Western blot analysis: lanes: (1) standard marker; (2) E12; (3) E14; (4) E15; (5) E16

2.3.3.3 Immunohistochemistry analysis

Mab 8F7 identified *M. marinum* in granulomas found in tissue sections prepared from fish infected with mycobacteriosis. They appeared brown and little non-specific staining of the tissue was evident (Fig 2.8 a-b). The same tissues stained in parallel with ZN appeared deep pink in colour presenting acid-fast bacteria (Fig 2.8 c).

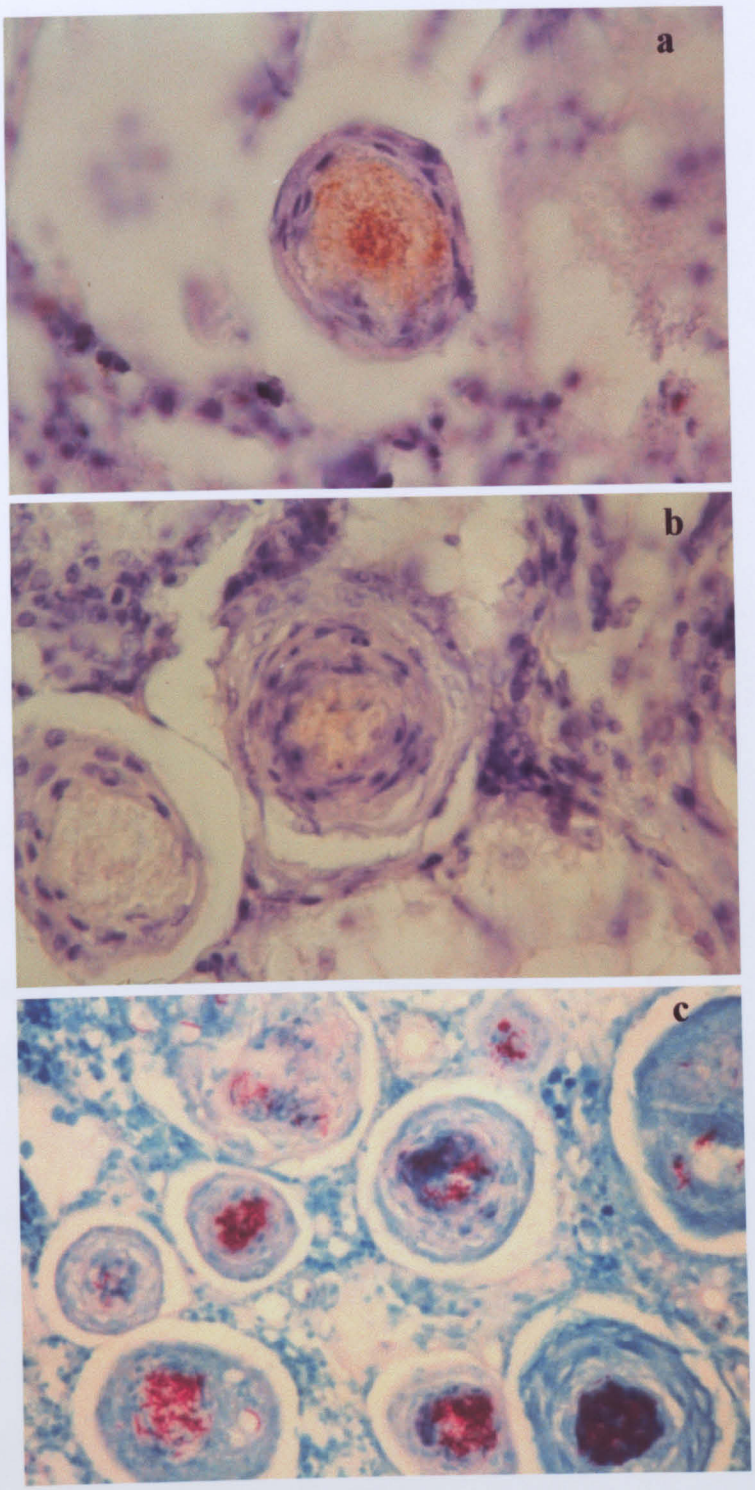

Fig 2.8 Reaction of Mab 8F7 to spleen tissue of Siamese fighting fish (*Betta splendens*) infected with *Mycobacterium* spp. by immunohistochemistry staining with: (a) Mab 8F7 (x100); (b) normal mouse serum (x100); (c) Ziehl-Neelsen staining (x 40)

2.4 Discussion and Conclusions

Mycobacterium spp are a distinctive group of Gram-positive bacteria. The envelope of mycobacteria comprises the plasma membrane and the wall. The cell wall of mycobacteria is constructed of three layers, an inner layer of peptidoglycan, a middle layer of arabinogalactan mycolate and an outer layer of lipopolysaccharide (Brennan & Draper, 1994). The peptidoglycan of their cell wall is associated mainly with lipids rather than proteins or polysaccharides as found in the peptidoglycan of non-mycobacteria in the gram-positive group (Leaderer *et al*, 1975; Brennan & Draper, 1994).

Bacteria are often prepared for immunisation of mice for antibody production by heat killing or sonication (Thorns & Morris, 1985; Adams *et al* 1995 & 1996). The cell wall of mycobacteria is very robust but can be broken by mechanical stress including sonication and grinding. However, Mabs obtained using these methods of preparation failed to identify the bacteria in immunohistochemical analysis (Adams *et al*, 1996). This might be because some of the antigenic determinants on the bacteria, especially the waxy coat were destroyed by heat during the disruption process. UV light was, therefore, used in this study to kill the bacteria prior to immunising the mice, avoiding the production of heat. The Mab produced using UV-killed bacteria reacted in IHC and successfully identified the bacteria within fish tissue. IHC is a simple test, which can be performed in parallel with ZN staining, on fixed tissue.

The granuloma, a typical inflammatory response to mycobacteriosis in fish, is mainly present in the liver and the spleen, although smaller granuloma can be found in the heart, digestive tract, peritoneal serosa, ovaries and testes of the animal (Gómez *et al*,

1993). The spleen of infected fish was examined by IHC, with particular attention focusing on the granulomas for the presence of the bacterium. The granulomas in the sample contained the pathogen appeared as golden brown stained bacteria. Absence of bacteria in the granulomas may occur in some cases, which has been previously reported (Hastings *et al*, 1982; Gómez *et al*, 1993 &1996).

The Mab identified epitopes on the surface of intact bacteria as shown by immunogold staining, where the gold probes appeared on the outer layer of the cells. It was however difficult to locate the three different layers of the wall, since differential staining is needed to visualise the individual layers (Brennan & Draper, 1994), and it was not possible to carry out such staining during the immunogold staining procedure.

Mab 8F7 was characterised by an indirect ELISA, performed on both whole and sonicated *Mycobacterium* cells. The ELISA results obtained were very similar between these two preparations, suggesting that the epitopes recognised by the Mab are on the surface of the cell rather than internally located. Adams *et al* (1996) reported that cell lysates gave a higher response than the whole cells in their ELISA since epitopes concealed in the cells were released upon lysis. The results obtained here by ELISA confirmed the staining obtained with the immunogold staining.

Some of the antigens previously described for mycobacteria are common to all mycobacteria, nocardiae and related genera (Stanford & Wong, 1974; Standford *et al*, 1975; Thorns & Morris, 1985). The specificity of the Mab 8F7 was tested against a variety of mycobacteria species and non-mycobacteria in this study, where it was shown that Mab 8F7 recognised only *M. marinum* (S267) and type strain *M. marinum*

(NCIMB1297), and not other *Mycobacterium* type species or non-mycobacteria including *Norcardia* sp. This implies that the Mab is specific for only *M. marinum.*

The reactivity pattern of Mab 8F7 divided the isolates from Thailand into two distinct groups when they were analysed by ELISA. One group reacted positively with the Mab while the other group was negative. The positive group was assumed to be *M. marinum* and the negative group other *Mycobacterium* species or possibly non-mycobacteria. Despite this, biochemical analysis suggested that the latter were *Mycobacterium* spp. When isolates from different geographical regions were analysed by ELISA, very few isolates, namely those from USA, Germany, Denmark and UK reacted positively with the Mab. Those from Israel and Greece did not react. However, reaction with the Mab alone does not provide sufficient evidence to conclusively establish that negative isolates are not *M. marinum* and further analysis using molecular probes is necessary for a definitive classification.

Mab 8F7 recognised the same high and low molecular weight bands (71 and 4-17 kDa) in the profiles of lysate *M. marinum* (S267), *M. marinum* (KIT) and *M. marinum* (NCIMB 1297) by Western blot analysis, indicating that the recognised epitopes are presented among *M. marinum* isolated from different hosts including, marine fish (NCIMB 1297), freshwater fish (S267) and man (KIT). Chen *et al* (1997) reported the protein profiles of ECP of S267 to have major bands at 65 kDa and <14 kDa and several minor bands between 60 and 16 kDa regions. The ECP examined here had a band around 18-22 kDa, which was reactive with Mab 8F7, and this may represent the band observed by Chen *et al* (1997). It is probably not a protein since it was still recognised by the Mab after treatment with the proteinase K. The 71 kDa band in both

M. marinum (NCIMB1297) and *M. marinum* (S267) in cell lysates disappeared after proteinase K treatment. The 4-17 kDa region of *M. marinum* (NCIMB1297) remained unaffected by proteinase K activity, whereas some of the 4-17 kDa region was digested in *M. marinum* (S267). This could be either due to differences in the composition of these molecules between the two strains, or else there was incomplete digestion of the protein by the enzyme, possibly due to insufficient enzyme being present. Thus, it appears that the epitope on *M. marinum* recognised by the Mab is not a protein, but is located on a molecule, which contains a protein moiety. It is known not to be a glycoprotein from analysis with the glycoprotein kit. The bands of *M. marinum* (NCIMB 1297 and S267) with and without proteinase K treatment detected by the glycoprotein detection kit are different to those recognised by the Mab.

The profiles of *M. marinum* (NCIMB1297) and *M. marinum* (S267) stained with lectins were similar, but the bands recognised by the lectins were different to those stained with Mab 8F7, except for the high molecular weight band at 132, 71 and 64 kDa. The bands at 132 and 64 kDa had not been previously noted in Western blot analysis with the Mab and possibly may be due to sample degradation in the previous analysis. The 4-17 kDa broad band recognised by the Mab is not a carbohydrate recognised by any of the lectins.

The 4-17 kDa band may possibly be a lipoprotein, and attempts were made to classify the lipid moiety. The band was first electro-eluted from the SDS-PAGE gel, then extracted with chloroform/methanol. This was analysed by high performance liquid chromatography (HPLC), but the result was inconclusive (data not presented).

The isolates from snakehead fish, S1, S2, S3, S4, S6, S9, S10, S15, S267 and S268 were positive in the ELISA and also had similar profiles in Western blot analysis with Mab with bands reacting at 4-17 and 71 kDa. Isolates that were negative in the ELISA, S5, S7, S8, S11, S12, S13, S14, S18 and S269 only produced a band at 71 kDa with the Mab and did not react with the low molecular weight material. The 71 kDa appeared to be a common antigen among mycobacteria species.

Of the isolates recovered from Siamese fighting fish, Mab 8F7 recognised TB7, TB42, TB45 and TB62 but not TB1, TB38, TB40, TB43, TB44 and TB73 by Western blot analysis, again confirming the Mab response obtained in the ELISA. The positive group of *M. marinum* had a similar reactivity pattern to that of the type strain with the Mab in Western blot analysis. However, these profiles differed slightly to those obtained for the positive strains isolated from snakehead. This suggests that they may be a different strain of *M. marinum*.

Only three of the isolates from different geographical regions, E4, E10 and E12 were recognised by the Mab by Western blotting with the same profiles appearing as the snakehead isolates. E4, E10 and E12 were isolated from USA, Germany and Denmark respectively. The Mab did not react with any of the strains obtained from Israel or Greece, and this could be due to them not being *M. marinum*, different strains of *M. marinum*, or in fact a different *Mycobacterium* species.

In conclusion, Mab (8F7) appeared strain specific for *M marinum*. It is reactive in IHC and is useful for diagnosis of mycobacteriosis from formalin-fixed tissue and as an immunological probe for ELISA, where *M. marinum* is the causative agent. However,

some isolates appeared negative with the Mab and it was not possible to establish if they were non-mycobacteria, other species of mycobacteria or different *M. marinum* strains, which the Mab was unable to recognise. Molecular analysis has been carried out to address this question and will be discussed in Chapter 4, while biochemical fingerprinting was performed in Chapter 3.

Chapter 3

Pyrolysis mass spectrometry (PyMS) analysis

3.1 Introduction

Pyrolysis mass spectrometry (PyMS) is an analytical technique which allows rapid and precise characterisation of bacteria within minutes, compared to conventional bacteriological analysis for which long culture periods are required for *Mycobacterium* spp. It has the added advantage that only small quantities of sample are needed for the analysis. This technique has been previously used to identify and characterise many different bacteria (Shute *et al*, 1984 & 1985; Magee *et al*, 1989a & 1989b; Freeman *et al*, 1990, 1991a & 1991b, Hindmarch *et al*, 1990; Winstanley *et al*, 1992; Manchester, *et al*, 1995) including *Mycobacterium* spp. (Wieten *et al*, 1981a, 1981b & 1983; Sisson *et al*, 1991; Freeman *et al*, 1993). PyMs has been successfully used to separate tuberculosis complex bacteria (*M. tuberculosis*, *M. bovis* and *M. bovis* BCG) from other *Mycobacterium* species (Weiten *et al*, 1981a). *M. africanum* has also been identified as one of the tuberculosis complex using PyMS (Wieten *et al*, 1983).

Pyrolysis mass spectrometry (PyMS) is a physico-chemical method for microbial identification. Pyrolysis is the thermal degradation of the organism in an inert atmosphere producing a series of low molecular weight volatile compounds. These volatile fragments are subsequently counted by a mass spectrometer to produce a complex spectrum (Gutteridge *et al*, 1985). The spectrum, based on their mass:charge ratio (m/z) are collected only at the mass range of m/z 51 to 200, where each spectrum corresponds to a particular biochemical molecule. The data below m/z 51 and over m/z 200 are discarded. That is because the mass below m/z 50 corresponds to general

organic compounds such as methane (m/z 15, 16), ammonia (m/z 16, 17), water m/z 17, 18), methanol (m/z 31, 32) and hydrogen sulphide (m/z 32-34), which originate from the pyrolysis of any biological materials, while the mass over m/z 200 contains less evidence for discrimination analysis (Berkeley *et al*, 1990). The data are subsequently analysed by a statistical software package, which displays principal component analysis (factor analysis), canonical variates analysis (discrimination analysis) and graphical rotation as outlined in Fig 3.1.

PyMS has been extensively applied to the field of microbial taxonomy. However, inter-laboratory reproducibility of the results is sceptical, since the pyrolysis mass spectra vary with the age and cultural conditions of the organism and ageing of the machine components such as the electron multiplier (Tempest & Neijssel, 1978; Boon *et al*, 1981; Sisson *et al*, 1992; Berkeley *et al*, 1990). Windig *et al* (1979) reported that the short term reproducibility of the average of pyrolysis mass spectra from glycogen and bovine serum albumin was at 6% and 8% after 34 days. Shute *et al* (1984) showed a mean of dissimilarity of 4% for pyrolysis spectra from replicate samples over eight weeks. However, if the organisms are prepared under identical conditions and analysed within a single machine batch, the information is highly reliable (Sisson *et al*, 1992).

In this Chapter, *Mycobacterium* spp. isolated from fish during outbreaks of mycobacteriosis world-wide were analysed by PyMS in an attempt to classify the bacteria to species level, and to distinguish *M. marinum*, *M. fortuitum* and *M. chelonae*.

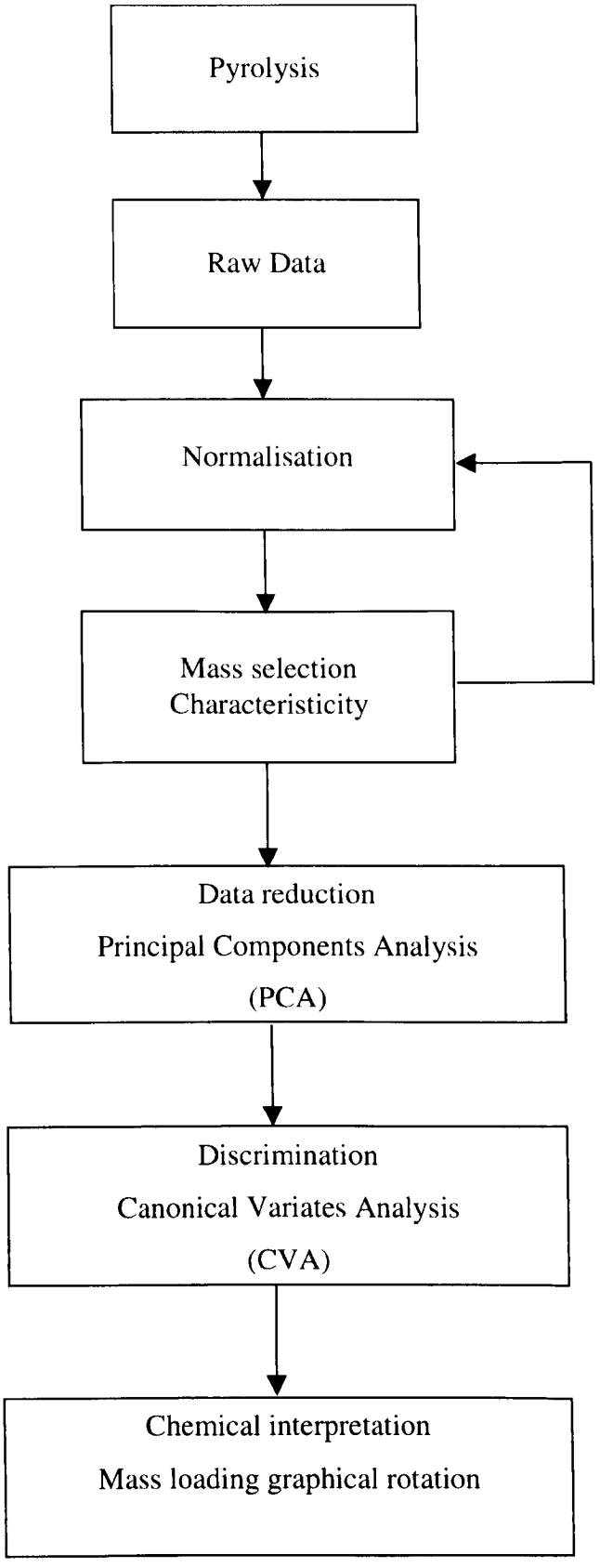

Fig 3.1 Flow diagram of PyMS data analysis (Berkeley *et al*, 1990)

3.2 Materials and methods

3.2.1 Bacterial isolates

The *Mycobacterium* strains isolated from Siamese fighting fish (*Betta splendens*) and snakehead fish (*Channa striata*) from Thailand, as well as the isolates from a variety of other geographical regions (Table 3.1) were compared to reference strains *M. marinum* (NCIMB 1297), *M. fortuitum* (NCIMB1294), and *M. chelonae* (NCIMB1474).

Table 3.1 Mycobacteria strains isolated from Thailand and a variety of other sources

Isolate	Fish	Origin
Mycobacterium marinum	Marine fish (non identified species)	NCIMB1297
Mycobacterium fortuitum	Chinook salmon (*Oncorhynchus tschawytscha*)	NCIMB1294
Mycobacterium chelonae	Tortoise	NCIMB1474
TB1	Siamese fighting fish (*Betta splendens*)	Thailand
TB40	Siamese fighting fish (*Betta splendens*)	Thailand
S267	Snakehead (*Channa striata*)	Thailand
S268	Snakehead (*Channa striata*)	Thailand
S269	Snakehead (*Channa striata*)	Thailand
S4	Snakehead (*Channa striata*)	Thailand
S5	Snakehead (*Channa striata*)	Thailand
S6	Snakehead (*Channa striata*)	Thailand
S7	Snakehead (*Channa striata*)	Thailand
S8	Snakehead (*Channa striata*)	Thailand
S9	Snakehead (*Channa striata*)	Thailand
S10	Snakehead (*Channa striata*)	Thailand
S11	Snakehead (*Channa striata*)	Thailand
S12	Snakehead (*Channa striata*)	Thailand
S13	Snakehead (*Channa striata*)	Thailand
S14	Snakehead (*Channa striata*)	Thailand
S15	Snakehead (*Channa striata*)	Thailand
S18	Snakehead (*Channa striata*)	Thailand
E1	Sheepshead (*Puntazzo puntazzo*)	Eilat, Israel
E2	Sea bass (*Dicentrarchus labrax*)	Eilat, Israel
E3	Sea bass (*Dicentrarchus labrax*)	Greece
E4	unknown	USA
E5	unknown	Germany
E6	Red Drum (*Sciaenops ocellatus*)	Eilat, Israel
E7	Butterfly fish (*Chaetodon fasciatus*)	Eilat, Israel
E8	Sea bass (*Dicentrarchus labrax*)	Greece
E9	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel
E10	unknown	Hernandez, Germany
E11	Sea bass (*Dicentrarchus labrax*)	Atlit, Israel
E12	Sea bass (*Dicentrarchus labrax*)	Denmark
E14	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel
E15	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel
E16	Sea bass (*Dicentrarchus labrax*)	Ein Yahav, Israel

3.2.2 Sample preparation for pyrolysis

The bacteria were cultured for 14 days at 30°C in modified Sauton's broth (MSB), then prepared for pyrolysis by centrifugation at 3000 xg for 10 min. The supernatant was removed and a small amount of the pellet was thinly smeared onto alloy foils (50% iron: 50% nickel). Triple samples were prepared for each isolate. The foils were individually placed in pyrolysis tubes and allowed to dry in an oven at 120°C for 20 min. The samples were stored overnight at 4°C and analysed by PyMS the following day.

3.2.3 Pyrolysis mass spectrometry

The reaction tubes containing the samples were inserted into a RAPyD 400 pyrolysis mass spectrometer (Horison Instruments Ltd, Heathfield, East Sussex, UK) and pyrolysed for three seconds at a Curie point temperature of 530°C. Each set of samples was analysed in sequence before analysing the next replication. The Fe/Ni foil was subjected to high frequency inductive heating whereupon reaching the Curie point the bacterial samples were pyrolysed into volatile products. These then diffused through an expansion chamber into the quadrupole mass spectrometer, where they were scanned over a mass range (m/z) ratio of 50 to 200 for 20 s and a mass spectrum produced for fragmentations in this mass range. Optimal loading of bacteria on the grids gave ion counts of between 3,000,000-6,000,000 ions.

3.2.4 Analysis of data

The data were analysed using the GENSTAT software package supplied by NAG Ltd., Oxford, UK and Sigma plot was used to illustrate the data.

3.3 Results

Canonical Variates Analysis (CVA) was obtained from the software package attached to the PyMS and the average of the three replicates samples determined. These were represented as a 3-D scatter plot illustrated in Fig 3.2. The plot shows that the mycobacterial isolates are divided into three distinct groups. The first group contains most of the isolates from Israel and Greece. In the second group, some of the Thai isolates are clustered together with isolates from USA and Denmark, as well as type strain *M marinum* (NCIMB1297). The remainder of the Thai isolates are found in the third group clustered together with both type strains *M. fortuitum* (NCIBM 1294) and *M. chelonae* (NCIMB1474). Most of the mycobacteria isolated from snakehead fish in Thailand are placed in the second group, while a few of these, and the isolates from Siamese fighting fish are located in the third group.

The hierarchical cluster analysis (HCA) in Fig 3.3, shows a similarity of 63% between the second and the third group, whereas the second and third group have a similarity of 51% with that of the first group. Slight differences in similarity occurred between replicate samples of a particular isolate. The similarity between the second and the third replicates of S267 is 99%, however they are similar to the first replicate by 96%. Likewise with replicates of isolates S267 and S4, second and third replicates are related at 99% while they are similar to their first replicates by 96%. Isolate TB1 was similar to its replicates at 99%, whereas S268 was similar to its replicate at 96%. Notably, the type strain *M. fortuitum* (NCIMB1294) clustered with the type strain *M. chelonae* (NCIMB1474) at 99% similarity. Isolates E3, E10, S5, S8, S11, S12 and S269 were found to be dissimilar to the three main groups of *Mycobacterium* and were therefore excluded from the HCA.

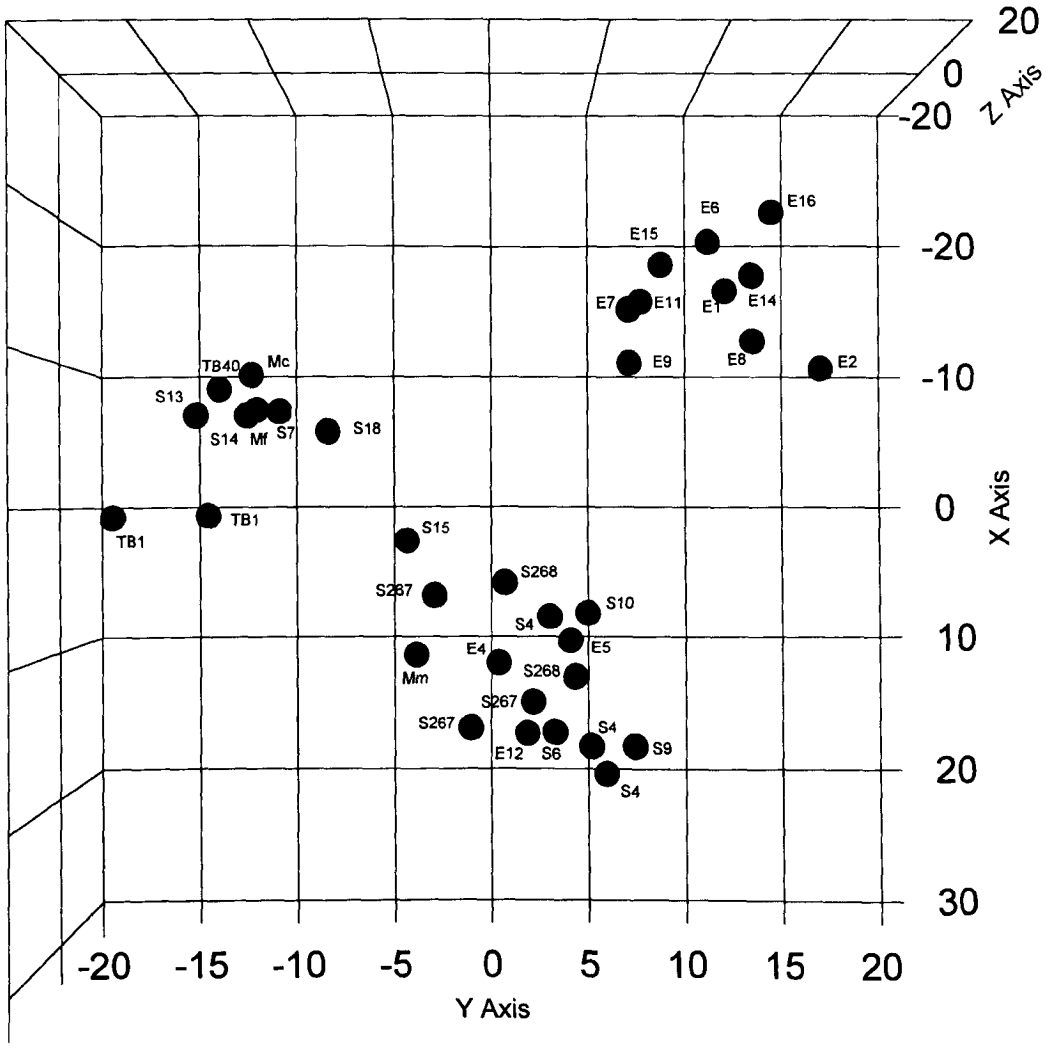

Fig 3.2 Canonical Variates Analysis (CVA) of *Mycobacterium* spp. isolates from different geographical regions. Mm: *M. marinum* (NCIMB1297), Mf: *M. fortuitum* (NCIMB1294), Mc: *M. chelonae* (NCIMB 1474).

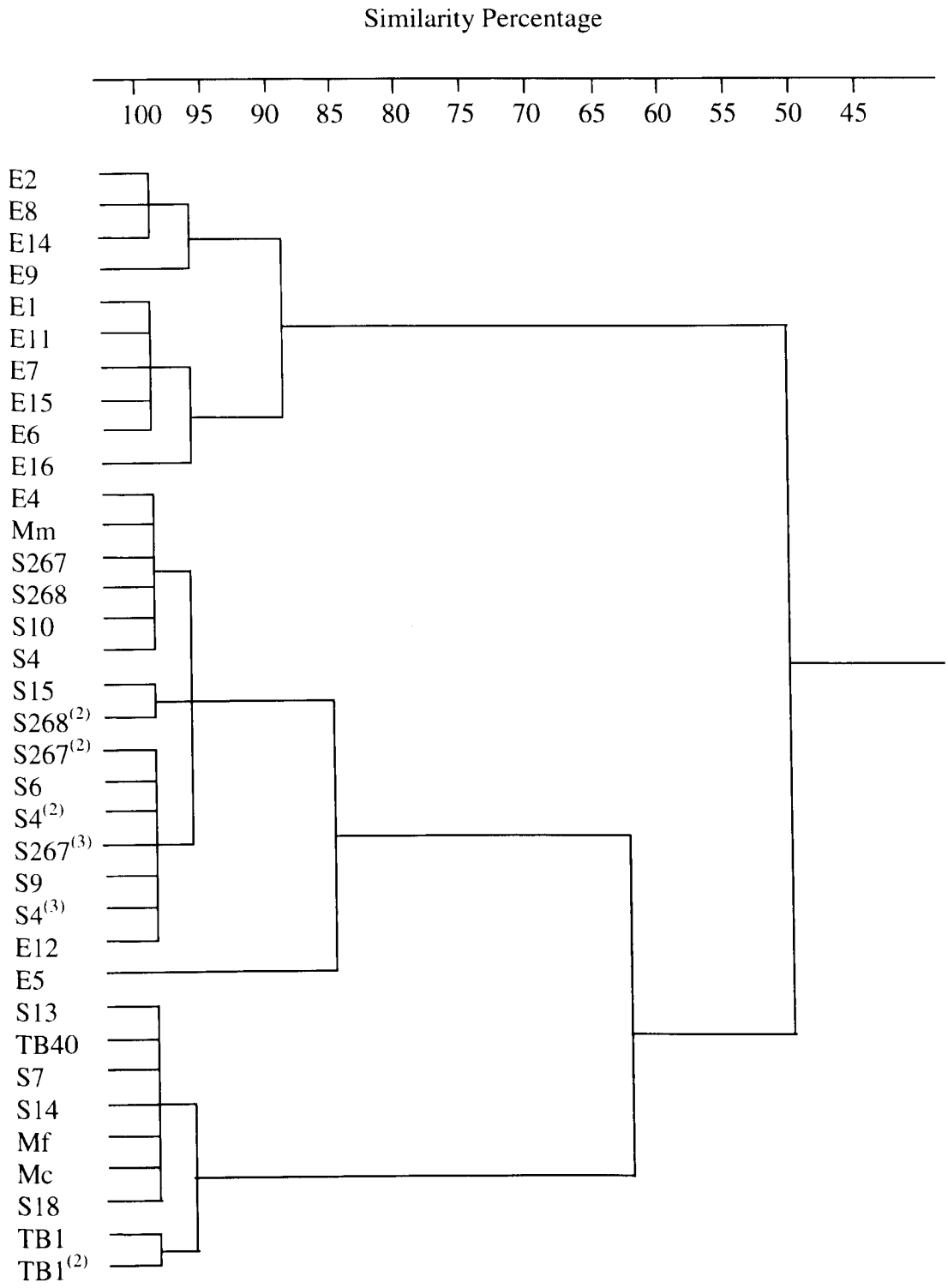

Figure 3.3 Hierarchical cluster analysis (HCA) dendrogram of *Mycobacterium* spp isolated from different geographical regions. $S267^{(2),\ (3)}$, $S4^{(2),\ (3)}$ and $TB1^{(2)}$ are replicate of isolates S267, S4 and TB1 respectively. Mm is *M. marinum* (NCIMB 1297), Mf is *M. fortuitum* (NCIMB1294) and Mc is *M. chelonae* (NCIMB1474).

3.4 Discussion and Conclusions

Construction of a mass pyrogram for *Mycobacterium* isolated from fish from different geological regions indicates that more than three different species of *Mycobacterium* exist between the isolates, and they are related to *M. marinum*, *M. fortuitum* or *M. chelonae*, or remain unclassified. The three type strains used as reference bacteria are represented in two distinct main groups by the PyMS analysis. One group contains *M. marinum* and another group contains *M. fortuitum-M. chelonae*. The third group of *Mycobacterium* does not contain an appropriate reference strain and therefore these bacteria remain as an unclassified *Mycobacterium* species. The isolates obtained from snakehead in Thailand (S4, S6, S9, S10, S15, S267 and S268) appear to be closely related to *M. marinum*. This agrees to previous work, in which S4, S10, S267 and S268 were found to be *M. marinum* by polymerase chain reaction (PCR) and restriction enzyme analysis (Puttinaowarat, 1995). Isolates E4, E12 and E5 are also included in the *M. marinum* cluster, however E5 has a higher dissimilarity within this group at the similarity index of 85%. This finding correlated with the results obtained in Chapter 2 where all isolates in this group were positive with Mab 8F7 except isolate E5.

More replicate samples of some isolates (S4, S267 and TB1) were introduced to check the variability of the mass spectrophotometer. Nine samples were included into the analysis for each of isolate S4 and S267 and six samples for isolate TB1, instead of only three as used for the remaining isolates. Replicates of S4 and S267 were related at a similarity index of 96%. This means that discrimination between bacteria above this point is unreliable (Lilley, 1997) and that PyMS is only capable of discriminating between identical strains at 96% similarity.

It is worth noting that the relationship between type strain *M. fortuitum* (NCIMB1294) and *M. chelonae* (NCIMB1474) is highly significantly similar. The results of PyMS suggest that they may possibly be identical, however biochemical analysis showed that the *M. fortuitum* was distinct from *M. chelonae* (David *et al*, 1981; Silcox *et al*, 1981; Lévy-Frébault *et al*, 1983). With respect to serology, there was no antigenic relation between the strains of *M. chelonae* and *M. fortuitum* (Tsang *et al*, 1984). Thus the method of analysis chosen to characterise the *Mycobacterium* isolates tends to influence the classification obtained. The biochemical analysis differs from that of the physico-chemical data obtained here. *M. africanum* from Rwanda and Burundi were previously related to *M. tuberculosis* based on biochemical analysis (David *et al*, 1978), however Wieten *et al* (1983) establised that they were closely related to *M. bovis* by PyMS.

Isolates TB1, TB40, S7, S13, S14 and S18 were also placed in the *M. fortuitum-M. chelonae* group and this supports the results obtained in Chapter 2 where these isolates gave a negative response with Mab 8F7. Unfortunately, it is not possible to classify them to species level and establish whether they are in fact *M. fortuitum* or *M. chelonae*.

Isolates from Israel and Greece are significantly different from the other two groups described above, and bacteria in this group gave a negative response with Mab 8F7 (Chapter 2). The reference strains, *M. marinum*, *M. fortuitum* and *M. chelonae* are not represented in this group and this suggests the presence of a completely distinct group of *Mycobacterium* isolated from fish. Moreover, this result supports the finding of Colorni (1992), who suggested that *Mycobacterium* spp. isolated from European sea bass (*Dicentrarchus labrax*) in Israel during outbreaks of mycobacteriosis are possibly a new

strain of *Mycobacterium* and no positive identification as to their classification has yet been reached.

Epidemiologically, European sea bass (*Dicentrarchus labrax*) in Italy, Belgium, Greece, and Israel appear to be susceptible to *Mycobacterium* spp (Ghittino 1970, Verdonck *et al* 1986, Colorni 1992; Knibb *et al* 1993), whereas there is no report of mycobacteriosis among sea bass (*Dicentrarchus labrax*) in Thailand. Instead, snakehead (*Channa striata*) and Siamese fighting fish (*Betta splendens*) have been reported as particularly susceptible to this disease (Chinabut *et al*, 1990; Pungkachonboon *et al*, 1992). This suggests that the pathogens isolated from different geographical regions differ in terms of their virulence or the host species, which they infect.

The remaining isolates examined, E3, E10, S5, S8, S11, S12 and S269 did not fall within the three groups of *Mycobacterium* and were statistically unrelated to reference strains, *M. marinum*, *M. fortuitum* or *M. chelonae* based on their physico-chemical composition. They may possibly be related to an other *Mycobacterium* species or may be one of the three type strains but differ in their biochemical compositions (Wieten *et al*, 1981a & 1983).

In general, the results obtained with PyMS confirm the findings in Chapter 2 in which bacteria were classified with the *M. marinum* specific Mab 8F7. The isolates from Thailand tended to cluster together in the same group and were distinctly different from the cluster of isolates, in which the majority came from mycobacteriosis outbreaks in Europe. PyMS appears to be a valuable method for the rapid identification of the *Mycobacterium* cultures without prior knowledge of their classification. Moreover, only

small amounts of the samples are required (5-20 μg organic material) (Weiten *et al* 1983), and the analysis is simple and straightforward. However, some isolates remained unclassified by PyMS analysis and were analysed by PCR and reverse cross blot hybridisation, as described in Chapter 4.

Chapter 4

Detection and identification of *Mycobacterium* spp. using polymerase chain reaction (PCR) and reverse cross blot hybridisation

4.1 Introduction

The amplification of DNA using the PCR is an alternative technique for the detection bacterial pathogens, such as mycobacteria in fish. PCR offers a more rapid, specific and sensitive test in comparison to classical bacteriology, and antibody-based tests. The development of PCR-based techniques has been intense over recent years, with the aim of producing a test to identify mycobacterial species causing tuberculosis in man. Kox *et al* (1995a) and Ngunyen *et al* (1996) found PCR-based methods to be the only techniques that could detect tuberculous meningitis in the cerebrospinal fluid of humans within 24 h. This compares with conventional bacteriology, which requires 4-8 weeks for bacterial cultures to grow. Since DNA homologies exist between human mycobacterial pathogens and those isolated from fish, it was possible that molecular biological tests developed for human diagnosis could also be applied to identify the different aquatic *Mycobacterium* species. Knibb *et al* (1993) pioneered PCR-based tests to study mycobacteriosis in fish, by developing primers based on the 16S r DNA of *M. marinum* from European sea bass (*Dicentrarchus labrax*).

Apart from agarose gel electrophoresis analysis, PCR is often combined with other tests for further identification of the amplified DNA. Such tests which employ the products of PCR amplification include restriction enzyme digestion (Knibb *et al*, 1993; Talaat *et al*, 1997), restriction fragment length polymorphorism (RFLP) (Plikaytis *et al*, 1992; Taylor *et al*, 1997; Otal *et al*, 1997), random amplified

polymorphic DNA analysis (RAPD) (Linton *et al*, 1994; Ross & Dwyer, 1993), single-stranded conformational polymorphism analysis (SSCP) (Telenti *et al*, 1993), pulsed-field gel electrophoresis (PFGE) (Wallace *et al*, 1993b; Hector *et al*, 1992; Kim & Kim, 1997), spoligotyping (Kamerbeek *et al*, 1997; Goyal *et al*, 1997; Sola *et al*, 1998) and reverse cross blot hybridisation (Kox *et al*, 1994 & 1995b; Sanguinetti *et al*, 1998). The latter was used in this study.

The choice of the target DNA sequence used for the amplification reaction is a crucial parameter for the success of the PCR. In human tuberculosis, many sequences have been designed for amplification including the insertion sequence IS*6110* (Eisenach *et al*, 1991; Thierry *et al*, 1990; Clarridge *et al*, 1993; Noordhoek *et al*, 1994; Hellyer *et al*, 1996), the direct repeat (DR) locus (Kamerbeek *et al*, 1997), the *mtp40* gene (Del Portillo *et al*, 1991; Liebana *et al*, 1996; Weil *et al*, 1996; Marchetti *et al*, 1998), a repetitive sequence MIRU (Magdalena *et al*, 1998) and 16S rRNA (Rogall *et al*, 1990a & 1990b; Böddinghaus *et al*, 1990; Hermans *et al*, 1990; Hughes *et al*, 1993). The 16S rDNA, a gene coding for 16S rRNA, is a highly conserved part of the bacterial genome containing sufficient information to allow both phylogenetic analysis and species identification (Woese, 1987; Dams *et al*, 1988; Edwards *et al*, 1989). Edwards *et al* (1989) reported the entire genes coding for the 16S rRNA of *Mycobacterium* using *M. kasasii* as a model. Some of the nucleic acid sequences used to study human mycobacterial disease are outlined in Table 4.1.

Bacteria isolated from fish during outbreaks of mycobacteriosis, previously characterised in Chapters 2 and 3, were examined here by means of molecular biology. Amplification of the 16S rDNA for various mycobacterial species, combined

with a reverse cross blot hybridisation assay, previously described by Kox *et al* (1994 & 1995b) was utilised to detect and identify aquatic *Mycobacterium* species.

Table 4.1 Reported primers for the amplification of *Mycobacterium* species

Authors	Target sequence	Primer #1	Primer #2	PCR product
Böddinghaus *et al*, 1990	DNA encoding mycobacterial 16S rRNA (16S rDNA)	5'-AGAGTTTGAT CCTGGCTCAG-3'	5'-TGCACACAGG CCACAAGGGA-3'	1,030 bp
Plikaytis *et al*, 1992	DNA encoding 65 kDa heat shock protein (hsp 65) of *M. tuberculosis*	5'-CGGAGGAATC ACTTCGCAATG-3'	5'-TTGAAGGCGA TCTGCTT-3'	1,380 bp
Soini *et al*, 1992	DNA encoding 32-kDa protein of *M. tuberculosis*	5'-GGCCAGTCAA GCTTCTACTCCGA CTGG-3'	5'-GCCGTTGCCGC AGTACACCCAGA CGCG-3'	423 bp
Takewaki *et al*, 1993	DNA of the *dnaJ* gene from *M. tuberculosis*	5'-GGGTGACGCG GCATGGCCCA-3'	5'-CGGGTTTCGTC GTACTCCTT-3'	236 bp
Kox *et al*, 1994	IS*6110* insertion sequence	5'-GTGCGGATGG TCGCAGAGAT-3'	5'-CTCGATGCCCT CACGGTTCA-3'	541 bp
Kox *et al*, 1995b	DNA encoding mycobacterial 16S rRNA (16S rDNA)	5'-GRGRTACTCG AGTGGCGAAC-3'[a]	5'-GGCCGGCTAC CCGTCGTC-3'	208 bp
Kolk *et al*, 1998	IS*6110* insertion sequence	5'-GAACCGTGAG GGCATCGAGG-3'	5'-GCGTAGGCGT CGGTGACAAA-3'	249 bp

[a] R was A or G

4.2 Materials and Methods

4.2.1 Bacteria samples

Reference strains *M. marinum* (NCIMB 1297), *M. fortuitum* (NCIMB1294), *M. chelonae* (NCIMB1474), *M. poriferae* (NCIMB12538) and *Mycobacterium* spp. isolated from different hosts and different geographical regions (see Section 4.3.3) were cultured on modified Sauton's agar (MSA) at 30°C for 1-4 weeks depending on the growth rate of each strain. Another two reference strains, *M. chelonae* (KIT11303) and *M. chelonae* (KIT4358), obtained as a solution of purified DNA from the Royal Tropical Institute (KIT), N.H. Swellengrebel Laboratory of Tropical Hygiene, Amsterdam were included. Non-mycobacteria (see Section 4.3.2) were cultured in different medium and at different temperatures as outlined in Chapter 2.

4.2.2 Polymerase chain reaction (PCR)

4.2.2.1 Preparation of DNA

Bacterial lysates

The non-mycobacteria and mycobacteria samples were lysed as described by Kox *et al* (1995b). Briefly, a small amount of bacteria (around 1 mg; 10^8 bacteria) was suspended in a 1.5 ml vial containing 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 0.5 ml of 150-212 nm glass beads (Sigma). The bacterial suspension was incubated at 75°C for 15 min and then vortexed at maximum speed for 1 min. The supernatant was diluted 1/100 with TE buffer and used as a DNA solution in the PCR.

DNA isolation

The DNA of reference strains *M. marinum* (NCIMB 1297) and *M. fortuitum* (NCIMB1294) was extracted by a modification of the method described by Hermans *et al* (1990). These DNA samples were then amplified by PCR and analysed by reverse cross blot hybridisation in order to determine the detection limit of each probe.

The bacteria were cultured in MSB for 2 weeks at 30°C and then adjusted to an OD of 0.8 at 610 nm. Four hundred ml of the suspension was taken and incubated in a water bath at 75°C for 15 min to kill the bacteria. The suspension was then centrifuged at 3000 xg for 20 min. The pellets were washed twice with TE buffer and resuspended in 5 ml of TE buffer. Lysozyme was added at a final concentration of 1 mg ml^{-1} and the bacteria were incubated for 90 min at 37°C. Proteinase K and sodium dodecyl sulfate (SDS) were then added at a final concentration of 0.1 $mgml^{-1}$ and 1% respectively, and the bacteria were incubated for a further 1 h at 60°C with continuous shaking. The mixture was added to Tris-saturated phenol, pH 8.0 (Sigma) at a ratio of 1:1 (v:v) and shaken on a rotator for 10 min. The bacterial suspension was then centrifuged at 3000 xg for 10 min. The upper aqueous layer was collected taking care not to disturb the interphase and phenol/chloroform was added to the aqueous fraction at a ratio 1:1 (v:v). The tubes were again shaken on a rotator for 10 min and centrifuged at 3000 xg for 5 min. The upper aqueous phase was transferred to a fresh tube and the procedure repeated with chloroform. DNA present in the final solution was precipitated by adding cold ethanol (-20°C) and NaCl at a final concentration of 70% and 0.2 M respectively, and incubating at -20°C for at least 1 h. The tubes were centrifuged at 3000 xg at -10°C for 30 min. The supernatant was discarded and 70%

cold ethanol was gently added to the pellets taking care not to resuspend them. The tubes were again centrifuged at 3000 xg at -10°C for 5 min and the supernatant was removed. The pellets were dried in a desiccator before re-dissolving them in 3 ml of TE buffer with an overnight incubation at 4°C. The concentration and purity of DNA was determined by measure the absorbance (A) at 260 nm and 280 nm. The ratio of A_{260} / A_{280} was between 1.8 and 1.9. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA ml^{-1}.

4.2.2.2 Primers

Two oligonucleotides sequenced by Kox *et al* (1995b) were used as primers in this study for the amplification of a part of DNA coding for mycobacterial 16S rRNA. This set of primers was specific for the genus *Mycobacterium*, one was a forward primer pMyc14-F (5'-GRGRTACTCGAGTGGCGAAC-3') (Pharmacia Biotech) and another one was a reverse primer pMyc7-R (5'-GGCCGGCTACCCGTCGTC-3') (Pharmacia Biotech). Both primers were labelled with biotin at the 5'end.

4.2.2.3 Reaction

To amplify 16S rDNA a PCR mixture was prepared for a 50 µl reaction, which contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$ (Promaga), 0.1% Triton X-100 (Sigma), 0.2 mM of each deoxynucleoside triphosphates (dATP, dGTP, dCTP and dUTP)(Pharmacia Biotech), 0.2 µM of each primer pMyc14-F-Bio and pMyc7-R-Bio, 0.5 U Super Taq polymerase (HT Biotechnology, Cambridge, U.K.), 0.4 U Uracil DNA glycosylase (UDG, HT Biotechnology, Cambridge, U.K.) and 10 µl of DNA sample. Mineral oil (Sigma) was layered on the top of the reaction mixture to prevent evaporation. Prior to the

amplification, all vials were incubated at 40°C for 30 min to remove the uracil residues from any contaminating uracil-containing amplicons (the amplified DNA fragments) and then subsequently heated to 94°C for 10 min for defragmenting the contaminating amplicon. The amplification was performed in a thermocycler (OmniGene, Hybriad) for 40 cycles as follows: 1.5-min denaturation at 94°C, 2-min annealing at 65°C and 3-min extension at 72°C. The temperature of the vials was held at 72°C until the next step was performed (Kox *et al*, 1994 & 1995b).

4.2.2.4 Gel electrophoresis analysis

Four g of agarose (Flowgen) was added to 200 ml TBE buffer (0.089M Tris, 0.089M boric acid, 0.002 M EDTA, pH 8.3) and then heated in microwave oven for 2 min 40 sec at 750 Watt. Twenty µl of 5 mg ml^{-1} ethedium bromide solution was added to the dissolved gel and the gel was then allowed to cool in waterbath to 60°C for 1 h. It was poured into a horizontal tray and combs inserted. The gel was allowed to set in the dark.

PCR product, 50 µl, was added directly to 12.5 µl of loading buffer (50% (v:v) glycerol in 10X TE buffer, 0.125% Orange G, 15 mM EDTA). Then, 20 µl of the mixture was added to a well of the gel and 5 µl of molecular weight marker (ØX/Hinc II, HT biotechnology, Cambridge) was also added to one well as a reference. The gel was subjected to 110 V for 50 min and the bands of DNA were viewed under UV transilluminator (UVP, Life Sciences, Cambridge, UK). Its image was recorded by photography.

4.2.3 Reverse cross blot hybridisation assay

4.2.3.1 Tailing of oligonucleotide probes with dTTP

The oligonucleotide probes outlined in Table 4.2 were tailed with dTTP. This facilitates the hybridisation of the probes which then add a spacer. Two hundred pmol of each oligonucleotide was added to 8.8 µl of tailing solution, which contained 1.6 µl of 5x tailing buffer, 1.6 µl of 25 mM $CoCl_2$, 2 µl of 10 mM dTTP (Pharmacia) and 0.2 µl of 25U TdT (Boehringer Mannheim). The mixture was incubated at 37°C for 2 h and then 4 µl of 0.2 M EDTA, pH 8.0 added to stop the reaction. The volume of dTTP-tailed oligonucleotide was made up to 400 µl with nanopure water giving a final concentration of 0.5 µM of dTTP-tailed oligonucleotide. The tailed probes were stored at -20°C until used.

Table 4.2 Oligonucleotide probes using for identification of 16S rDNA PCR products

Code	bp-position	Specific for	Nucleotide sequences[a]
pMyc5a	201-184	*Mycobacterium* spp.	5'-GGGCCCATCCCACACCGC-3'
pMar2	148-168	*M. marinum* and *M. ulcerans*	5'-CGGGATTCATGTCCTGTGGT-3'
pChe3	167-146	*M. chelonae*	5'-CCACTCACCATGAAGTGTGTG-3'
pFor1	168-148	*M. fortuitum* and *M. senegalense*	5'-ACCACACACCATGAAGCGCG-3'

(a) Kox *et al*, 1995b &1997

4.2.3.2 Membrane preparation

A nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell) was placed on an accutran cross unit (Schleicher & Schuell), which was assembled in the following

order: a rubber mat, 2 blotting papers (GB 002, Schleicher & Schuell), the nitrocellulose membrane, and a 14-longitudinal-slot mould on the top. Fifty pmol of each dTTP-tailed probe was diluted in 1 ml of 10x Saline-sodium citrate buffer (SSC: 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Each diluted oligonucleotide probe was then added to each slot of the mould and incubated overnight at 20°C on a rotary shaker. The membrane was taken out of the apparatus and wrapped with a piece of cling film. The probes were fixed to the membrane by exposuring it to UV light (BDH) at 312 nm until 1.5 J cm^{-2} was reached. The membrane was washed twice with 10x SSC and then incubated in hybridisation solution (5x SSC, 1% blocking agent (Boehringer Mannheim), 0.1% N-laurylsarcosine, 0.02% SDS) for 5 mins. The membrane was allowed to air-dry and kept at 4°C until the next step of the process.

4.2.3.3 Hybridisation assay

The hybridisation assay used here was previously described by Kox *et al* (1995b). The accutran cross unit was assembled accordingly by first placing a rubber mat, then the membrane prepared in Section 4.2.3.2 and finally a vertical slot mould on top of this. To each slot was added 100 µl of hybridisation solution and the membrane was incubated on a rotator mixer at 20°C for 5 min. Fifteen µl of PCR product (Section 4.2.2.3) was placed into 1.5 ml screwcap vials and boiled at 100°C for 5 min. The vials were placed on ice immediately after boiling and 100 µl of hybridisation solution was added to each vial. The hybridisation solution was removed from each slot and replaced with the DNA mixture. The unit was incubated at 50°C for 1 h. The DNA mixture was discarded from each slot using vacuum pump and the membrane was then removed from the unit. It was briefly rinsed with 0.1% SDS in 2x SSC and then incubated at 50°C for 5 min in fresh 0.1% SDS in 2x SSC. The

membrane was washed briefly with 100 ml of washing buffer (0.1 M Tris HCl, 0.15 M NaCl, pH 7.5) and then incubated in 100 ml of a blocking buffer [0.5% (w:v) blocking reagent (Boehringer Mannheim) in washing buffer] at 20°C for 30 min on a rotary shaker. The membrane was washed as above then incubated with 10 ml of streptavidin conjugated alkaline phosphatase (0.1U ml^{-1}) (Boehringer Mannheim) in washing buffer for 30 min at 20°C. Unbound conjugate was removed by incubating the membrane in 100 ml washing buffer for 30 min. The membrane was equilibrated with 20 ml of a substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 2 min. Finally, it was incubated in 10 ml of substrate solution [45 µl of 4-nitroblue tetrazolium chloride (NBT, Boehringer Mannheim), 35 µl of 5-bromo-4-chloro-3-indyl phosphate (X-phosphate, Boehringer Mannheim), 10 ml substrate buffer] until the colour completely developed. The reaction was stopped by rinsing the membrane with distilled water.

4.2.4 Sensitivity determination

DNA prepared from reference strains *M. marinum* (NCIMB1297), *M. fortuitum* (NCIMB1294) and *M. chelonae* (KIT4358) was diluted to 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} g DNA ml^{-1} with TE buffer; these weights correspond to 2 $x10^8$, 2 $x10^7$, 2 $x10^6$, 2 $x10^5$, 2 $x10^4$ and 2 $x10^3$ mycobacteria ml^{-1} respectively (Kox *et al*, 1994 & 1995b). Ten µl of each DNA solution was added to 40 µl PCR mixture and amplified as described in Section 4.2.2.3. The amplified DNA was then detected by gel electrophoresis (Section 4.2.2.4) and reverse cross blot hybridisation as described in Section 4.2.3.

4.2.5 Specificity determination

The DNA of non-mycobacteria and reference mycobacteria strains outlined in Section 4.3.2 was prepared as described in Section 4.2.2.1. Then DNA of each sample was amplified by PCR (Section 4.2.2.3) and examined by agarose gel electrophoresis (Section 4.2.2.4) and reverse cross blot hybridisation (Section 4.2.3).

4.2.6 Detection and identification of *Mycobacterium* spp. by PCR-reverse cross blot hybridisation

DNA from mycobacterial isolates obtained from different geographical regions, outlined in Section 4.3.3, were prepared as described in Section 4.2.2.1. Ten µl of each lysate sample was added to 40 µl of PCR mixture and amplified as described in Section 4.2.2.3. The amplified DNA was then analysed both by agarose gel electrophoresis (Section 4.2.2.4) and reverse cross blot hybridisation (Section 4.2.3).

4.3 Results

4.3.1 Sensitivity

The sensitivity of the primers pMyc14-F and pMyc7-R (for amplification of 16S rDNA) was determined by both agarose gel electrophoresis and reverse cross blot hybridisation assay. In agarose gel electrophoresis, the positive bands at 208 bp were visualised for up to 1 pg of DNA, which was equivalent to 2 $x10^2$ mycobacteria (Fig 4.1, 4.2 and 4.3). This detection limit was the same for all reference strains. In the reverse cross blot hybridisation assay, the detection limit of probes pMar2, pChe3 and pFor1 (which respond with *M. marinum*, *M. chelonae* and *M. fortuitum* respectively) had detection limits at 100 fg DNA, which is equivalent to 20 bacteria as illustrated in Fig 4.4, 4.5 and 4.6.

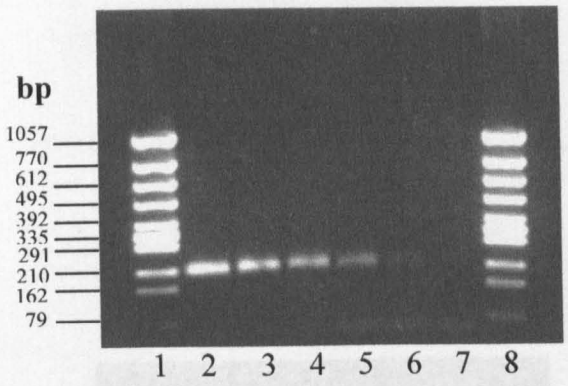

Fig 4.1 Sensitivity determination of PCR for *M. marinum* (NCIMB1297) by agarose gel electrophoresis (2%). Lanes:(1) Molecular size markers (ØX/HincII), (2)10 ng DNA, (3) 1ng DNA, (4) 100 pg DNA, (5) 10 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA, (8) Molecular size markers (ØX/HincII)

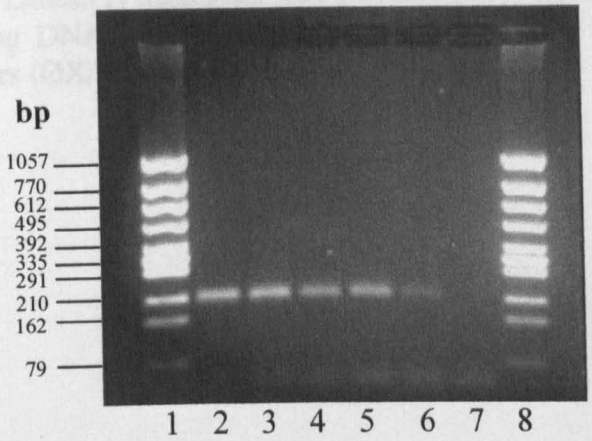

Fig 4.2 Sensitivity determination of PCR for *M. chelonae* (KIT 4350) by agarose gel electrophoresis (2%). Lanes:(1) Molecular size markers (ØX/HincII), (2)100 pg DNA, (3) 50 pg DNA, (4) 10 pg DNA, (5) 5 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA, (8) Molecular size markers (ØX/HincII)

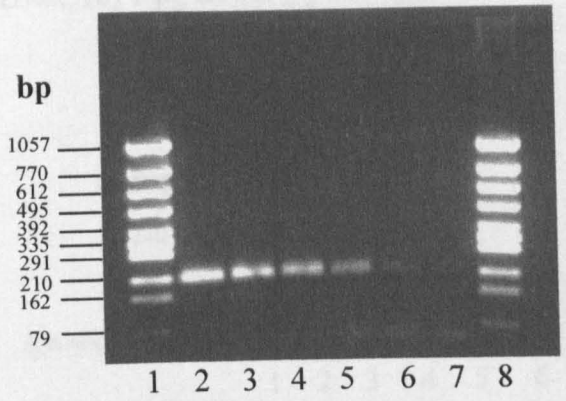

Fig 4.3 Sensitivity determination of PCR for *M. fortuitum* (NCIMB1294) by agarose gel electrophoresis (2%). Lanes:(1) Molecular size markers (ØX/HincII), (2)10 ng DNA, (3) 1ng DNA, (4) 100 pg DNA, (5) 10 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA, (8) Molecular size markers (ØX/HincII)

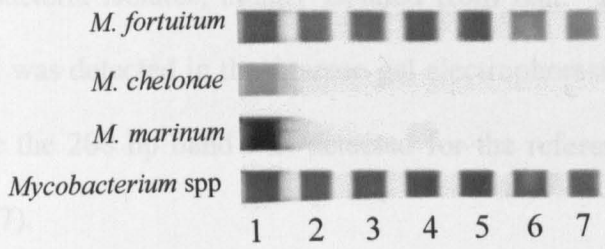

Fig 4.4 Sensitivity of pFor1 probe in reverse cross blot hybridisation with PCR products of *M. fortuitum* (NCIMB1294). Lanes: (1) DNA pool, (2) 10 ng DNA, (3) 1 ng DNA, (4) 100 pg DNA, (5) 10 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA

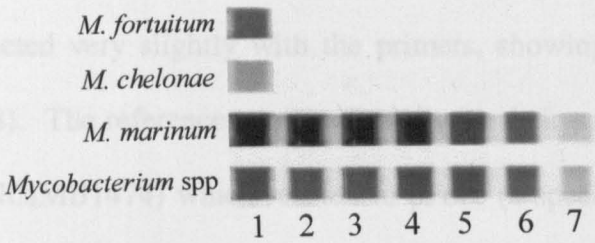

Fig 4.5 Sensitivity of pMar2 probe in reverse cross blot hybridisation with PCR products of *M. marinum* (NCIMB1297). Lanes: (1) DNA pool, (2) 10 ng DNA, (3) 1 ng DNA, (4) 100 pg DNA, (5) 10 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA

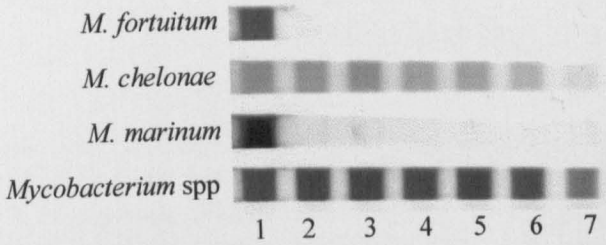

Fig 4.6 Sensitivity of pChe3 probe in reverse cross blot hybridisation with PCR products of *M. chelonae* (KIT4350). Lanes: (1) DNA pool, (2) 100 pg DNA, (3) 50 pg DNA, (4) 10 pg DNA, (5) 5 pg DNA, (6) 1 pg DNA, (7) 100 fg DNA

4.3.2 Specificity

The specificity of PCR with primers pMyc 14-F and pMyc7-R was tested against 24 different non-mycobacteria isolates, mainly isolated from fish. The results showed that no PCR product was detected in the agarose gel electrophoresis with the DNA of these bacteria, while the 208 bp band was detected for the reference strains used as control DNA (Fig 4.7).

The specificity of all four probes used in reverse cross blot hybridisation was also tested against the DNA of 24 non-mycobacteria and reference strains. DNA as shown in Table 4.2, only from *Norcardia asteroides* (NCIMB1290) at the concentration of 10^6 cell ml^{-1} cross reacted very slightly with the primers, showing a faint band at pMyc5a probe (Fig 4.8). The reference strains reacted with their appropriate probes, except *M. chelonae* (NCIMB1474) which reacted to pFor1 (a species-specific probe of *M. fortuitum*) instead of pChe3 as illustrated in Fig 4.8.

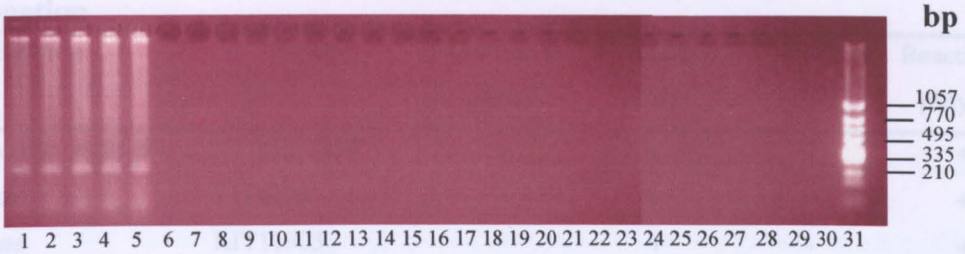

Fig 4.7 Agarose gel electrophoresis analysis of non-mycobactiria and reference strain mycobacteria. Lanes: 1 *M. marinum* (NCIMB1297), 2 *M. chelonae* (KIT 4350), 3 *M chelonae* (NCIMB1474), 4. *M. fortuitum* (NCIMB1294), 5 *M. poriferae* (NCIMB12538), 6 *Nocardia asteroides* (NCIMB 1290), 7 *N. seriolae*, 8 *Aeromonas salmonicida* (Weymouth), 9 *A. salmonicida* (NCIMB1102), 10 *A. hydrophila* (IOA), 11 *A. hydrophila* (WCS), 12 *Aeromonas* sp (IOA), 13 *Bacillus subtilis* (IOA), 14 *Citrobacter freundii* (B96221), 15 *Escherichia coli* (NCTC10418), 16 *Edwardsiella ictaruli* (IOA), 17 *E. tarda* (IOA), 18 *Cytophaga psychrophila* (NCIMB1947), 19 *Pasteurella aerugiris* (IOA), 20 *P. piscicida* (IOA), 21 *Pasteurella* sp. (IOA), 22 *Pseudomonas flurescens* (NCIMB1953), 23 *Serratia* sp (IOA), 24 *Streptococcus faecalis* (IOA), 25 *Listonella anguillarum* (NCIMB571), 26 *L. anguillarum* (NCIMB6), 27 *V. ordalii* (NCIMB2167), 28 *Yersinia ruckeri* (IOA), 29 *Y. ruckeri* (NCIMB1316), 30 TE buffer, 31 Molecular weight makers (∅X/HincII)

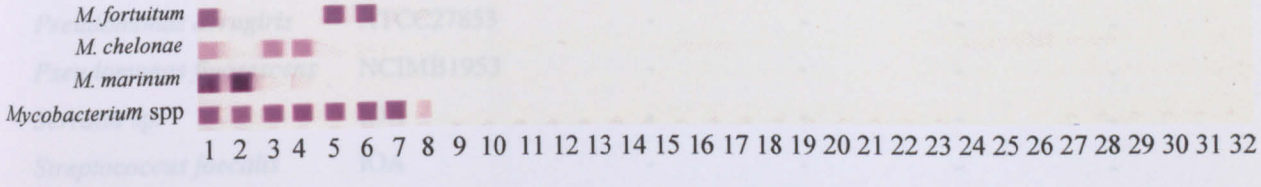

Fig 4.8 PCR-reverse cross blot hybridisation of non-mycobactiria and reference strain mycobacteria. Lanes:1 DNA pool, 2 *M. marinum* (NCIMB1297), 3 *M. chelonae* (KIT 4350), 4 *M. chelonae* (KIT11303), 5 *M chelonae* (NCIMB1474), 6. *M. fortuitum* (NCIMB1294), 7 *M. poriferae* (NCIMB12538), 8 *Nocardia asteroides* (NCIMB1290), 9 *N. seriolae*, 10 *Aeromonas salmonicida* (Weymouth), 11 *A. salmonicida* (NCIMB1102), 12 *A. hydrophila* (IOA), 13 *A. hydrophila* (WCS), 14 *Aeromonas* sp (IOA), 15 *Bacillus subtilis* (IOA), 16 *Citrobacter freundii* (B96221), 17 *Escherichia coli* (NCTC10418), 18 *Edwardsiella ictaruli* (IOA), 19 *E. tarda* (IOA), 20 *Cytophaga psychrophila* (NCIMB1947), 21 *Pasteurella aerugiris* (IOA), 22 *P. piscicida* (IOA), 23 *Pasteurella* sp. (IOA), 24 *Pseudomonas flurescens* (NCIMB1953), 25 *Serratia* sp (IOA), 26 *Streptococcus faecalis* (IOA), 27 *Listonella anguillarum* (NCIMB571), 28 *L. anguillarum* (NCIMB6), 29 *V. ordalii* (NCIMB2167), 30 *Yersinia ruckeri* (IOA), 31 *Y. ruckeri* (NCIMB1316), 32 TE buffer

Table 4.3 Reaction of DNA from mycobacteria reference strains and non-mycobacteria with primers pMyc14-F-Bio and Myc7-R-Bio in the polymerase chain reaction

Bacteria	Source	Reaction to pMar2	Reaction to pChe3	Reaction to pFor1	Reaction to pMyc5a
Mycobacterium marinum	NCIMB 1297	+	-	-	+
M. chelonae	KIT4358	-	+	-	+
M. chelonae	KIT11303	-	+	-	+
M. chelonae	NCIMB 1474	-	-	+	+
M. fortuitum	NCIMB 1294	-	-	+	+
M. poriferae	NCIMB 12538	-	-	-	+
Aeromonas sp.	IOA	-	-	-	-
A. salmonicida	NCIMB 1102	-	-	-	-
A. hydrophila	NCIMB 1118	-	-	-	-
A. hydrophila	IOA	-	-	-	-
A. salmonicida	Weymouth	-	-	-	-
Bacillus subtilis	IOA	-	-	-	-
Citrobacter freundii	IOA	-	-	-	
Cytophaga psychrophila	NCIMB1947	-	-	-	-
Escherichia coli	NCTC10418	-	-	-	-
Edwardsiella ictaruli	IOA	-	-	-	-
E. tarda	NCIMB 2034	-	-	-	-
Norcardia asteroides	NCIMB 1290	-	-	-	+
N. seriolae	NCIMB 13256	-	-	-	-
Pasteurella sp.	IOA	-	-	-	-
P. piscicida	IOA	-	-	-	-
Pseudomonas aerugiris	ATCC27853	-	-	-	-
Pseudomonas fluorescens	NCIMB1953	-	-	-	-
Serratia sp.	IOA	-	-	-	-
Streptococcus faecalis	IOA	-	-	-	-
Listonella anguillarum	NCIMB571	-	-	-	-
L. anguillarum	NCIMB6	-	-	-	-
Vibrio ordalii	NCIMB2167	-	-	-	-
Yersinia ruckeri	IOA	-	-	-	-
Y. ruckeri	NCIMB1316	-	-	-	-

NCTC: National Collections of Type Cultures
NCIMB: National Collection of Industrial and Marine Bacteria, Scotland
IOA: Institute of Aquaculture, University of Stirling, Scotland
KIT: The Royal Tropical Institute, Amsterdam, The Netherlands
ATCC: American Tissue Culture Centre

4.3.3 Characterisation of *Mycobacterium* spp. isolated from different geographical regions

4.3.3.1 Detection of amplified DNA by agarose gel electrophoresis

Amplification products of DNA from all of the isolates obtained from either Thailand (Table 4.4) or the other different geographical regions (Table 4.5) resulted in a band at 208 bp, whereas no PCR products were detected for the negative control as illustrated in Fig 4.9, 4.10 and 4.11.

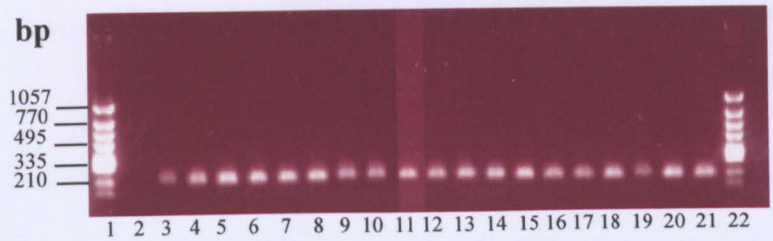

Fig 4.9 Agarose gel electrophoresis analysis of PCR product of *Mycobacterium* spp isolates from snakehead fish (*Channa straitus*). Lanes: (1) Molecular weight markers (ØX/HincII), (2) TE buffer, (3) S1, (4) S2, (5) S3, (6) S4, (7) S5, (8) S6, (9) S7, (10) S8, (11) S9, (12) S10, (13) S11, (14) S12, (15) S13, (16) S14, (17) S15, (18) S18, (19) S267, (20) S268, (21) S269, (22) Molecular weight markers

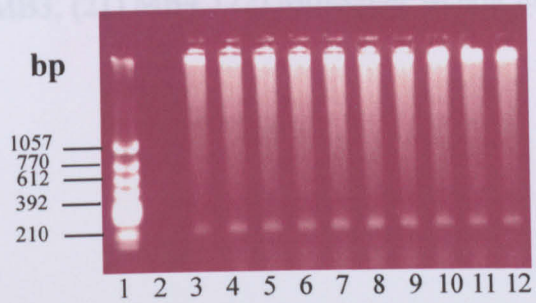

Fig 4.10 Agarose gel electrophoresis analysis of PCR product of *Mycobacterium* spp isolates from Siamese fighting fish (*Betta splendens*). Lanes: (1) Molecular weight markers (ØX/HincII), (2) TE buffer, (3) TB1, (4) TB7, (5) TB38, (6) TB40, (7) TB42, (8) TB43, (9) TB44, (10) TB45, (11) TB62, (12) TB73

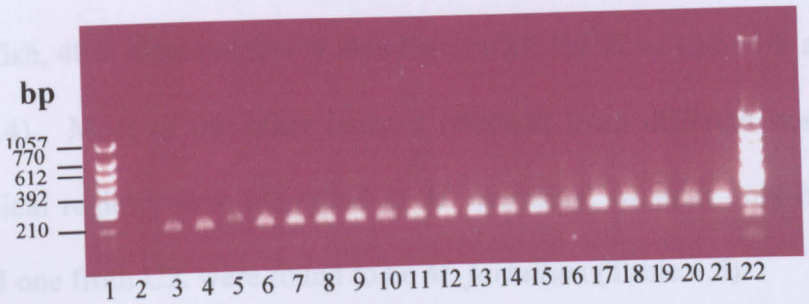

Fig 4.11 Agarose gel electrophoresis analysis of PCR product of *Mycobacterium* spp isolates from a variety of fish in different geographical regions. Lanes: (1) Molecular weight marker (ØX/HincII), (2) TE buffer, (3) E1, (4) E2, (5) E3, (6) E4, (7) E5, (8) E6, (9) E7, (10) E8, (11) E9, (12) E10, (13) E11, (14) E12, (15) E14, (16) E15, (17) E16, (18) MB1, (19) MB2, (20) MB3, (21) MB4, (22) Molecular weight marker (ØX/HincII)

4.3.3.2 Detection of amplified DNA by reverse cross blot hybridisation

The isolates from both snakehead fish and Siamese fighting fish in Thailand, as well as the isolates from different geographical regions, were positive with either the pMar2 or the pFor1 probe, specific for *M. marinum* and *M. fortuitum*, respectively. Every isolate tested was positive with the pMyc5a probe, which was specific to the genus *Mycobacterium* sp as illustrated in Fig 4.12, 4.13 and 4.14.

Of the mycobacteria isolated from snakehead fish in Thailand, approximately 53 % were positively identified as *M. marinum* and 47% were positively identified as *M. fortuitum* by reverse cross blot hybridisation. Of the isolates obtained from Siamese fighting fish, 40% were positively identified as *M. marinum* and 60% as *M. fortuitum* (Table 4.4). Most of the other isolates obtained from different hosts in different geographical regions were identified as *M. marinum*, whereas only one strain from Chile and one from UK were found to be *M. fortuitum* (Table 4.5).

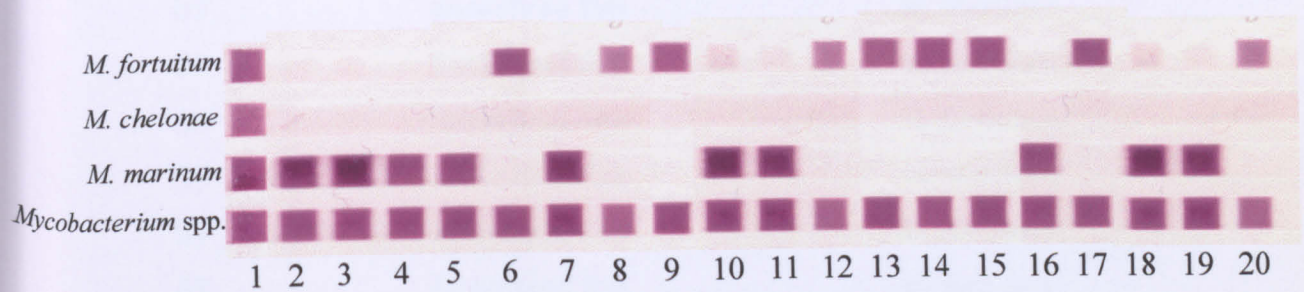

Fig 4.12 Characterisation of *Mycobacterium* spp isolated from snakehead fish (*Channa straitus*) in Thailand by PCR-reverse cross blot hybridisation. Lanes: (1) DNA pool; (2) S1; (3) S2; (4) S3; (5) S4; (6) S5; (7) S6; (8) S7; (9) S8; (10) S9; (11) S10; (12) S11; (13) S12; (14) S13; (15) S14; (16) S15; (17) S18; (18) S267; (19) S268; (20) S269.

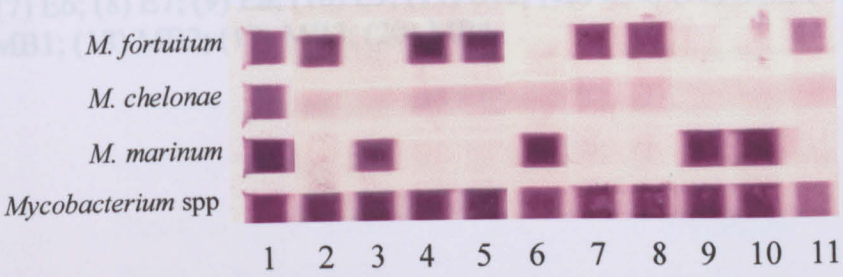

Fig 4.13 Characterisation of *Mycobacterium* spp isolated from Siamese fighting fish (*Betta splendens*) in Thailand by PCR-reverse cross blot hybridisation. Lanes: (1) DNA pool; (2) TB1; (3) TB7; (4) TB38; (5) TB40; (6) TB42; (7) TB43; (8) TB44; (9) TB45; (10) TB62, (11) TB73

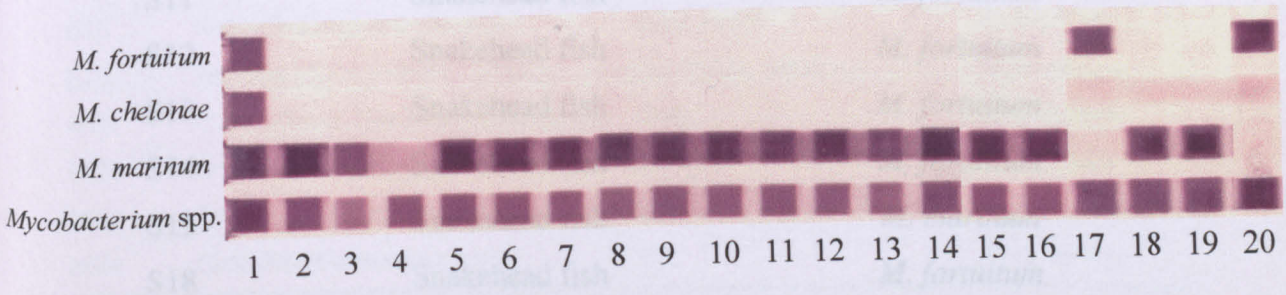

Fig 4.14 Characterisation of *Mycobacterium* spp obtained from fish in different geographical regions by PRC-reverse cross blot hybridization. Lanes: (1) DNA pool; (2) E1; (3) E2; (4) E3; (5) E4; (6) E5; (7) E6; (8) E7; (9) E8; (10) E9; (11) E10; (12) E11; (13) E12; (14) E14; (15) E15; (16) E16; (17) MB1; (18) MB2; (19) MB3; (20) MB4.

Table 4.4 Characterisation of *Mycobacterium* spp. obtained from Thailand by PCR-reverse cross blot hybridisation

Isolates	Fish	Species identification
S1	Snakehead fish	*M. marinum*
S2	Snakehead fish	*M. marinum*
S3	Snakehead fish	*M. marinum*
S4	Snakehead fish	*M. marinum*
S5	Snakehead fish	*M. fortuitum*
S6	Snakehead fish	*M. marinum*
S7	Snakehead fish	*M. fortuitum*
S8	Snakehead fish	*M. fortuitum*
S9	Snakehead fish	*M. marinum*
S10	Snakehead fish	*M. marinum*
S11	Snakehead fish	*M. fortuitum*
S12	Snakehead fish	*M. fortuitum*
S13	Snakehead fish	*M. fortuitum*
S14	Snakehead fish	*M. fortuitum*
S15	Snakehead fish	*M. marinum*
S18	Snakehead fish	*M. fortuitum*
S267	Snakehead fish	*M. marinum*
S268	Snakehead fish	*M. marinum*
S269	Snakehead fish	*M. fortuitum*
TB1	Siamese fighting fish	*M. fortuitum*
TB7	Siamese fighting fish	*M. marinum*
TB38	Siamese fighting fish	*M. fortuitum*
TB40	Siamese fighting fish	*M. fortuitum*
TB42	Siamese fighting fish	*M. marinum*
TB43	Siamese fighting fish	*M. fortuitum*
TB44	Siamese fighting fish	*M. fortuitum*
TB45	Siamese fighting fish	*M. marinum*
TB62	Siamese fighting fish	*M. marinum*
TB73	Siamese fighting fish	*M. fortuitum*

Table 4.5 Characterisation of *Mycobacterium* spp obtained from fish in different geographical regions by PCR-reverse cross blot hybridisation

Isolates	Fish	Origin	Species identification
E1	Sheepshead (*Puntazzo puntazzo*)	Eilat, Israel	*M. marinum*
E2	Sea bass (*Dicentrarchus labrax*)	Eilat, Israel	*M. marinum*
E3	Thalassa (*Dicentrarchus labrax*)	Greece	*Mycobacterium* sp.
E4	unknown	USA	*M. marinum*
E5	unknown	Germany	*M. marinum*
E6	Red Drum (*Sciaenops ocellatus*)	Eilat, Israel	*M. marinum*
E7	Butterfly fish (*Chaetodon fasciatus*)	Eilat, Israel	*M. marinum*
E8	Thalassa (*Dicentrarchus labrax*)	Greece	*M. marinum*
E9	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	*M. marinum*
E10	unknown	Hernandez, Germany	*M. marinum*
E11	Sea bass (*Dicentrarchus labrax*)	Atlit, Israel	*M. marinum*
E12	Sea bass (*Dicentrarchus labrax*)	Denmark	*M. marinum*
E14	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	*M. marinum*
E15	Rabbitfish (*Siganus rivulatus*)	Eilat, Israel	*M. marinum*
E16	Sea bass (*Dicentrarchus labrax*)	Ein Yahav, Israel	*M. marinum*
MB1	Coho salmon (*Onchorhyncus kisutch*)	Chile	*M. fortuitum*
MB2	Frog (*Rana rugulosa*)	Thailand	*M. marinum*
MB3	Rosy barb (*Barbus conchonius*)	UK	*M. marinum*
MB4	Gold fish (*Carassius auratus*)	UK	*M. fortuitum*

4.4 Discussion and Conclusions

The reproducibility and accuracy of the PCR method is determined by many factors including the choice of primers, the preparation of the DNA, the amount of target DNA used in each reaction, the presence of natural or induced inhibitors and DNA contamination. The source of contamination can be either cross-contamination between samples or amplified target DNA carried over form the previous PCR. The latter can be a significant problem as it can be re-amplified with the same set of primers, and thus yield a false-positive reaction. The false positive reaction was controlled in this study.

The enzyme uracil DNA glycosylase (UDG) was introduced into the PCR mixture used here in order to control any contaminating amplicons from previous experiments. The UDG acts on both single and double stranded DNA containing uracil, which may be synthesised *in vitro*, such as by PCR incorporating dUTP rather than dTTP (Duncan, 1981; Longo *et al*, 1990). It removes any uracil residues from the amplicon, which in turn makes the amplicon labile to heat, while leaving natural DNA unaffected (Longo *et al*, 1990). The PCR mixture here was therefore designed to use dUTP instead of dTTP in order to obtain an amplified DNA which can be degraded by UDG (Nguyen *et al*, 1996). Prior to amplification, the mixtures were incubated at 40 °C to allow the UGD to incise the contaminating DNA and then subsequently incubated at 94 °C for defragmenting the contaminating DNA. Kox *et al* (1994) reported that using dUTP instead of dTTP did not affect the overall efficiency of the PCR and the use of UDG did not affect its sensitivity. They found that 0.01U of UDG was able to break down 1.5×10^{-12} g of 541-bp amplicon of *M. tuberculosis*. However, in the beginning of this study, 0.2U of UDG was added to 50 µl reaction,

but positive bands were detected in some of the negative controls in both agarose gel electrophoresis and reverse cross blot hybridisation (not shown data). Finally, 0.4U of UDG was applied after which no PCR products were detected in the negative control either in agarose gel electrophoresis or in reverse cross blot hybridisation.

The presence of inhibitors in the PCR assay may produce false-negative reactions. Thus, Kolk *et al* (1994) used recombinant *M. smegmatis* 1008 DNA with an insert of modified IS*6110* element as an internal control in their PCR. This modified DNA was combined in the multiplex PCR for the detection of *M. tuberculosis* complex since they yielded different molecular weight products (see Chapter 6). This modified DNA was used either before or after DNA purification to monitor the presence of inhibitors in DNA purification process or the amplification reaction respectively. In this study, DNA of known origin, *M. marinum* (NCIMB 1297), *M. fortuitum* (NCIMB1294) and *M. chelonae* (KIT11303 or KIT4358) were used as positive controls since they are inexpensive and simple to prepare.

The method of DNA preparation is crucial to reduce inhibitors which may be present in the sample. The DNA used in the PCR described here was prepared from two sources; directly from bacterial lysates or from purified DNA. The use of bacterial lysates was simple and quick for strain identification, and did not require a known amount of DNA. At the high concentrations of DNA however, the presence of the inhibitors resulted in poor amplification (not shown data). Dilution of the bacterial lysates was therefore necessary to reduce the level of inhibition. To determine the level of sensitivity, the purified DNA was used.

The sensitivity of the PCR was determined by agarose gel electrophoresis and reverse cross blot hybridisation. In general, the species-specific probes were able to identify up to 100 fg DNA, which was equivalent to 20 mycobacterial cells. The level of sensitivity obtained when the PCR product was examined by agarose gel electrophoresis was less than from the reverse cross blot hybridisation as it was able to identify up to 1 pg, equivalent to 200 mycobacteria cells. For diagnostic purposes, the reverse cross blot hybridisation has the advantage of not only being more sensitive, but the identification of mycobacteria to species level is also possible. Examination of PCR products by agarose gel electrophoresis, however, is much less expensive and is simpler to perform. This can detect *Mycobacterium* only to the genus level. Knibb *et al* (1993) reported the different sets of primers for the amplification of mycobacterial 16S rDNA and identified *M. marinum* of the PCR product by restriction enzyme analysis. The authors reported the sensitivity to be 20 bacterial cells in agarose gel electrophoresis. A similar study was reported on *M. fortuitum* and *M. chelonae*, however the sensitivity was not determined (Talaat *et al*, 1997). The primers used in this Chapter were previously reported to have a sensitivity of 100 fg of *M. tuberculosis* DNA equivalent to 20 bacteria in 2% agarose gel electrophoresis (Kox *et al*, 1995b).

The specificity of primers in the PCR was examined by both agarose gel electrophoresis and reverse cross blot hybridisation. Primers pMyc14-F and pMyc 7-R, amplifying part of a gene coding for 16S rRNA, were specific for genus *Mycobacterium* spp and yeilded a 208 bp fragment. However, the primers cross-reacted slightly with *Nocardia asteroides* (NCIMB1290) resulting in a faint band on pMyc probe in reverse cross blot hybridisation assay, but a positive band at 208 bp

was not detected in agarose gel electrophoresis. The genus *Nocardia* is closely related to the genus *Mycobacterium* (Frerichs, 1993; Laurent *et al*, 1999). This may pose a problem if *N. asteroides* was present in very high numbers since *N. asteroides* is a known fish pathogen (Valdez & Conroy, 1963; Snieszko *et al*, 1964; Campbell & MacKelvie, 1968; Kawatsu *et al*, 1976). No cross-reactivity was evident with any of other probes used in reverse cross blot hybridisation assay. However, *Nocardia* spp. infection can be confirmed by PCR using species specific primers amplified 16S rRNA of *Nocardia* spp. as described by Laurent *et al* (1999).

The identification of isolates from different geographical regions revealed that only two species of mycobacteria appeared to be involved in fish mycobacteriosis examined here, *M. marinum* and *M. fortuitum*. None of the isolates was positive with the *M. chelonae*-species probe. It is not surprising since *M. chelonae* has been more commonly isolated from cold water fish species such as salmonid (Frerichs, 1993). The causative agents of mycobacteriosis in snakehead fish and Siamese fighting fish in Thailand were identified as either *M. marinum* or *M. fortuitum*. Moreover, all strains which were positively identified as *M. marinum* were also positive with Mab 8F7, and all the strains which were identified as *M. fortuitum* were negative with Mab 8F7 (Chapter 2). Identification of isolates here correlated not only with the results obtained in Chapter 2, but also with those from Chapter 3 analysed by PyMS.

Mycobacterium species have been classified by several different criteria including growth rate and pigmentation such as Runyon classification (Wayne & Kubica, 1986). The Runyon classification categorised *Mycobacterium* species into four groups: photochromogenic, scotochromogenic, nonchromogenic and rapid growth. However,

Bottger (1989) and Rogall *et al* (1990b) discovered that with 16S rRNA analysis a phylogenetic relatedness within a slow-growing species did not reflect the Runyon classification. Pungkachonboon *et al* (1992) has previously characterised the isolate TB1 and another nine isolates from Siamese fighting fish by means of biochemical and physiological properties and reported that these isolates were similar to *M. piscicida*. With respect to the work of Pungkachonboon *et al* (1992), TB1 was identified as *M. fortuitum* in this study based on PCR-reverse cross blot hybridisation and PyMS (Chapter 3).

One of the isolates from snakehead (S7) was also previously analysed by Tortoli *et al* (1996) who reported it to be *M. poriferae* based on the HPLC profile of cell wall mycolic acid of the bacterium. Reference strain *M. poriferae* (NCIMB12538) was included in the present study to examine the cross reactivity of the probes, the results of which revealed no cross reaction with the species-specific probes. In fact, the reference strain *M. poriferae* (NCIMB12538) was negative for the pMar2, pChe3 and pFor1 probes and was positive to the genus probe (pMyc5a). Isolate S7 was previously identified as *M. poriferae* by mycolic acid profile and biochemical tests (Tortoli *et al*, 1996). The result obtained here confirmed that S7 was *M. fortuitum*. Moreover, S7 did not react with Mab 8F7 (Chapter 2) and it clustered in the *M. fortuitum-chelonae* group in the PyMS analysis outlined in Chapter 3.

It should be noted that all reference strains analysed here by PCR and reverse cross blot hybridisation were positive with the appropriate probes except *M. chelonae* (NCIMB 1474) which also reacted positively with the *M. fortuitum* probe. However, Rogall *et al* (1990b) reported that *M. fortuitum* was genetically similar to *M.*

chelonae. According to the results obtained in PyMS analysis (Chapter 3), *M. chelonae* (NCIMB1474) was closely related to *M. fortuitum* (NCIMB1294). The probes pChe3 and pFor1 used here successfully differentiated between *M. chelonae* and *M. fortuitum* respectively (Kox *et al* 1995b & 1997). It was therefore concluded that *Mycobacterium* isolate (NCIMB1474) classified as *M. chelonae* appeared to be *M. fortuitum*. To confirm the reaction of the pChe3 probe, *M. chelonae* (KIT11303) and *M. chelonae* (KIT4358) were tested and they were shown to be positive to the pChe3 probe. Further studies with other isolates of *M. chelonae* are required to reconfirm these results.

Most of the isolates obtained from different geographical regions (E1-E16) were positively identified as *M. marinum* and only one isolate E3 from Greece was not positive for any of the species-specific probes, but did react with the genus probe pMyc5a. With regard to epidemiology, only the Thai isolates and isolates E4, E10 and E12 isolated from the USA, Germany and Denmark, respectively, were positive with the *M. marinum* specific monoclonal probe, 8F7, (Chapter 2). This suggests that the other isolates from Israel (E1, E2, E6, E7, E9, E11 & E14-E16), Greece (E8) and Germany (E5) although also identified as *M. marinum* by PCR, are different in their antigenic make-up. These results agreed with the gene sequencing of 16S rDNA, which showed that isolate E12 was identical to 16S r DNA of *M. marinum* described by Rogall *et al* (1990a & 1990b) while E1, E2, E5-E9, E11 and E14-E16 were different (M. Ucko, pers. comm.). Further analysis of *M. marinum* Thai and Israeli stains by the DNA-based techniques, particularly spoligotyping and RFLP, should be considered. Goyal *et al* (1997) and Kamerbeek *et al* (1997) reported the use of spoligotyping and RFLP for differentiation of *M. tuberculosis* isolates to determine

disease transmission. Use of these techniques with *M. marinum* isolates from Thailand and Israel may provide useful epidemiological information. As a general conclusion, the results obtained in this Chapter confirmed the physico-chemical analysis (PyMS) in Chapter 3, as the isolates clustered together in either the *M. marinum* group or in the *M. fortuitum-M. chelonae* group.

Chapter 5

In situ Hybridisation

5.1 Introduction

The molecular-based technique, *in situ* hybridisation, developed in the late 1960s involves labelling pathogen DNA present in fixed tissue sections with specific oligonucleotide probes, and subsequently allows visualisation of the reaction of probes within the infected tissue (Gall & Pardue, 1969; John *et al*, 1969; Buongiorno-Nardelli & Amaldi, 1970). *In situ* hybridisation, normally performed on tissue sections 4 to 7 μm thick, has an advantage over other molecular methods such as Northern blot and Southern blot analysis, in that substantially more material is required for the latter methods than with the *in situ* hybridisation technique (Chevalier *et al*, 1997).

In situ hybridisation resembles immunohistochemistry (IHC) in that the pathogen can be detected *in situ* within infected tissue sections. However, the sensitivity of IHC may be low if the epitopes on the bacteria are masked or destroyed during sample preparation. The pathogen may be present but inappropriate expression of antigens may prevent IHC from working or species-specific antibodies may not be available. Moreover, background staining may be high in IHC compared with *in situ* hybridisation since the latter involves stringency washing (Biering & Bergh, 1996).

The main advantage of the *in situ* hybridisation technique is its ability to locate the precise position of the pathogens within infected tissue which makes it particularly useful in diagnosis and for studying the pathogenesis of organisms (McNicol &

Farquharson, 1997). The technique is also useful for examining gene structure and expression (Forozan *et al*, 1997; Hopman *et al*, 1997), or for investigating the morphology of cells such as tumor cells or cells loaded with viruses (Speel *et al*, 1994; Speel *et al*, 1999)

Until now, no reports have been made of *in situ* hybridisation being used to diagnose mycobacteriosis in fish. Generally, formalin-fixed tissue samples are used for the identification of the etiological agent involved in this disease, with samples being examined with either immunological probes in IHC or Ziehl-Neelsen (ZN) stain. The monoclonal antibody probe (Mab 8F7) developed in Chapter 2 did not detect all *M. marinum* species involved in mycobacteriosis, however, a negative reaction did not necessarily mean that *Mycobacterium* was not present. Therefore, *in situ* hybridisation was employed in this Chapter using the same oligonucleotide primers that were used in the PCR detailed in Chapter 4. The primers were biotinylated and used as probes, and were specific for the genus *Mycobacterium*.

5.2 Materials and Methods

5.2.1 Sample preparation

Half of samples were stained with ZN staining (Section 2.2.3.3) prior to examining them for the presence of mycobacteria. The remaining samples were analysed by ISH.

5.2.1.1 Preparation of slides

Slides were treated to increase their adhesiveness so as to prevent sample loss during the assay procedure. Briefly, the slides were incubated in 3% (v:v) APES (3-

aminopropyltriethoxysilane, Sigma) in acetone for 5 min, incubated in 100% acetone for 5 min and finally rinsed with distilled water. The slides were then incubated at 37°C overnight.

5.2.1.2 Preparation of tissue sections

Tissue samples from goldfish (*Carassius auratus*) infected with mycobacteriosis were fixed in 10% neutral buffered formalin (0.03 M NaH_2PO_4, 0.05 M Na_2HPO_4, 10% formaldehyde) for 24 h and then embedded in wax. Tissue sections, 7 µm-thick, were cut and placed on the treated slides. The sections were incubated overnight at 60°C.

Two other infected fish samples were also examined. These were samples from Siamese fighting fish (*Betta splendens*) and rabbit fish (*Siganus rivulatus*) obtained as paraffin blocks from Thailand and Israel respectively.

5.2.2 Oligonucleotides

Two sets of biotinylated oligonucleotides, specific for 16S rDNA from the genus *Mycobacterium*, were used as probes (Kox *et al*, 1995b). They were pMyc14-F (5'-GRGRTACTCGAGTGGCGAAC-3'), where R was either A or G, and pMyc7-R (5'-GGCCGGCTACCCGTCGTC-3') (Pharmacia Biotech). Both oligonucleotides were biotinylated at the 5' end of the molecule by the manufacturer (Pharmacia Biotech).

5.2.3 Hybridisation

The sections were dewaxed in xylene for 5 min and rehydrated: 5 min in 100% ethanol, 3 min in 70% ethanol and 3 min in distilled water. The slides were then immersed in 0.2 M HCl for 20 min and rinsed in 2x SSC (2x SSC: 0.3 M NaCl, 0.03

M sodium citrate, pH 7.0) for 3 min. The sections were incubated with 0.3% Triton X-100/PBS for 5 min and then rinsed briefly in TBS (0.05 M Tris base, 0.15 M NaCl, pH 7.4). The sections were incubated with 5 μg ml^{-1} proteinase K at 37°C for 1 h, and subsequently rinsed twice with 0.2% glycine/PBS, 3 min each wash. The slides were again rinsed with PBS and incubated in 0.4% paraformaldehyde/PBS at 4°C for 20 min, after which they were rinsed with distilled water. The sections were incubated with 10% H_2O_2/methanol at 25°C for 10 min and then rinsed with TBS.

Prehybridisation mixture [4x SSC, 0.5% bovine serum albumin (BSA), 0.5% ficoll, 0.5% polyvinylopyrrolidone, 1 μg ml^{-1} salmon sperm DNA] was applied to the sections for 1 h at 42°C. The prehybridisation mixture was removed from the sections and the hybridisation mixture (prehybridisation mixture containing 2 μg ml^{-1} of each probe) was applied, except for the negative control, where only prehybridisation mixture was added. Coverslips were then placed on top of the mixture. The samples were incubated in a thermal control machine (Hybaid OmniSlide) at 96°C for 10 min, then overnight at 42°C. The coverslips were removed the following morning by immersing the slides into 2x SSC for 20 min. The slides were transferred into 0.1x SSC and incubated at 42°C for 30 min. The slides were finally rinsed with TBS.

5.2.4 Visualisation of signal

Non-specific binding sites were blocked with 10% normal goat serum/TBS for 10 min at 25°C. The serum was removed from sections and streptavidin conjugated with horseradish peroxidase (Scottish Antibody Production Unit; Lanarkshire, UK) diluted 1/50 (v:v) in TBS was placed onto the sections. The slides were incubated at 25°C for 2 h and then washed three times with TBS.

Two different chromogen systems were utilised to visualise the reaction and optimise the method. Firstly, the slides were incubated with DAB substrate solution, (0.15 mg ml^{-1} of 3'3' diaminobenzidine tetrahyrochloride/TBS, 0.02% H_2O_2) for 10 min at 25°C. The reaction was stopped by immersing the slides in distilled water and then counterstained with haematoxylin for 4 min. The slides were placed under running water for 10 min and then dehydrated in alcohol series: 3 min in 70% ethanol and 10 min in 100% ethanol. The slides were rinsed twice in xylene and then mounted with Pertex (Cellpath, UK). The slides were observed using a microscope and the product of the reaction was brown. Alternatively, commercially prepared HistoMark TrueBlue (KPL; Maryland, USA) was used as substrate for the peroxidase. HistoMark TrueBlue was applied to the sections for 10 min at 25°C. The slides were washed twice with TBS and then Contrast Red (KPL; Maryland, USA) was applied for a further 3 min. The slides were rinsed in distilled water and dehydrated in alcohol series as described above. The slides were finally mounted with Pertex and observed under the microscope. The product of the reaction was blue.

5.3 Results

5.3.1 Comparison of *in situ* hybridisation and ZN staining on mycobacteriosis-infected fixed tissue sections

Infected kidney tissues from goldfish and Siamese fighting fish were analysed by *in situ* hybridisation and ZN staining and these methods were compared for pathogen identification. With ZN staining, mycobacteria stained a deep pink colour, and were evident inside most of the granulomas examined in the tissue sections of both fish (Fig 5.1a, 5.2a). *In situ* hybridisation analysis of goldfish tissue using DAB as a substrate resulted in staining of bacteria brown. Fewer stained bacteria were evident

in the granulomas than were observed with the ZN staining (Fig 5.1b). No positive reaction was obtained with the *in situ* hybridisation of infected Siamese fighting fish (Fig 5.2b). The negative controls for both fish remained clear (Fig 5.1c and 5.2c).

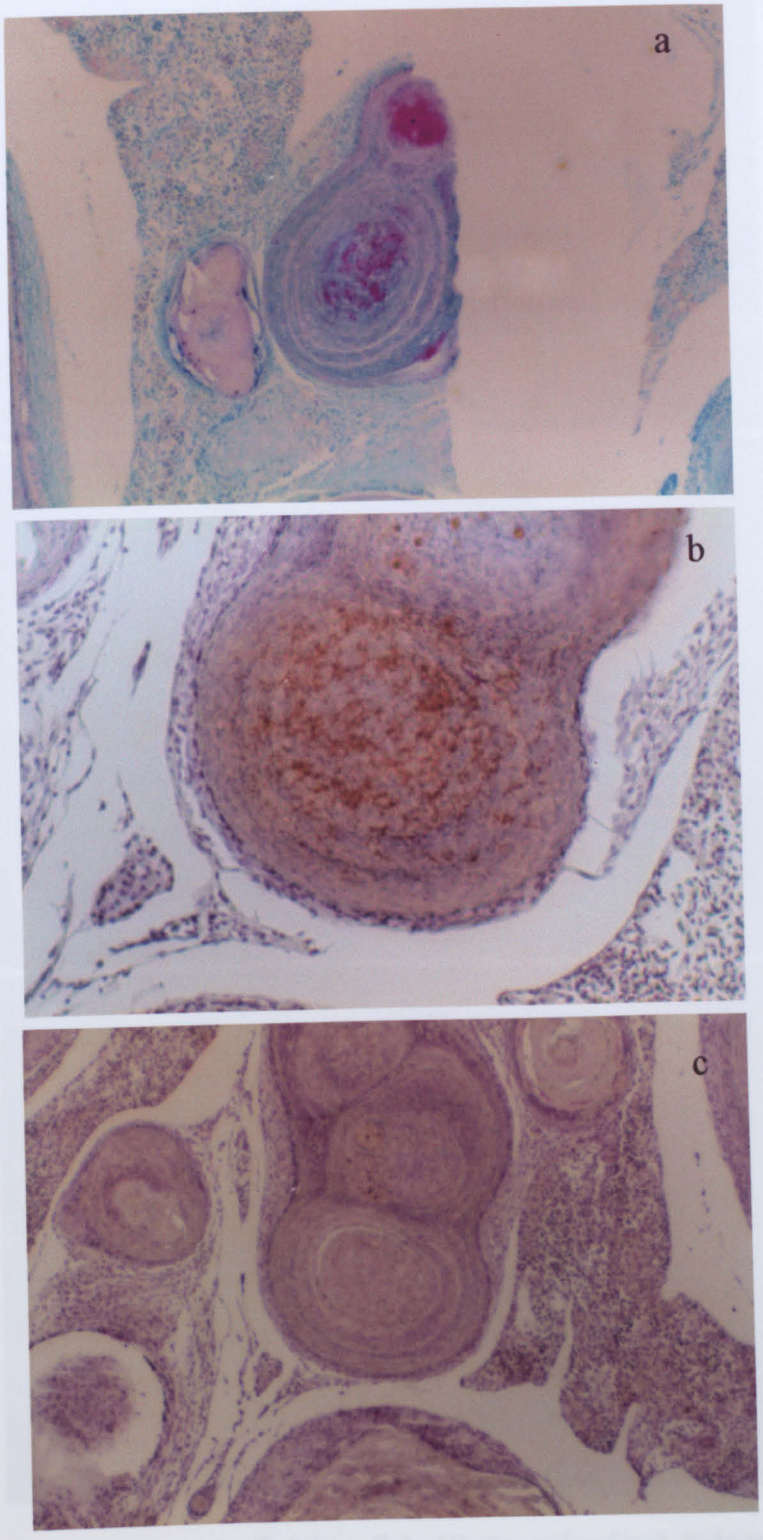

Fig 5.1 Examination of goldfish (*Carassius auratus*) kidney infected with *Mycobacterium* spp with *in situ* hybridisation and Ziehl-Neelsen (ZN) staining: (a) ZN staining (x10); (b) *in situ* hybridisation analysis (x20); (c) negative control for *in situ* hybridisation (x10).

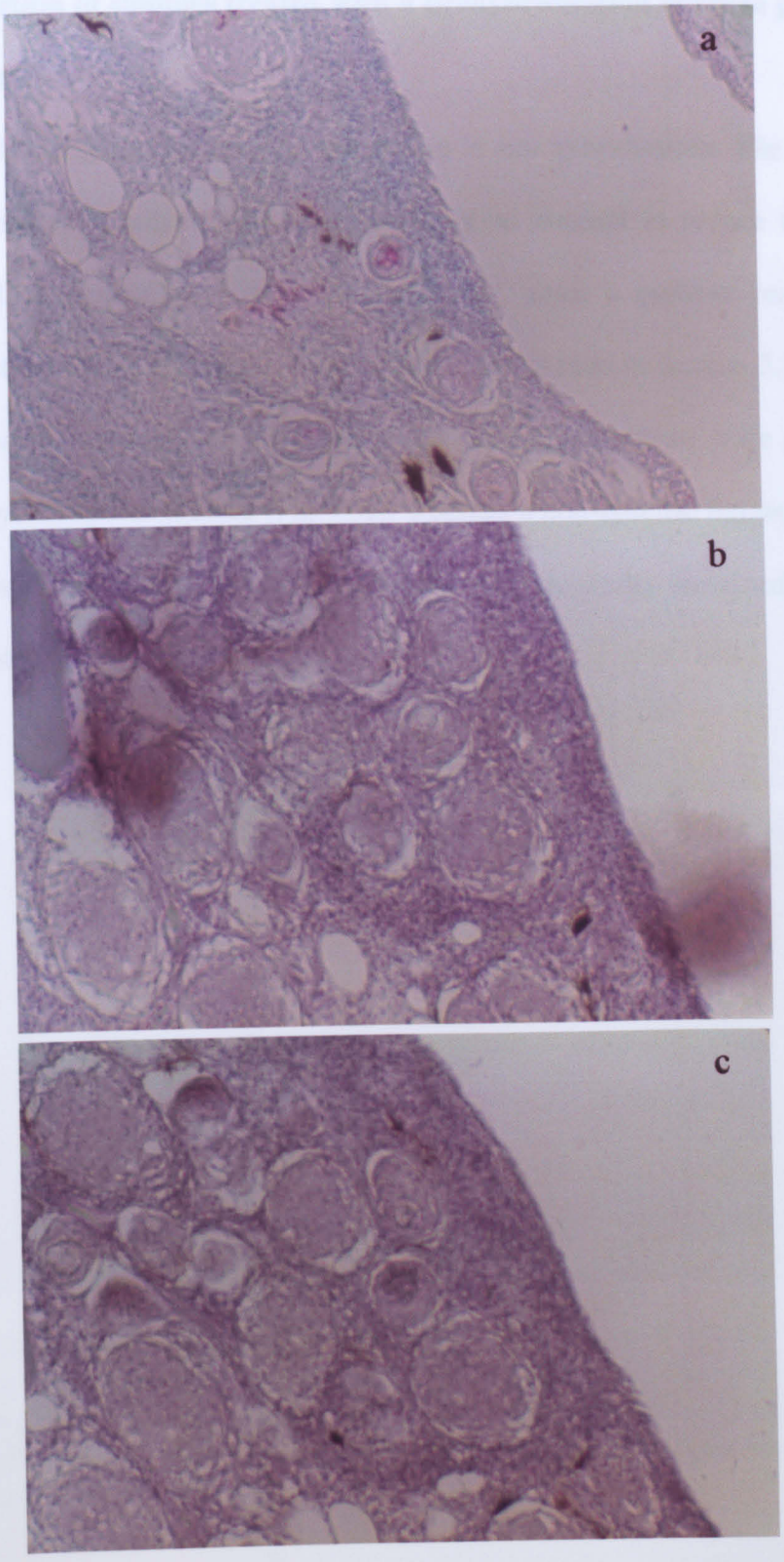

Fig 5.2 Examination of Siamese fighting fish (*Betta splendens*) kidney infected with *Mycobacterium* spp with *in situ* hybridisation and Ziehl-Neelsen (ZN) staining: (a) ZN staining; (b) *in situ* hybridisation analysis; (c) negative control for *in situ* hybridisation (x20).

5.3.2 Comparison of samples treated with a prehybridisation mixture prior to *in situ* hybridisation

Non-specific binding occurred sometimes during *in situ* hybridisation. The procedure of prehybridisation treatment was investigated in an attempt to reduce this effect. Infected goldfish tissues were used for this study, since a positive reaction was previously obtained with this tissue in the *in situ* hybridisation in Section 5.3.1. Clear, positively stained bacteria were obtained when the tissue sections were pre-treated with the prehybridisation mixture (Fig 5.3a), whereas a non-specific binding occurred when it was not used (Fig 5.3b). The negative control generally remained clear with both treatments (Fig 5.3c-5.3d).

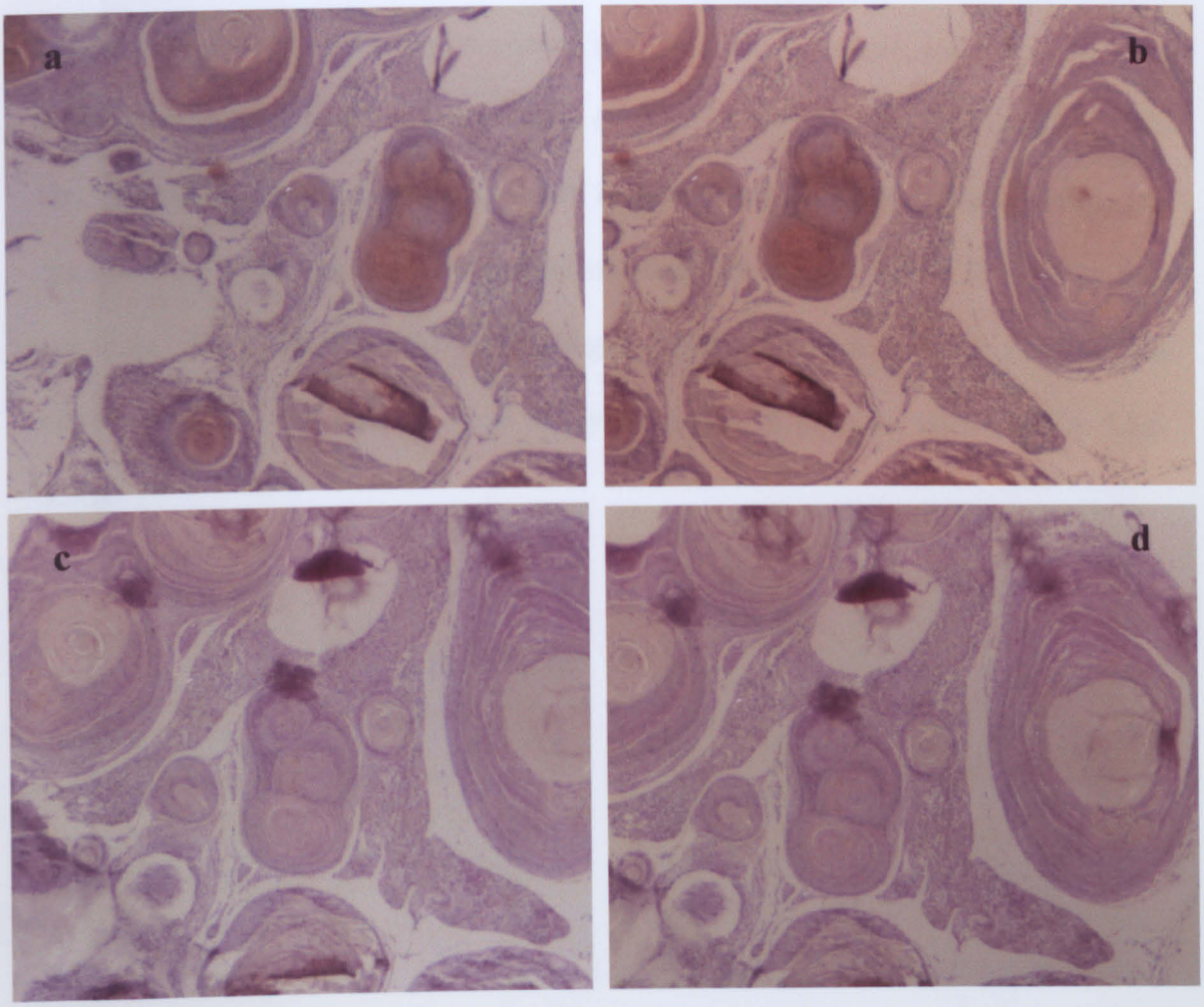

Fig 5.3 The effects of treatment of goldfish (*Carassius auratus*) kidney infected with *Mycobacterium* spp. with prehybridisation mixture prior to *in situ* hybridisation: (a) treatment with prehybridisation mixture; (b) no treatment with prehybridisation mixture; (c) treatment of negative control with prehybridisation mixture; (d) treatment of negative control without prehybridisation mixture (x 4).

5.3.3 Comparison of chromogens

Sections of infected rabbit fish spleen were used to compare the two different chromogen systems, DAB and HistoMark TrueBlue. A positive reaction stained brown with the DAB substrate, and bacteria were clearly seen within granulomas, while the negative control remained clear (Fig 5.4a-5.4d). Brown melanin, however, was also observed in the negative control and the positive sample, and it was difficult to differentiate between this and bacteria (Fig 5.4b and 5.4d). Positive staining with the HistoMark TrueBlue resulted in dark blue coloured bacteria appearing inside the granulomas, while the negative control remained clear (Fig 5.5a-5.5b). With this substrate, the melanin within macrophages remained brown both in positive and negative section and bacteria were easily identified in positive sections (Fig 5.5a-5.5b).

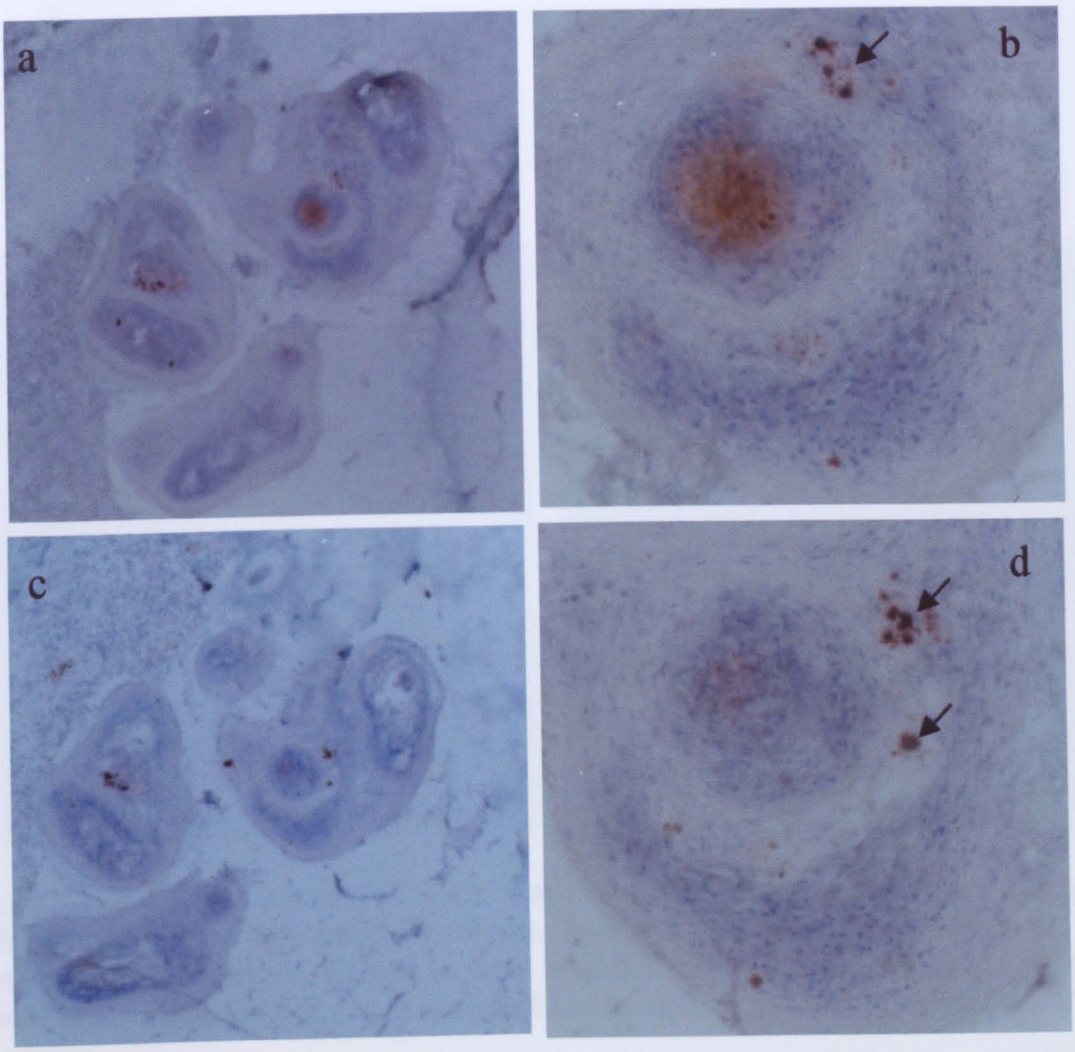

Fig 5.4 *In situ* hybridisation of spleen tissue of rabbit fish (*Siganus rivolatus*) infected with *Mycobacterium* spp. using DAB as a substrate. a: positive staining inside granuloma (magnification x10); b: positive staining inside granuloma and necrosis (arrow) (magnification x40); c: negative control (magnification x10); d: negative control and necrosis (magnification x40).

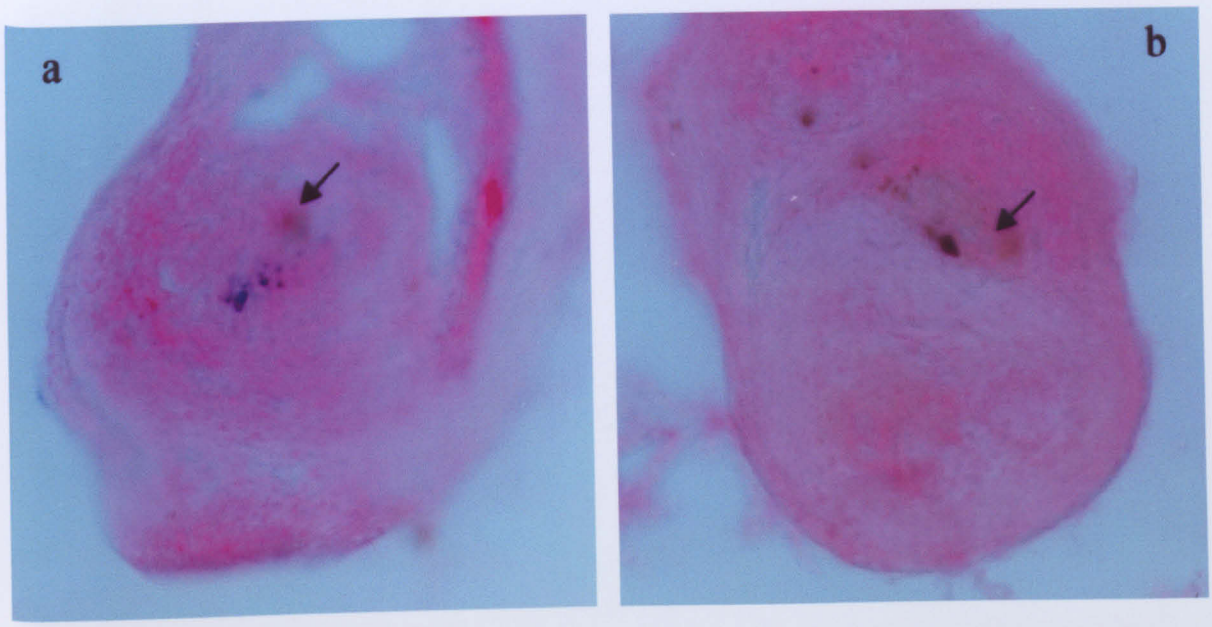

Figure 5.5 *In situ* hybridisation of spleen tissue of rabbit fish (*Siganus rivolatus*) infected with *Mycobacterium* spp. using HistoMark TrueBlue as a substrate solution. a: the bacteria inside granuloma stained in deep blue for the positive staining and necrosis stained in brown (arrow); b: negative control and necrosis (arrow) (magnification x 40).

5.4 Discussion and Conclusions

In situ hybridisation has proved a useful tool for both disease diagnosis and in gene studies, and over the course of the last 10 years a great deal of research has focused on improving the sensitivity and the specificity of the method. The sensitivity and efficiency of *in situ* hybridisation depends on a number of factors. These include the hybridisation conditions of the reaction, the nature of the probe construction, the type of label incorporated into the probe, how the tissues are prepared and the method used to visualise the signal (McQuaid & Allan, 1992; Chevalier *et al*, 1997).

The fixative used for tissue preservation is an important consideration since it may result in cross-linking of the nucleic acid into a complex matrix, thus hindering access of the probe to the target DNA. Fixatives commonly used for *in situ* hybridisation are 4% paraformaldehyde, 4% formaldehyde or 1% gluteraldehyde. However, the choice of fixative used depends on the purpose of the study. Wilkinson (1992) reported that stronger fixation yields a better preservation of cellular morphology. However, this increases the cross-linking, which in turn, lowers the accessibility of the probe to target DNA. In the present study, the samples were fixed in 10% neutral buffered formalin for 24 h. Neutral buffered formalin is a common fixative used in fish disease histology laboratories. Nuovo & Richart (1989) reported that 10% neutral buffered formalin was the preferred fixative for *in situ* hybridisation, whereas Hartman's and Bouin's fixative (71% saturated picric acid, 24% formalin, 5% acetic acid, pH 1.39) was not suitable. The Siamese fighting fish samples obtained from Thailand, which were positive to mycobacteriosis by bacterial culture (T. Somsiri, pers. comm.), showed no positive reaction in *in situ* hybridisation, although ZN staining was strongly positive. Perhaps, this is due to the lengthy fixation time (over 24 h) (T.

Somsiri, pers. comm.) in comparison to the other blocks examined which were fixed for 24 h., or else the target DNA had degraded. The samples were embedded in paraffin wax and kept at room temperature for three years period. Emson & Gait (1992) suggested that embedded samples should be kept at -70°C to stop DNA degradation. This may explain why some of the sections appeared positive in *in situ* hybridisation, but later appeared negative when re-examined a year later (data not shown). The fixation time is also crucial for good sample preparation. Greer *et al* (1991) reported that 1-24 h in 10% neutral buffered formalin was optimal for sample fixation. Nevertheless, Chevalier *et al* (1997) suggested that the tissue samples should be freshly prepared by cryostat and then fixed. This method results in good nucleic acid preservation, however, the morphology of the tissues is not well preserved. Clearly the method must be standardised so that consistent results are obtained and further studies are required to determined the time limits for fixation of mycobacteria-infected tissues in 10% buffered formalin.

Sometimes no positive reaction was obtained with *in situ* hybridisation, even although heavy bacterial infections were observed in granulomas with ZN staining. This may be because the target DNA was not accessible to the probes and hybridisation could therefore not take place (Bendayan, 1984; Chevalier *et al*, 1997). Pre-treatment of the tissue sections with proteolytic enzymes makes them more accessible to the probes by permeabilising the tissues thus helping to improve the efficiency of *in situ* hybridisation reaction (Lin *et al*, 1993; Mandry *et al*, 1993; Morey, 1995). In this study, proteinase K was used to permeabilise the sample, and still no reaction was observed with some of the Siamese fighting fish samples.

The results obtained showed very low non-specific binding as long as the prehybridisation was performed. However, it is worth noting that problems can occur due to endogenous biotin or endogenous peroxidase activity, resulting in false positive reactions (Kuhn, 1988; Kirkeby *et al*, 1993; Varma *et al*, 1994). Kirkeby *et al* (1993) reported that endogenous biotin was normally found in mitochondria-rich tissues such as cardiac and skeletal muscle, while Wilchek & Bayer (1990) suggested that avidin might bind to lectins which are normally present in liver, kidney, pancreas and brain (Duhamel & Whitehead, 1990).

Necrosis and melanin, present in some of the sections, made it difficult to determine which samples were actually positive when DAB was used as the substrate in the reaction, as both bacteria and melanin appeared brown and were similar in size. HistoMark TrueBlue, on the other hand, produced a blue product thus allowing easy identification of the bacteria within the granuloma, and distinction from melanin granules.

The oligonucleotides used as probes in the current study have been used as a set of primers in PCR for the detection of tuberculosis in man (Kox *et al*, 1994; 1995b), and in PCR for the identification of mycobacteria in fish (Chapter 4 & 6). The primers were biotinylated at the 5'ends and used as probes. This labelling position has been used by a number of groups (Mougin *et al*, 1990; Le Moine *et al*, 1990), however, Bardin *et al* (1993) suggested that probes labelled at the 3'-end with a biotin had higher sensitivity than 5'-end-labelling. The 5'-end-biotin labelling, however, has an advantage over the 3'-end-biotin labelling in that the former offers labelling with a

higher number of biotin molecules, which in turn increased the sensitivity of the reaction (Emson & Gait, 1992). Larsson & Hougaard (1990) suggested that adding 3-4 biotin residues at the 3'-end may confer optimal sensitivity, whereas Le Moine *et al* (1990) reported that 10 biotin residues at the 5'-end produced the same level of sensitivity as obtained with radioactive labelling. The probes used here were labelled with a single biotin, which may explain why some reactions appeared weak. To improve the sensitivity of the reaction, McQuaid & Allan (1992) suggested that multiple steps should be incorporated into the assay, using a Mab directed against biotin to amplify the reaction. Multiple-step antibody reactions appear to improve the hybridised signal. Recently, Speel *et al* (1999) suggested that the signal could be amplified using catalyzed reporter deposition (CARD) which incorporates tyramide labels.

It is interesting to compare the results obtained with IHC in Chapter 2 with the *in situ* hybridisation results described here. The monoclonal antibody (Mab 8F7) used in IHC performed well on formalin-fixed samples. The detection by IHC using Mab 8F7 appeared as sensitive as the results obtained here with *in situ* hybridisation and appeared unaffected by the length of fixation time. Biering & Bergh (1996) also reported that detection of virus in Atlantic halibut (*Hippoglossus hippoglossus*) by *in situ* hybridisation did not appear to be more sensitive than IHC. Mab 8F7, however, is species-specific for *M. marinum* and this limits screening of other *Mycobacterium* species by IHC. The *in situ* hybridisation was performed here using a genus probe to provide a wide range of screening. Moreover, Mab 8F7 was only specific to *M. marinum* species isolated from fish obtained from Thailand, Denmark, Germany and USA. The other Mabs available, which were developed to *Mycobacterium* spp

(Adams *et al*, 1995) did not effectively identify the bacteria in the infected fish tissue by IHC. Thus, *in situ* hybridisation has an advantage over IHC in that the oligonucleotide probes may be used detect a whole-range of *Mycobacterium* species. Since the Mabs specific to *M. fortuitum* or *M. chelonae* are not yet available for use as species specific probes in IHC, the *M. fortuitum* and *M. chelonae* specific probes used in the reverse cross blot hybridisation described in Chapter 4 may possibly identify individual *Mycobacterium* species by *in situ* hybridisation.

In conclusion, *in situ* hybridisation investigated here successfully identified *Mycobacterium* spp in sections of tissue from fish infected with mycobacteriosis using the labelled probes previously employed as primers for the PCR in Chapter 4. However, probes conjugated with different labels such as digoxigenin, radioactive isotopes and immunological probes should also be tried in order to improve the sensitivity and species specific probes used to increase the specificity of the reaction. Unfortunately, the reaction was not always reproducible possibly due to accessibility of the DNA, or degradation of the DNA due to over fixation, or for other reasons discussed above. Further work is therefore required to optimise the parameters of the *in situ* hybridisation assay used here to detect *Mycobacterium* spp. in fixed tissue samples.

Chapter 6

Environment and public health

6.1 Introduction

Of the 54 known species of *Mycobacterium* spp, many are able to infect humans. *M. tuberculosis* complex, consisting of *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG and *M. microti* (Kox 1995; Githui *et al*, 1999) is the most recognised, since these *Mycobacterium* spp. cause the lethal disease, tuberculosis, which is particularly prevalent in the developing world (Youmans, 1979; Peltola *et al*, 1994; Dye *et al*, 1998). Over 2.5 million people are reported to die annually from this disease (Murray *et al*, 1990). Leprosy, the etiological agent of which is *M. leprae*, is another serious disease caused by this genus of bacteria (Van der Vliet *et al*; 1993; Abulafia & Vignale 1999).

M. marinum, *M. fortuitum* and *M. chelonae* are known fish pathogens, causing mycobacteriosis in a wide range of fish (Nigrelli & Vogel, 1963; Giavenni, 1980; Arakawa & Fryer, 1984; Chinabut *et al*, 1990; Lansdell *et al*, 1993; Adams *et al*, 1996). They are known as atypical or nontuberculous mycobacteria which infected man (Wallace *et al*, 1993a; Escalonilla *et al* 1998; Bruno *et al*, 1998; Kullavinijaya, 1999; Hadjiliadis *et al*, 1999). Escalonilla *et al* (1998) described that cutaneous infections were commonly caused by *M. marinum*, *M. fortuitum*, *M. chelonae* and *M. ulcerans* and among these, *M. marinum* was the predominant isolate.

In Thailand, Kullavanijaya *et al* (1993) reported 18 cases of *M. marinum* cutaneous infections among people who have jobs or hobbies related to fish, whereas no cases of

M. fortuitum or *M. chelonae* infection were reported. Cases of *M. fortuitum* and *M. chelonae* infection were later reported, however, the patients did not have occupations or hobbies involving fish or water (Kullavanijaya, 1999).

M. fortuitum, a common mycobacterial species present in water and the environment, has also been reported as an atypical human pathogen since 1938 (Nigrelli & Vogel, 1963; Wayne & Sramek, 1992). Escalonilla *et al* (1998) reported nine cases of cutaneous infections where patients were infected by *M. fortuitum* and *M. chelonae*, however the sources of infection were not reported.

In this chapter, two types of fish farming were surveyed and samples collected included fish, water, sediment and fish food. Fish farmers were also interviewed. Some fish, which were sent for routine checking at laboratories at the IOA and AAHRI (the Aquatic Animal Health Research Institute, Bangkok), and water samples around Bangkok were also examined to determine the presence of mycobacteria.

Prior to analysis of the field samples from Thailand, DNA extraction was performed in order to investigate the capability of the method for recovering the DNA from samples, as DNA extraction was crucial for PCR in this study. As the study was set at the Royal Tropical Institute in Amsterdam, muscle tissue of fish (whiting) obtained from a local market was used, whereas the mycobacterial DNA samples were *M. tuberculosis* complex and *M. smegmatis* 1008.

In the study of DNA extraction, two target DNAs of 16S rDNA and IS*6110* were amplified in PCR. IS*6110* is a sequence of DNA present only in strains belonging to

the *M. tuberculosis* complex. The copy number of IS*6110* present in the genome varies among the different strains. There is no IS*6110* sequence present in the native genome of *M. smegmatis*, and thus native *M. smegmatis* was manipulated by inserting IS*6110* sequence into its genome obtaining a new strain called *M. smegmatis* 1008 (Kolk *et al*, 1994). The authors introduced the *M. smegmatis* 1008 as an internal control in the PCR.

The aims of the current chapter were to examine the presence of mycobacterial fish pathogens in the fish-farming environment and to investigate the prevalence of their transmission to man. A survey of Siamese fighting fish (*Betta splendens*) farms and snakehead fish (*Channa striata*) farms was performed for this purpose. Siamese fighting fish and snakehead fish are two of the most economically important fish in Thailand. The former is an ornamental fish, which is exported worldwide, while the latter is a popular cultured food fish.

Siamese fighting fish farms are a common family business in Nakhon Pathom Province, Thailand (Fig 6.1). The farmers collect the brood stock from the wild, buy them from local markets, obtain them from their neighbours or sometimes breed offspring themselves. The farms are normally set up in front and back yard areas, and consist of many flat-sided whisky bottles into which are placed individual fish (Fig 6.2 a). The water is changed every 3-5 days either by hand or using a locally designed tool, which can hold up to 20 bottles at the same time (Fig 6.2 b). Hand changing water or filling the apparatus involves the farmers handling the bottles unprotected. They often obtain cuts on their fingers from broken bottles, thus increasing the risk of developing a mycobacterial infection. The farmers also walk on the bottles with bare

feet as bottles are often stored tightly packed on the ground (Fig 6.2 a). Thus they also experience cuts on their feet.

Snakehead fish farming is also a common business in Suphan Buri Province, Thailand (Fig 6.1). Snakehead fish are cultured in earth ponds, normally located next to the farmer's house. The fingerling fish are obtained from market or collected from the wild. The fish are generally fed with mixed trash fish or processed offal for a six to seven month period during which time the water is regularly changed using an electronic pump. The water is obtained from nearby rivers or irrigation canals, while the water from ponds is allowed to drain back into the river. The farmers do not generally have direct contact with the water or the fish until final harvesting.

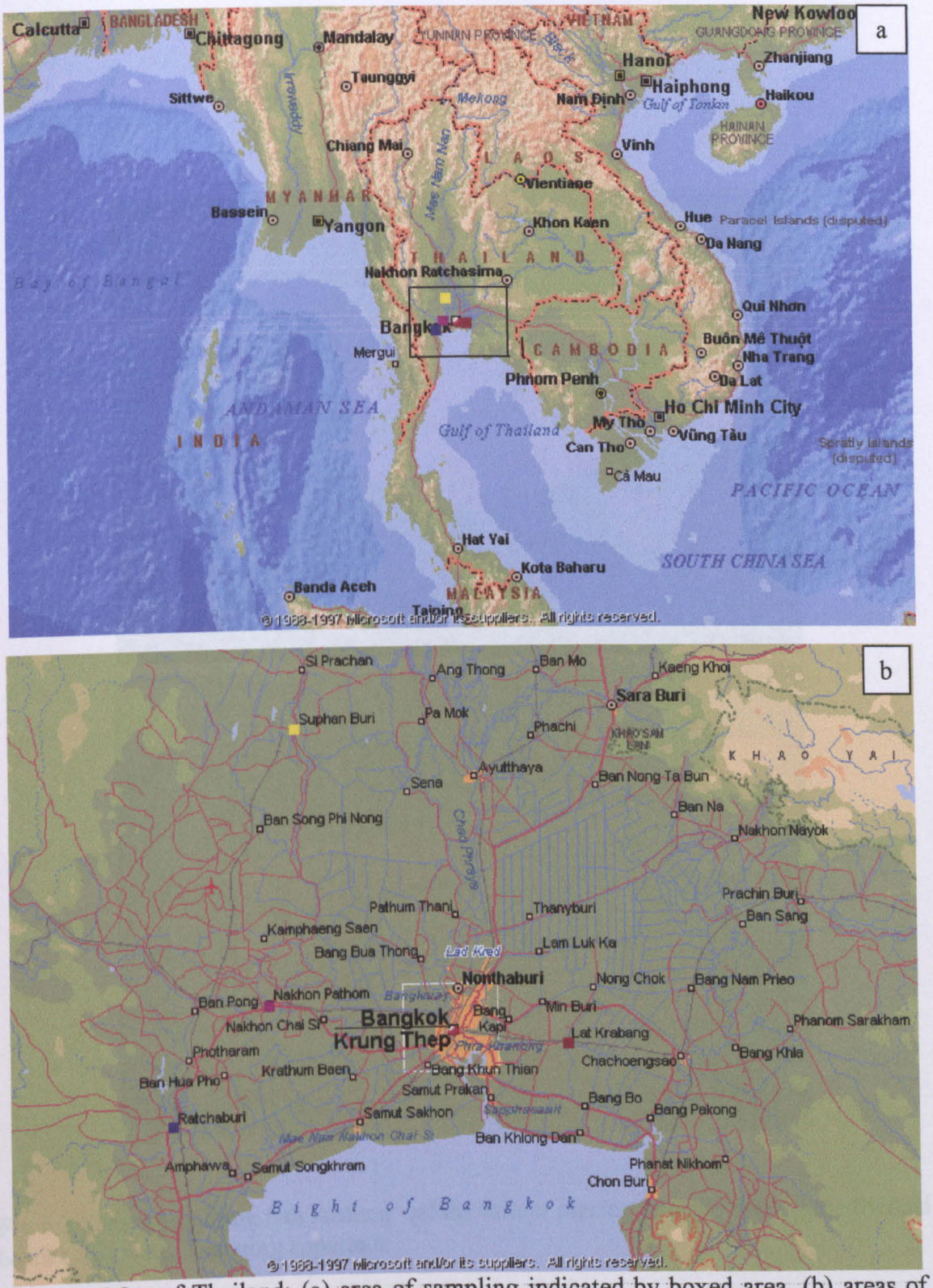

Fig 6.1 Map of Thailand: (a) area of sampling indicated by boxed area, (b) areas of fish samplings: Bangkok (red round bullet), Suphan Buri (yellow), Nakhon Pathom (purple), Ratchaburi (blue), Lat Krabang (maroon).

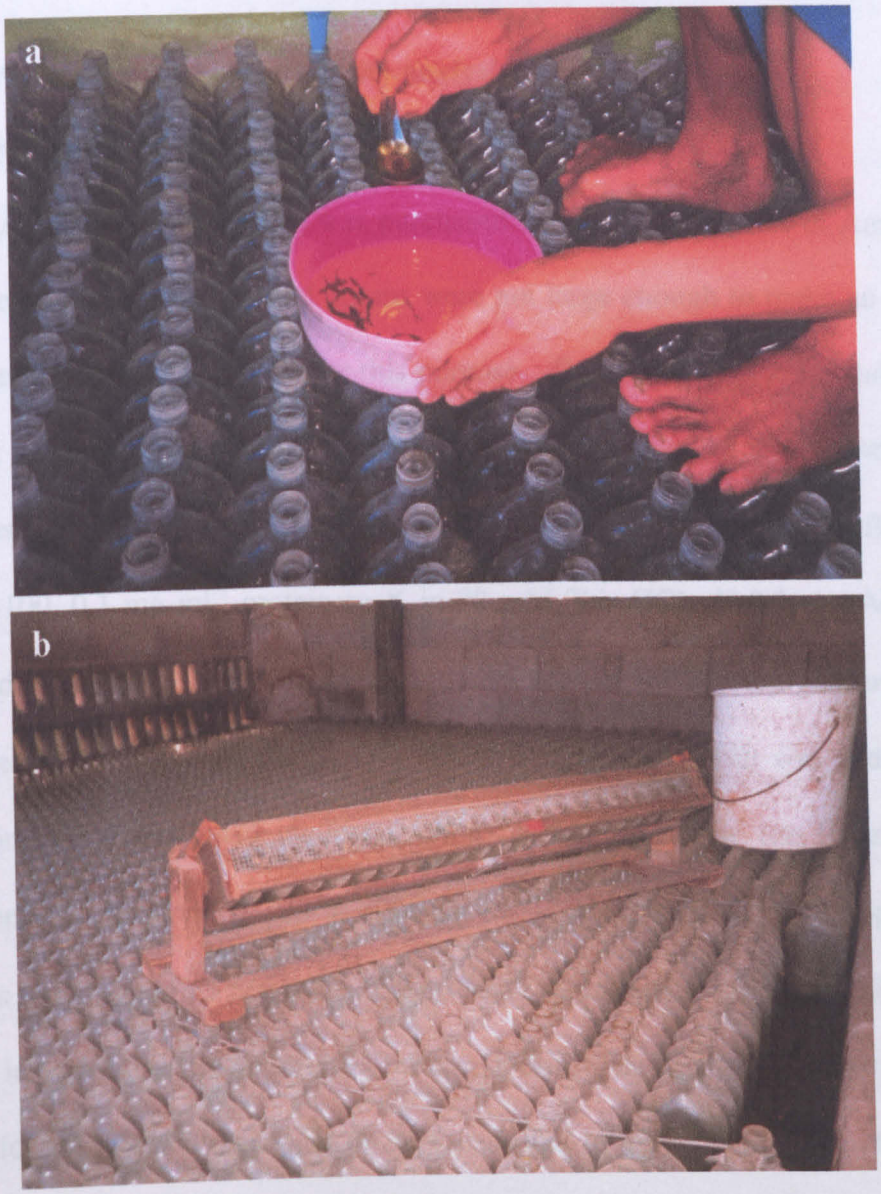

Fig 6.2 Siamese fighting fish farming: (a) the farmer placing juvenile fish into individual bottles; (b) water changing apparatus.

6.2 Materials and Methods

DNA extraction methods were examined in order to determine their capacity for recovering DNA from field samples, and in particular fish tissue samples.

6.2.1 DNA purification and sensitivity analysis

6.2.1.1 DNA purification

Whiting (*Merlangius merlangus*) was purchased from a fishmonger's in Amsterdam, The Netherlands. As the visceral organs were not large enough to make an identical replication, the muscle was used for this purpose. The muscle of the animal was cut into 1.5 g pieces and placed into individual 15 ml sterile tubes. Samples were spiked with either 10^3, 10^2, 10 or no *M. bovis* BCG, and 300 µl of digestion buffer (0.5% Triton X-100, 0.1 mg ml^{-1} proteinase K in 20 mM Tris-HCl pH 8.3, 1 mM EDTA) was added to each tube. The samples were incubated at 60°C overnight, or until the tissues were completely digested. Tris-saturated phenol (pH 8.0) was added to the suspension at 1:1 (v:v) and incubated on a high speed mixer for 10 min. The suspension was then centrifuged at 3000 xg for 10 min at 20°C. The upper liquid phase was transferred to a new tube. Chloroform was again added to the solution 1:1 (v:v) and incubated on the mixer for a further 10 min. The mixture was centrifuged at 3000 xg for 10 min and the upper phase transferred to a new tube. The chloroform extraction was repeated, and the upper phase was then precipitated with NaCl and iso-propanol at a concentration of 0.2 M and 40% respectively. The solution was incubated at 4°C for 1 h and then centrifuged at 3000 xg for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol of -20°C without resuspending the pellet. The supernatant was removed and the pellet was treated, as previously described by Boom *et al* (1990). Briefly, 1 ml of lysis buffer

[5M guanididine thiocyanate (Acros), 1% Triton X-100, 50 mM Tris-HCl (pH 6.4), 20 mM EDTA] and 20 µl of diatom suspension [10 g Celite (Acros), 50 ml of H_2O, 500 µl of HCl] were added directly to the pellet. The mixture was incubated on a high-speed mixer for 10 min, vortexed for 5 sec and then centrifuged 12000 xg for 15 sec. The supernatant was removed and the pellets were washed twice with 1 ml of washing buffer [5 M GuSCN in 0.1 M Tris-HCl (pH 6.4)] as described above. The supernatant was removed and then washed twice with 70% ethanol, and once with acetone. After removing the supernatant, the pellet was dried at 56°C in a water bath. The DNA was then eluted from the diatom by incubating the pellet with 100 µl of Tris-EDTA buffer (TE: 1 mM EDTA in10 mM Tris HCl, pH 8.0) at 56°C for 20 min.

6.2.1.2 Multiplex PCR assay

Ten µl of each DNA solution from Section 6.2.1.1 was added to the PCR mixture set up in duplicate. One hundred fg of *M. smegmatis* 1008 DNA was added to one of the replicates as a positive control before the amplification was performed. A series of dilutions of *M. tuberculosis* DNA was made corresponding to 100 fg, 50 fg, and 10 fg, which were used to determine the detection limit of the PCR. Two target DNA were simultaneously amplified: IS*6110*, a specific sequence belonging to the *M. tuberculosis* complex, which was amplified by primers Pt18-F (5'-GAACCG TGAGGGCATCGAGG-3') and INS2-R (5'-GCGTAGGCGTCGGTGACAAA-3') and 16S rDNA which was amplified by primers pMyc14-F (5'-GRGRTA CTCGAGTGGCGAAC-3') and pMyc7-R (5'-GGCCGGCTACCCGTCGTC-3'). Primers INS2-R, pMyc14-F and pMyc7-R were labelled with biotin at the 5'end. The basic PCR mixture was similar to the one described in Section 4.2.2.3. The amplification was carried out as described in Section 4.2.2.3.

6.2.1.3 PCR product identification

The amplified DNA were identified both by gel electrophoresis (Section 4.2.2.4) and reverse cross blot hybridisation (Section 4.2.3). Fourteen probes (Table 6.1) were used to analyse the PCR product in the reverse cross blot hybridisation.

Table 6.1 Oligonucleotide sequences for hybridisation of 16S rDNA PCR product

Code	bp-position	Species specific	Nucleotide sequence[a]
pTub1	168-148	*M. tuberculosis* complex	5'-ACCACAAGACATGCATCCCG-3'
pAvi7	167-146	*M. avium, M. paratuberculosis*	5'-CCAGAAGACATGCGTCTTGAG-3'
pInt5	169-149	*M. intracellulare*	5'-CACCTAAAGACATGCGCCTAA-3'
pInt7	169-147	*M. intracellulare*	5'-CACCAAAAGACATGCGTCTAA-3'
pKan7	164-144	*M. kansasii, M. scrofulaceum, M. gastri, M. simiae*	5'-CAAGGCATGCGCCAAGTGGT-3'
pXen1	188-148	*M. xenopi*	5'-ACCACCCCACATGCGCAGAA-3'
pFor1	168-148	*M. fortuitum, M. senegalense*	5'-ACCACACACCATCAAGCGCG-3'
pChe3	167-146	*M. chelonae*	5'-CCACTCACCATGAAGTGTGTG-3'
pGen1	167-148	*M. genavense*	5'-CCACAAAACATGCGTTCCGTG-3'
pGor5	156-135	*M. gordonae*	5'-TGTGTCCTGTGGTCCTATTCG-3'
pMar2	148-168	*M. marinum, M. ulcerans*	5'-CGGGATTCATGTCCTGTGGT-3'
Pt3	IS*6110*	*M. tuberculosis* complex	5'-GAACGGCTGATGACCAAACT-3'
pSme3	158-139	*M. smegmatis*	5'-CATGCGACCAGCAGGGTGTA-3'
pMyc5a	201-184	*Mycobacterium* spp.	5'-GGGCCCATCCCACACCGC-3'

(a) Kox *et al*, 1995b & 1997

6.2.2 Survey of fish farming and sample analysis

A survey of snakehead fish and Siamese fighting fish farms was carried out, whereby fish and fish farm environmental samples from the farms were collected and analysed for the presence of *Mycobacterium* spp. Farmers were also interviewed using a questionnaire (see Appendix 2) to establish the incidence of *Mycobacterium* infection among the farmers. Biopsies were later taken by a clinician from some of the farmers and analysed by Dr. Arend H.J. Kolk (the Royal Tropical Institute, Amsterdam).

6.2.2.1 Fish samples

Fifty-four samples of snakehead fish were randomly collected from 7 farms in three different regions of Thailand, illustrated in Fig 6.1, and outlined in Table 6.2. The internal organs of the fish were removed and stored in sterile eppendorfs at -70°C.

Twenty-seven samples of Siamese fighting fish were collected from 9 farms at sites where farmers were known to have experienced lesions on their skin (Table 6.3). Eighty-eight samples of Siamese fighting fish were also collected from 6 export aquaria around Bangkok, Thailand (Table 6.4). The fish were collected in sterile eppendorfs and stored at –70 °C until analysis.

6.2.2.2 Environmental samples

Samples of water from the inlet, outlet and the pond itself were collected from different snakehead fish farms and samples of sediment were also taken (Table 6.2). Water samples were also collected from 9 Siamese fighting fish farms in total (Table 6.3). Environmental samples were further collected from random Siamese fighting fish farms around Bangkok and included moina (mosquito larvae), mosquito, reservoir water (inlet water) and bottle water (Table 6.5).

6.2.2.3 Clinical tissue samples

Farmers and co-workers of both Siamese fighting fish and snakehead fish farms were interviewed (see Appendix 2) and any lesions present on their skin observed. Some biopsies of the lesions were taken by a doctor from a hospital in Bangkok (Dr. Preeya Kullavanijaya) and were later analysed at the Institute of Dermatology, Bangkok,

Thailand and by Dr. A. Kolk at the Royal Tropical Institute, Amsterdam, The Netherlands (Table 6.6).

6.2.2.4 DNA extraction from samples

6.2.2.4.1 DNA isolation from fish samples

Internal organs including liver, spleen, kidney and heart were digested with 1:1 (w:v) digestion buffer (0.5% Triton X-100, 0.1 mg ml^{-1} proteinase K in 20 mM Tris-HCl pH 8.3, 1 mM EDTA) by incubating at 60°C overnight, or until the tissues were completely digested. Then the following steps were carried out as described in Section 6.2.1.1.

6.2.2.4.2 DNA isolation from fish pond water

Fifty ml of water was centrifuged at 3000 xg for 30 min and the supernatant was discarded. The pellet was treated as described by Boom *et al* (1990) (Section 6.2.1.1).

6.2.2.4.3 DNA isolation from pond sediment

Twenty g of pond sediment was re-suspended in 20 ml of sterile distilled water. Tris-saturated phenol (pH 8.0) was added at a ratio of 1:1 (v:v) to the suspension and incubated on a high-speed mixer for 10 min. The tube was then centrifuged at 3000 xg for 10 min. The upper phase was transferred to a new tube and (v:v) phenol chloroform was added at a ratio of 1:1 before incubating for a further 5 min with mixing. The suspension was centrifuged at 3000 xg for 5 min and the upper phase removed to a new tube. Chloroform was added 1:1 (v:v) to the solution and again incubated for 10 min on the mixer. The tube was centrifuged at 3000 xg for 3 min and the upper layer removed to a new tube. DNA was precipitated from the solution

with the addition of 0.2 M NaCl and 40% 2-propanol (final concentration) overnight at 4°C. The tube was centrifuged at 3000 xg for 20 min and the supernatant was then discarded. The pellet was purified by the method described by Boom *et al* (1990) (Section 6.2.1.1).

6.2.2.5 Polymerase chain reaction

Ten µl of the extracted DNA was added to the PCR mixture and amplified by a primer set of pMyc14-F and pMyc7-R as described in Section 4.2.2.3.

6.2.2.6 Reverse cross blot hybridisation

The PCR products were analysed by reverse cross blot hybridisation with 4 probes of pMyc5a, pMar2, pChe 3 and pFor1, which respond with genus *Mycobacterium* spp., *M. marinum*, *M. chelonae*, and *M. fortuitum* respectively, as described in Section 4.2.3.3

6.3 Results

6.3.1 DNA extraction and detection limit

Agarose gel electrophoresis analysis

The primers Pt18-F and INS2-R amplified IS*6110* sequence of strains belonging to *M. tuberculosis* complex to yield a product at 249 bp, whereas amplification of IS*6110* of *M. smegmatis* 1008 yielded a product of 305 bp. The lowest level detectable using the DNA extraction methods was 10^3 cells of *M. bovis* BCG (Fig 6.3). The 249 bp product of *M. bovis* BCG at 10^3 cells is represented in lane 8, while no product can be found in lanes 2, 4 and 6 where lower levels of bacteria were analysed (Fig 6.3). Lanes 1, 3, 5 and 7 show the amplified IS*6110* product of *M. smegmatis* 1008 at 305

bp. When only *M. smegmatis* 1008 was used for a positive control, two products were seen at 305 bp and 208 bp which were the amplified product of IS*6110* and 16S rDNA respectively (lane 9). The detection limit of the PCR multiplex was 10 fg DNA of *M. tuberculosis*, which is equivalent to 2 cells as showed at lane 12 (Fig 6.3).

Reverse Cross blot hybridisation analysis

Most of the probes detected the 16S rDNA amplicon, but only probe Pt 3 was able to detect the IS*6110* amplicon. *M. smegmatis* 1008 DNA containing both 16S rDNA and IS*6110* sequenes was positive with all three probes: genus probe (pMyc5a), species probe (pSme3) and IS*6110* probe (Pt3) as shown in lane 1, 3, 5, 7 and 9 (Fig 6.4). The limit of the DNA extraction methods was also shown to be 10^3 *M. bovis* BCG by reverse cross blot hybridisation (Fig 6.4). At 10^2 cells of *M. bovis* BCG, the positive band was only faintly visible with the genus probe (pMyc5a) as shown in lane 6, but bands were visualised with both the species specific probe (pTub1) and the genus probe (pMyc5a) when 10^3 cells of *M. bovis* BCG were used (lane 8). The detection limit of IS *6110* probe (Pt3) was at 10 fg of *M. tuberculosis* DNA, equivalent to 2 cells (lane 12), while the limit detection of 16S rDNA amplicon detected by pMyc5a and pTub1 was 50 fg of the DNA, equivalent to 10 cells of *M. tuberculosis* (lane 11).

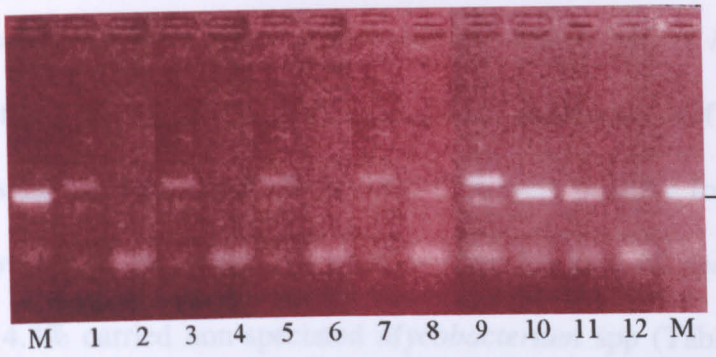

Fig 6.3 Agarose gel electrophoresis analysis of PCR products from DNA extraction from whiting (*Merlangius merlangus*) spiked with *M. bovis* BCG. Lanes: (M) molecular weight marker (249 bp), (1) DNA extracted from fish muscle without spiking and 100 fg of *M. smegmatis 1008* DNA, (2) DNA extracted from fish tissue, (3) DNA extracted from fish tissue spiked with 10 cells of *M. bovis* BCG and 100 fg of *M. smegmatis 1008* DNA, (4) DNA extracted from fish tissue spiked with 10 cells of *M. bovis* BCG, (5) DNA extracted from fish tissue spiked with 100 cells of *M. bovis* BCG and 100 fg of *M. smegmatis* 1008 DNA, (6) DNA extracted from fish tissue spiked with 100 cells of *M. bovis* BCG, (7) DNA extracted from fish tissue spiked with 1000 cells of *M. bovis* BCG and 100 fg of *M. smegmatis 1008* DNA , (8) DNA extracted from fish tissue spiked with 1000 cells of *M. bovis* BCG, (9) 100 fg of *M. smegmatis 1008* DNA, (10) 100 fg of *M. tuberculosis* DNA, (11) 50 fg of *M. tuberculosis* DNA, (12) 10 fg of *M. tuberculosis* DNA.

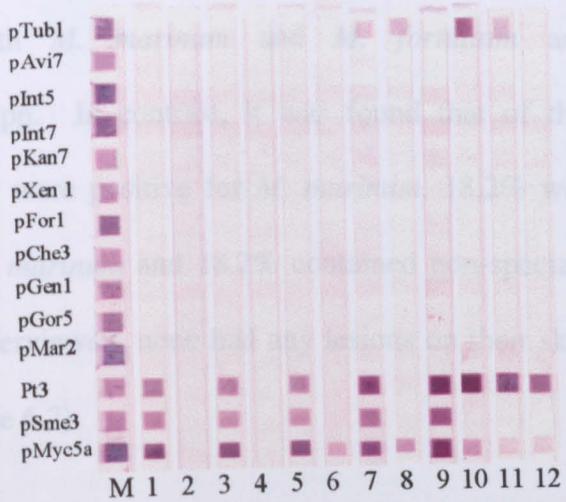

Fig 6.4 PCR-reverse cross blot hybridisation of DNA extraction from whiting (*Merlangius merlangus*) spiked with *M. bovis* BCG. Lanes: (M) DNA pool, (1) DNA extracted from fish muscle without spiking and 100 fg of *M. smegmatis 1008* DNA, (2) DNA extracted from fish tissue, (3) DNA extracted from fish tissue spiked with 10 cells of *M. bovis* BCG and 100 fg of *M. smegmatis 1008* DNA, (4) DNA extracted from fish tissue spiked with 10 cells of *M. bovis* BCG, (5) DNA extracted from fish tissue spiked with 100 cells of *M. bovis* BCG and 100 fg of *M. smegmatis* 1008 DNA, (6) DNA extracted from fish tissue spiked with 100 cells of *M. bovis* BCG, (7) DNA extracted from fish tissue spiked with 1000 cells of *M. bovis* BCG and 100 fg of *M. smegmatis 1008* DNA , (8) DNA extracted from fish tissue spiked with 1000 cells of *M. bovis* BCG, (9) 100 fg of *M. smegmatis 1008* DNA, (10) 100 fg of *M. tuberculosis* DNA, (11) 50 fg of *M. tuberculosis* DNA, (12) 10 fg of *M. tuberculosis* DNA.

6.3.2 Snakehead fish farms

The results revealed that there were no mycobacteria-free farms, as *Mycobacterium* spp. were identified either/and in fish, water and sediment. Of the fifty-four snakehead fish sampled from the 19 farm sites, 13% were positive for *M. fortuitum*, 5% were positive for *M. marinum*, 5% were positive for both *M. fortuitum* and *M. marinum*, and 14.8% carried non-speciated *Mycobacterium* spp (Table 6.2). Of 16 inlet waters sampled, 56.3% were positive for *M. fortuitum*, 12.5% were positive for *M. marinum*, 6.3% contained both *M. marinum* and *M. fortuitum* and 12.5% contained non-speciated *Mycobacterium* spp. When the pond water was analysed, 50% of the 12 samples were found to be positive for *M. fortuitum*, 8.3% for *M. marinum* and 25% for both *M. marinum* and *M. fortuitum*. The 15 outlet water samples showed a similar trend with 40% positive for *M. fortuitum*, 6.7% positive for *M. marinum*, 6.7% positive for both *M. marinum* and *M. fortuitum* and 20% non-speciated *Mycobacterium* spp. In contrast, it was found that of the 11 sediment samples examined, 36.4% were positive for *M. marinum*, 18.2% were positive for both *M. fortuitum* and *M. marinum* and 18.2% contained non-speciated *Mycobacterium* spp. Of 45 farmers interviewed, none had any lesions on their skin or previous history of skin lesions (Table 6.2).

Table 6.2 Identification of *Mycobacterium* spp. by PCR-reverse cross blot hybridisation in snakehead fish farm environments from different areas of Thailand

Farms[a]	Fish samples[b]										Water samples			Sediment samples	Farmers[c]				
	1	2	3	4	5	6	7	8	9	10	Inlet	Pond	Outlet		1	2	3	4	5
1	M	-	F	F	-	M	F	F	F	Myc	nd	nd	nd	nd	-	-	nd	nd	nd
2	M,F	-	-	-	Myc	-	Myc	-	-	-	F	M, F	nd	nd	-	-	nd	nd	nd
3	M	-	-	Myc	-	-	-	Myc	Myc	-	F	nd	M, F	nd	-	-	nd	nd	nd
4	-	-	nd	nd	nd	nd	nd	nd	nd	nd	F	nd	-	nd	-	-	-	-	-
5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	F	nd	F	nd	-	-	-	-	-
6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	nd	-	nd	-	-	nd	nd	nd
7	-	-	nd	nd	nd	nd	nd	nd	nd	nd	F	nd	-	nd	-	-	-	nd	nd
8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	F	M, F	F	-	-	-	nd	nd	nd
9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Myc	F	Myc	M	-	-	nd	nd	nd
10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	F	F	F	-	-	-	nd	nd	nd
11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-	Myc	M, F	-	-	nd	nd	nd
12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	F	M, F	M	M	-	-	nd	nd	nd
13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Myc	F	-	M	-	-	nd	nd	nd
14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	M, F	-	Myc	M, F	-	-	nd	nd	nd
15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	M	F	F	Myc	-	-	nd	nd	nd
16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	M	F	F	M	-	-	nd	nd	nd
17	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	F	F	F	Myc	-	-	nd	nd	nd
18	M,F	-	-	-	Myc	-	Myc	-	-	-	nd	M	nd	-	-	-	nd	nd	nd
19	-	-	-	M,F	-	F	F	-	-	-	nd	nd	nd	nd	-	-	nd	nd	nd

(a) Farms 1-17 were located in Ampur Sonpinong, Suphan Buri Province; farm 18 was located in Ampur Kampangsan, Nakhon Pathom Province; farm 19 was located in Ampur Lat Krabang, Bangkok Thailand

(b) M: *M. marinum*; F: *M. fortuitum*; Myc: *Mycobacterium* spp; -: no *Mycobacterium* spp identified; nd: no data obtained

(c) Interview and examination of farmers: -: no lesion observed; nd: no data

6.3.3 Siamese fighting fish

Samples from the Siamese fighting fish farms were collected from both fish farms and export ornamental fish aquaria.

6.3.3.1 Fish farms

The results revealed that among 9 sampled farms there were no mycobacteria-free farms. *Mycobacterium* spp were detected either in fish or in water or both samples. Of 27 fish samples collected from 9 farms (Table 6.3), 29.6% were positive for *M. fortuitum*, 11.1% were positive for *M. marinum* and 11.1% were non-speciated *Mycobacterium* spp. With the water samples obtained from 9 bottles containing fish, 77.8% were positive for *M. fortuitum* and 11.1% were detected both *M. fortuitum* and *M. marinum*. Very few inlet and outlet water samples were obtained from these farms. Of the twenty-six farmers interviewed, 15.4% had lesions present on their skin ranging from fingers, hands, wrists, arms, ankles, feet and knees, while another 11.5% had previously had lesions and had since been cured. The lesions found were nodular-like, as illustrated in Fig 6.5a- 6.5d.

Table 6.3 Identification of *Mycobacterium* spp in Siamese fighting fish (*Betta splendens*) and their environments, analysed by PCR-reverse cross blot hybridisation

Farms [a]	Fish number [b]					Water samples			Farmers [c]					
	1	2	3	4	5	Inlet	Bottle	Outlet	1	2	3	4	5	6
1	-	-	-	-	-	nd	F	F	-	-	-	-	-	-
2	M	-	Myc	F	F	nd	F	nd	-	+	nd	nd	nd	nd
3	M	-	nd	nd	nd	F, M	F	-	+	-	nd	nd	nd	nd
4	Myc	-	nd	nd	nd	nd	F, M	nd	-	-	+/-	nd	nd	nd
5	Myc	-	-	nd	nd	nd	F	nd	+/-	-	-	nd	nd	nd
6	F	-	nd	nd	nd	nd	F	nd	+	-	nd	nd	nd	nd
7	F	M	-	nd	nd	nd	F	nd	-	+	nd	nd	nd	nd
8	F	nd	nd	nd	nd	-	-	-	-	-	nd	nd	nd	nd
9	F	F	-	F	nd	nd	F	nd	-	+/-	-	-	nd	nd

(a) Samkwaipuke District, Nakhon Pathom Province, Thailand

(b) M: *Mycobacterium marinum*; F: *M. fortuitum*; Myc: *Mycobacterium* spp; nd: no data; - is negative

(c) Observation of lesions on skin of the farmers: - no lesion; + lesion present on skin; +/- previous history of lesion(s).

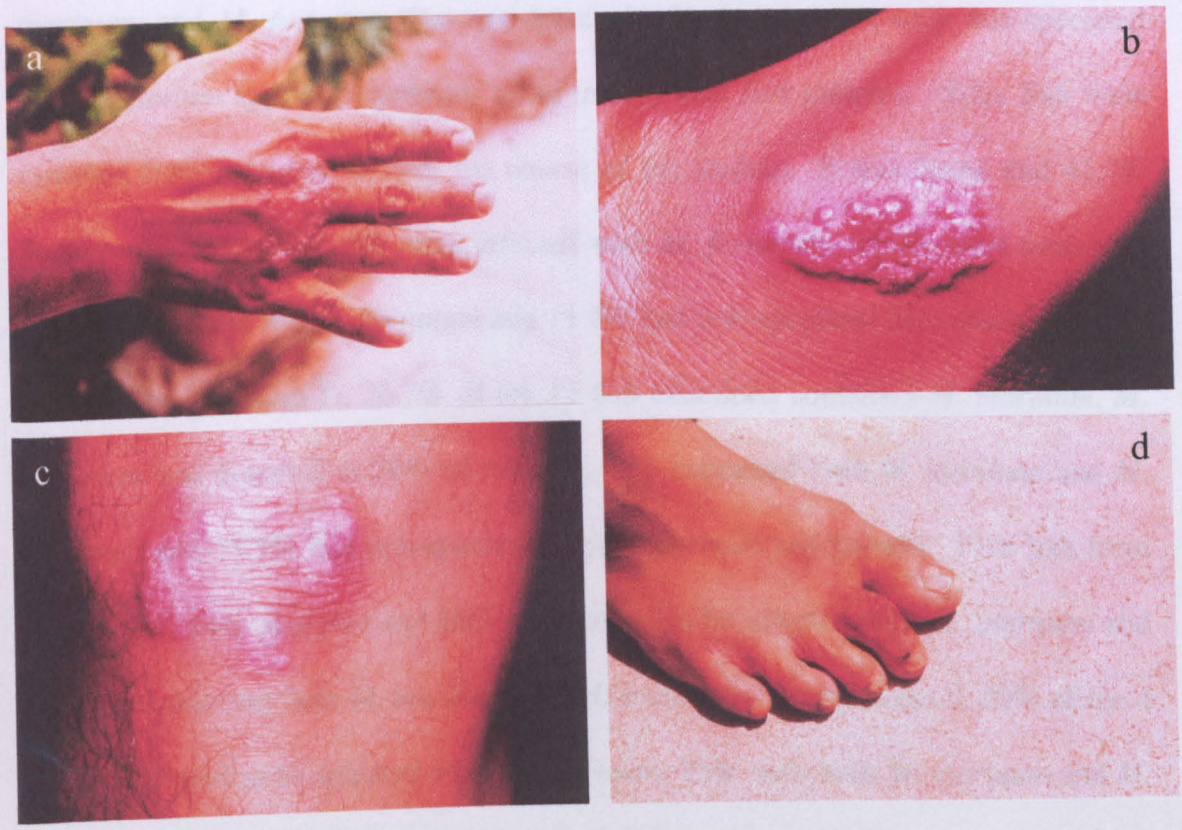

Fig 6.5 Lesions present on the skins of Siamese fighting fish farmers: (a) lesion on a hand; (b) lesion on an ankle; (c) lesion on a knee; (d) lesion on a toe (Courtesy of Dr. S. Chinabut)

6.3.3.2 Aquaria exporting ornamental fish

Eighty-eight samples of Siamese fighting fish were collected from 7 aquaria in Bangkok, namely A, B, C, D, E, F, and G (Table 6.4). The majority of samples (39.8%) contained *M. marinum*, 7.9% had *M. fortuitum* present, 20.5% contained both *M. marinum* and *M. fortuitum*, 13.6% contained non-speciated *Mycobacterium* spp and 18.2% were free of *Mycobacterium* spp. No water samples were collected or analysed from the aquaria. Two samples from aquarium A were infected with *M. marinum*, and *M. fortuitum* was not detected. In aquarium B, only non-speciated *Mycobacterium* spp. was detected, again in only two fish samples. Sixty fish were sampled from aquarium C over the course of the studies. It was found that 41.7% contained *M. marinum*, 5% were infected with *M. fortuitum*, 23.3% were contained both *M. marinum* and *M. fortuitum* and 11.7% had non-speciated *Mycobacterium* spp present. In aquarium D, 26.7% of the 15 fish examined contained *M. marinum*, *M. fortuitum* was detected in 20% of the fish, 13% contained both *M. marinum* and *M. fortuitum* and 13.3% had non-speciated *Mycobacterium* spp present. Five fish from aquarium E were analysed and 60% were found to be infected with *M. marinum* and 20% were detected both *M. marinum* and *M. fortuitum*. At aquarium F, 50% of the 4 fish were found to be infected with *M. marinum*, 25% with both *M. marinum* and *M. fortuitum* and 25% were non-speciated *Mycobacterium* spp. In general, *Mycobacterium* spp presented in all aquaria studied here.

Table 6.4 Identification of *Mycobacterium* spp. in Siamese fighting fish from aquaria around Bangkok, Thailand by PCR-reverse cross blot hybridisation

Aquarium	Sample number[a]	Reaction on reverse cross blot hybridisation[b]			
		pMar2[c]	pFor1	pChe3	pMyc5a
A	I/1	+	-	-	+
	I/2	+	-	-	+
B	I/1	-	-	-	+
	I/2	-	-	-	+
C	I/1	+	+	-	+
	II/1	+	-	-	+
	II/2	+	-	-	+
	II/3	+	-	-	+
	II/4	+	-	-	+
	II/5	+	-	-	+
	III/1	+	+	-	+
	III/2	+	+	-	+
	III/3	-	-	-	-
	III/4	+	+	-	+
	IV/1	+	-	-	+
	IV/2	+	+	-	+
	IV/3	+	-	-	+
	IV/4	+	+	-	+
	V/1	+	-	-	+
	V/2	+	+	-	+
	V/3	+	-	-	+
	V/4	-	-	-	-
	V/1	+	-	-	+
	V/2	-	-	-	-
	V/3	+	-	-	+
	V/4	-	-	-	+/-
	V/5	+	+	-	+
	V/6	+	-	-	+
	V/7	+	-	-	+
	V/8	-	-	-	+/-
	VI/1	+	+	-	+
	VI/2	+	+	-	+
	VI/3	+	-	-	+
	VI/4	+	+	-	+
	VI/5	+	-	-	+
	VI/6	-	-	-	+/-
	VI/7	+	-	-	+
	VII/1	-	-	-	+/-
	VII/2	+	-	-	+
	VII/3	+	-	-	+
	VII/4	-	+	-	+
	VII/5	+	+	-	+
	VII/6	+	-	-	+

Aquariums	Sample number[a]	Reaction on reverse cross blot hybridisation[b]			
		pMar2[c]	pFor1	pChe3	pMyc5a
C	VIII/1	-	-	-	-
	VIII/2	+	-	-	+
	VIII/3	-	+	-	+
	VIII/4	+	-	-	+
	VIII/5	-	-	-	-
	VIII/6	+	-	-	+
	VIII/7	-	-	-	-
	VIII/8	-	-	-	-
	VIII/9	-	-	-	-
	IX/1	-	-	-	-
	IX/2	-	-	-	-
	IX/3	-	-	-	-
	IX/4	-	+	-	+
	IX/5	+	-	-	+
	IX/6	+	+	-	+
	IX/7	-	-	-	+
	IX/8	+	-	-	+
	IX/9	+	-	-	+
	IX/10	-	-	-	+/-
	IX/11	+	+	-	+
	IX/12	-	-	-	+/-
D	I/1	+	+	-	+
	I/2	-	-	-	-
	II/1	-	+	-	+
	II/2	-	-	-	-
	II/3	+	-	-	+
	II/4	-	-	-	+/-
	III/1	+	+	-	+
	III/2	+	-	-	+
	III/3	-	+	-	+
	III/4	-	-	-	-
	III/5	-	+	-	+
	III/6	+	-	-	+
	III/7	-	-	-	-
	III/8	-	-	-	+/-
	III/9	-	+	-	+
E	I/1	+	+	-	+
	I/2	+	-	-	+
	II/1	+	-	-	+
	II/2	+	-	-	+
	II/3	-	-	-	-
F	I/1	+	-	-	+
	I/2	+	+	-	+
	II/1	+	-	-	+
	II/2	-	-	-	+/-

(a) The samples were collected at different times as shown by the Roman number and the fish number were shown in Arobic number

(b) – negative; + positive; +/- weakly positive

(c) pMar2, pFor1, pChe3 and pMyc5a probes respond to species *M. marinum*, *M. fortuitum*, *M. chelonae* and genus *Mycobacterium* spp respectively.

6.3.3.3 Environmental samples from Siamese fighting fish farms in the Bangkok area

Pond water was sampled at 5 Siamese fighting fish farms around Bangkok. Inlet water, moina (mosquito larvae) and mosquito were also sampled at some of these sites. *Mycobacterium* spp, detected in the samples, are indicated in Table 6.5. Of 5 pond water samples tested, 1 sample was positive for *M. marinum*, 2 were positive for *M. fortuitum* and 1 contained both *M. marinum* and *M. fortuitum* whereas one of three inlet samples contained *M. fortuitum* and another was non-speciated *Mycobacterium*. Four of the moina collected from 5 farms contained *M. fortuitum* and no *M. marinum* was detected in these samples. Only two mosquito samples were collected from farm K, one was positive for both *M. fortuitum* and *M. marinum* and the other for a non-speciated *Mycobacterium*.

Table 6.5 Detection of *Mycobacterium* spp. in environmental samples of Siamese fighting fish by PCR-reverse cross blot hybridisation

Farm[a]	Samples	Reaction on reverse cross blot hybridisation[b]			
		pMar2[c]	pFor1	pChe3	pMyc5a
G	pond water	+	+	-	+
	moina	-	+	-	+
H	pond water	-	-	-	-
	moina	-	-	-	-
I	inlet water	-	-	-	+
	pond water	-	+	-	+
	moina	-	+	-	+
J	inlet water	-	+	-	+
	pond water	-	+	-	+
	moina	-	+	-	+
K	inlet water	-	-	-	-
	pond water	+	-	-	+
	moina	-	+	-	+
	mosquito	+	+	-	+
	mosquito	-	-	-	+

(a) Samples were collected from Siamese fighting fish farms around Bangkok
(b) – negative; + positive
(c) pMar2, pFor1, pChe3 and pMyc5a probes respond to species *M. marinum*, *M. fortuitum*, *M. chelonae* and genus *Mycobacterium* spp respectively.

6.3.4 Biopsy analysis of Siamese fighting fish farmers

Fresh biopsies sampled from Siamese fighting fish farmers with lesions on their skin were taken by a Thai doctor (Dr. Preeya Kullavanijaya). The samples were tested for the presence of mycobacteria by bacterial culture at the Institute of Dermatology, Bangkok, Thailand and also by PCR-reverse cross blot hybridisation by Dr. Arend Kolk at N.H. Swellengrebel Laboratory of Tropical Hygiene (KIT), Royal tropical Institute, Amsterdam, The Netherlands. The results showed that of the 10 patients sampled, 50% were positive for *Mycobacterium* confirmed by bacterial culture. By PCR-reverse cross blot hybridisation, 40% were positive for *M. fortuitum*, while 50% of the samples were positive for a non-identified *Mycobacterium* spp and none was positive for *M. marinum* (Table 6.6).

Table 6.6 Clinical diagnosis of patients with skin granulomas

Patients	Bacterial culture[a]	Reaction in PCR-reverse cross blot hybridisation[b]			
		pMar2[c]	pFor1	pChe3	pMyc5a
1	+	-	-	-	+
2	+	-	-	-	+/-
3	+	-	-	-	+
4	+	-	+/-	-	+
5	+	-	+/-	-	+
6	-	-	-	-	+
7	-	-	-	-	-
8	-	-	-	-	+
9	-	-	+	-	+
10	-	-	+	-	+

(a) The data obtained from Dr. Preeya Kullavanijaya, Institute of Dermatology, Thailand and Dr. Arend H.J. Kolk, Royal Tropical Institute (KIT), N.H. Swellengrebel Laboratory of Tropical Hygiene, Amsterdam, The Netherlands.

(b) The samples were tested with 14 probes, which mainly respond to human pathogens. No significant reaction occurred with probes, other than the ones shown here: (–) negative; (+) positive; (+/-) weakly positive

(c) pMar2, pFor1, pChe3 and pMyc5a probes respond to *M. marinum*, *M. fortuitum*, *M. chelonae* and genus *Mycobacterium* spp respectively.

6.3.5 Detection of *Mycobacterium* spp in a variety of ornamental fish

Samples sent from different regions for routine screening were analysed (Table 6.7). Eight goldfish were collected from fish farms in Ratchaburi Province and Bangkok. No *Mycobacterium* species were detected in the samples obtained from Ratchaburi Province. Of the 5 samples obtained in Bangkok, one was positive for *M. marinum* and one contained of non-speciated *Mycobacterium* spp. No *Mycobacterium* spp. were detected in the 2 guppies obtained from Ratchaburi Province. Four disc cichlid fish were collected in Bangkok, and none were positive for *Mycobacterium* spp. Angel fish were obtained from Bangkok and East Anglia, UK. One fish from Bangkok contained *M. marinum*, while one of the fish obtained from UK was positive for both *M. marinum* and *M. fortuitum.* Of the 5 oscars collected in Bangkok, one was positive for both *M. marinum* and *M. fortuitum*, and one for a non-speciated *Mycobacterium* spp. The rest were negative with of all the probes used.

Table 6.7 Diagnosis of *Mycobacterium* spp in a variety of ornamental fish by PCR-reverse cross blot hybridisation

Fish	Origin	Sample number[a]				
		1	2	3	4	5
Goldfish (*Carassius auratus*)	Ratchaburi, Thailand	-	-	-	nd	nd
Goldfish (*Carassius auratus*)	Bangkok, Thailand	Myc	-	-	M	-
Guppy (*Poecilia reticulatus*)	Ratchaburi, Thailand	-	-	nd	nd	nd
Disc Cichlid (*Symphysodon discus*)	Bangkok, Thailand	-	-	-	-	nd
Angel fish (*Pterophyllum scalar*)	Bangkok, Thailand	-	-	-	-	M
Oscar (*Astronotus acellatus*)	Bangkok, Thailand	M, F	Myc	-	-	-
Angel fish (*Pterophyllum scalar*)	East Anglia, UK	M, F	-	nd	nd	nd

(a) M: *M. marinum*; F: *M. fortuitum*; Myc: non-speciated *Mycobacterium* spp.; nd: no data obtained.

6.4 Discussion and Conclusions

The level of DNA, which could be extracted from the sample and detected by PCR, was quantified prior to examining the samples from the survey. This was carried out using fish tissue spiked with known quantities of DNA. The DNA extraction appears to recover the bacterial DNA at 10^3 bacteria in total concentration from the spiked fish samples. The presence of bacteria at a level of less than 10^3 cells could not be recovered as shown by the results both in the agarose gel electrophoresis and reverse cross blot hybridisation. The sensitivity of PCR was also examined by amplification of *M. tuberculosis* DNA. It was clear that the detection limit was 50 fg DNA (10 cells) by both gel electrophoresis and reverse cross blot hybridisation.

The multiplex PCR using two sets of primers to simultaneously amplify two different target DNAs was studied in this Chapter. The primers, Pt18-F and INS2-R, are specific for IS*6110* resulting in a 249 bp product with the *M. tuberculosis* complex and 305 bp with *M. smegmatis* 1008. The primers, pMyc14-F and pMyc7-R, amplified 16S rDNA of *Mycobacterium* spp. resulting in a 208 bp product.

The DNA extraction methods used here involved many steps including, protein digestion, organic solvent extraction, alcohol precipitation, followed by the "Boom" extraction method (Boom *et al*, 1990) with which 50% of genomic nucleic acid can be recovered. Omitting some steps of the extraction procedure may reduce the loss of DNA, however, it may effect the purity of the DNA yield, thus resulting in a poor amplification reaction. Here, the DNA obtained by the multistep purification had no inhibitors present since the positive controls of *M. smegmatis* 1008 were completely

amplified and the positive product was evident in both agarose gel electrophoresis and reverse cross blot hybridisation assay.

Thus, the method used to extract the DNA here appears to be effective. However, the survey samples varied in size and composition, so the DNA extraction used was adjusted to suit individual samples. Basically, all samples were pre-treated with proteinase-K, extracted with organic solvent, precipitated with ethanol and finally purified by the method described by Boom *et al* (1990). This method was suitable for small-scale purification of samples such as fish tissue, moina and mosquito. However, large tissue samples and some water samples were treated with phenol and chloroform extraction followed by alcohol precipitation prior to performing the Boom extraction. Sediment samples, particularly, required phenol-chloroform treatment since they were enriched in organic material.

Snakehead fish farms

Snakehead fish have been reported to be susceptible to mycobacteriosis infection in Thailand (Chinabut *et al*, 1990). Fish fed on trash fish and processed offal are often contaminated with a variety of pathogens, including *Mycobacterium* spp. Nigrelli & Vogel (1963) suggested that ingestion of the pathogen directly from water or though contaminated feed was an important route of infection for mycobacteriosis in fish. Ross (1970) reported that feeding Pacific salmon with pasteurised offal reduced the incidence of mycobacteriosis. In the present study, environmental samples from fish farms including mosquito and moina from Siamese fighting fish farms were tested, however the food of the snakehead, which was usually the trash fish, was not obtained.

The results showed that *M. fortuitum* was the most common species detected in fish and water samples collected from the fish ponds, and the inlet and outlet water. *M. marinum* was most frequently detected in the sediment samples. On one farm, *M. fortuitum* was presented in water samples and it was also detected in the fish. However, on some farms no *M. fortuitum* could be detected in the fish, although the bacterium was detected in the pond water. *M. fortuitum* has been commonly reported in water and the environment (Woods & Washington, 1987). Stress and injury can make the fish more susceptible to pathogens in their surroundings (Nigrelli & Vogel, 1963; Austin & Austin, 1993; Smith, 1996). This may explain why the pathogen is either present in the fish environment but not detectable in the fish and vice versa.

M. marinum was the second most frequent species detected in sampled fish and in their environment, but unlike *M. fortuitum*, when *M. marinum* was detected in the fish environment it was also always detected within the fish. Non-identified *Mycobacterium* were also detected in some fish and also in the corresponding environmental samples. It was not possible to speciate these fish mycobacteria as only four probes were analysed in the reverse cross blot hybridisation here and the four probes were pMar2, pFor1, pChe3 and pMyc5a which detect *M. marinum*, *M. fortuitum*, *M. chelonae* and genus *Mycobacterium* spp respectively. Further analysis with the nine *Mycobacterium* probes described by Kox *et al* (1995b & 1997) may be useful. These detect a variety of human mycobacterial pathogen species or other opportunistic pathogen *Mycobacterium* species. However, the low level of bacteria present in the samples may be a problem, since the small amount of DNA extracted from them is only weakly positive with the genus probe (pMyc5a), and may not be sufficient to be recognised by species specific probes. For instance, 10 fg of *M.*

tuberculosis DNA equivalent to 2 bacterial cells was positive only with the genus probe (pMyc5a), and could not be detected by the species probe (pTub1) (Fig 6.4). In such a situation, the *M. tuberculosis* might be classified as a non-speciated *Mycobacterium* spp. whereas in fact it is *M. tuberculosis* but at too low a level for detection.

None of the snakehead fish farmers interviewed during the sample collection had lesions on his/her skin, possibly because the farmers are in direct contact with the fish and water only during harvesting when the farmer enters the water to catch his stock. Unfortunately, it was not always possible to obtain a complete set of samples. One problem was that the farmer did not want his stock to be sampled if they were large fish. Snakehead fish are particularly sensitive to disturbance and it can take a week before the animals begin to eat again after sampling, thus resulting in economic loss.

Siamese fighting fish farms

Siamese fighting fish farmers were observed to have lesions on their skin. *M. fortuitum* was the main species detected in the fish and in the water samples, and *M. marinum* was less frequently detected. In the farms, where either *M. fortuitum* or *M. marinum* was detected in the water and fish samples, lesions were observed on the fish farmers. Unfortunately, no human biopsies could be obtained without a qualified medical practitioner being present at the time of the interview. The farmers were subsequently sampled at a hospital and analysis of these biopsies revealed that the main pathogen present in the lesions was *M. fortuitum*, the main species identified in both the fish and the environment samples. The farmers sampled were not always exactly the same people as interviewed at the time of field sampling. This work

suggests that *M. marinum* is not the only species to infect the fish farmers as reported in a number of papers (Philpott *et al*, 1963; Jolly & Seabury, 1972; Kirk &Kaminski, 1976; Barrow & Hewitt, 1971; Huminer *et al*, 1986; Gray *et al*, 1990; Lawler, 1994). *M. fortuitum* was first isolated from a cold abscess in man in 1938 (da Costa Cruz, 1938; Westmoreland *et al*, 1990), and it has recently been reported to also cause skin lesions in man (Herndon *et al*, 1972; Wolinsky, 1979; Westmoreland *et al*, 1990).

Only Siamese fighting fish farmers had skin lesions, while none of the snakehead fish farmers, which was none of the farmers interviews suffering with skin lesions. This was probably due to the nature of the husbandry practised between the two fish species. The former involves contact with fish and their environment when changing the water from the bottles every 3-5 days, or when collecting insect larvae from a pond. The insect larvae ponds are usually fermented with pig excrement to attract insects laying their eggs. Nakhon Pathom Province Public Health Office (1997) reported that among the Siamese fighting fish farmers, the people who were responsible for collection the insect larvae had a significantly higher incidence of skin lesions.

It is noteworthy that some Siamese fish farmers do not keep only one type of fish, but often integrate these with other ornamental fish including goldfish and swordtail fish. Nakhon Pathom Province Public Health Office (1997) studied the relationship between the ornamental fish species and farmers suffering skin lesions amoung the fish farmers in Samkuipuk District, Nakhon Pathom Province. The species of fish farmed did not appear to correlate with fish farmers suffering with skin lesions.

The analysis of Siamese fighting fish samples obtained from different exporting aquaria for general diagnostic purposes revealed that *M. marinum* was the main etiological agent detected, however, the water samples or history of skin lesion on the aquaria workers were not obtained. *M. fortuitum* was also present in the samples of this group and both *M. fortuitum* and *M. marinum* were frequently detected in the same samples. This agreed with the results obtained from Chapter 4 which indicated that were two species involved in mycobacteriosis in Siamese fighting fish.

In general, *M. marinum* and *M. fortuitum* were present in all types of samples examined in the study. This should be a consideration for all owners of private and public aquaria. It should also be noted for public aquaria, particularly for children who are allowed to touch the fish and the water. *M. marinum* and *M. fortuitum* have been identified in fish and water obtained from "touch pools" from public aquaria in the UK (data not shown).

In conclusion, PCR-reverse cross blot hybridisation is a useful tool for detecting *Mycobacterium* spp both in fish and their environment. Even although there were often problems in collecting a complete set of samples, the results obtained indicate the presence of both *M. marinum* and *M. fortuitum* in farmed fish and in their environment. None of the farms was *Mycobacterium* free. *Mycobacterium* spp, were always detected on all snakehead and Siamese fighting fish farms tested, either in fish samples or environmental samples, or both. Unfortunately, the method used here, meant that the fish had to be sacrificed for the analysis, which was not acceptable with high value stock. Analysis of blood would be an ideal test for future work, especially since Nigrelli & Vogel (1963) proposed that skin lesions on fish may be a possible

route of entry for the pathogen, via the lymphatic system or blood stream, of the animals.

Chapter 7

Final Discussion and Conclusions

Mycobacteriosis is known to infect a wide range of marine and freshwater fish, including both edible and ornamental fish. The etiological agent infects not only fish, but also can be transmitted to man. In Thailand, mycobacteriosis was first described in snakehead fish (*Channa striata*) in 1983 (Limsuwan *et al*, 1983). Since the first report other fish species such as Siamese fighting fish (*Betta splendens*) have been reported to be infected (Pungkachonboon *et al*, 1992), and more infections in snakehead have also been documented (Chinabut *et al*, 1990; Adams *et al*, 1996). A variety of mycobacteria isolated from Siamese fighting fish were identified as *Mycobacterium* spp by bacterial culture and biochemical tests, and it was suggested from these studies that they were most similar to *M. marinum* (Pungkachonboon, 1994).

During a 10-year period (1981-1990), *M. marinum* infections were predominantly reported among patients with cutaneous infections at the Institute of Dermatology, Bangkok (Kullavanijaya *et al*, 1993). Among these patients, over 60% had occupations or hobbies associated with fish. These findings in Siamese fighting fish (Pungkachonboon, 1994) and man suggested that the main etiological agent of mycobacteriosis in fish transferring to man was *M. marinum*.

In this present study, it was found that two mycobacterial species were involved in the infections of Siamese fighting fish in Thailand. These were *M. fortuitum* and *M. marinum*. *M. fortuitum* was more often found in both fish and their environment. It has been reported that *M. fortuitum* is a common mycobacterial species present in

water (Viallier & Viallier, 1973; Wayne & Sramek, 1992; Iivanainen *et al*, 1999). The findings presented here revealed that *M. fortuitum* was the dominant etiological agent of mycobacteriosis in fish and it appeared to be transferred to workers who were exposed to the infected fish or contaminated water. Although *M. marinum* was detected in fish and water samples, this species was not identified from any of the skin biopsies from Siamese farm workers. As only 10 biopsies were sampled, however, this does not rule out the transfer of *M. marinum* in water and fish to man on the Siamese fighting farms.

As with Siamese fighting fish, both *M. fortuitum* and *M. marinum* were detected in the infections in snakehead. The proportion of these two species present was not notably different. In contrast to the Siamese fighting fish farmers, none of the snakehead fish farmers suffered from skin lesions. This may be due to the nature of fish husbandry required as the snakehead fish farmers are less exposed to fish or water than Siamese fighting fish farm workers.

In Israel, *M. marinum* appeared to be only one species infecting a variety of fish including sea bass (*Dicentrarchus labrax*). Characterisation of these isolates compared with *M. marinum* isolates from Thailand showed that they reacted differently to Mab 8F7 and PyMS analysis.

The *M. marinum* species-specific probe Mab 8F7 was developed in this study due to the original belief that *M. marinum* was the main species infecting fish and man in Thailand, as described above. ELISA analysis of *M. marinum* isolates from Israel with Mab 8F7 gave a negative reaction, whereas the *M. marinum* isolates from

Thailand were positive. Clearly the isolates from Israel lack the epitope to which Mab 8F7 binds. This finding agreed with PyMS analysis, a technique based on biochemical component analysis (Gutteridge *et al*, 1985; Berkeley *et al*, 1990). However, to further characterise this difference between Thai and Israel isolates may give some insight into the pathogenic differences between the two groups of *M. marinum*, and may help to answer the question why sea bass in Israel are more susceptible to the disease than in Thailand.

As antibiotic treatment is expensive and often long term, diagnosis in the early stages of the infection is very important for control of the disease. Early diagnosis not only allows for effective farm management, but also aids in preventing spread of the pathogen.

M. marinum is slow growing and bacterial culture methods are not always the most appropriate method for identification. Identification of *Mycobacterium* spp using biochemical tests (Kent & Kubica, 1985; Lansdell *et al*, 1993) tends to be limited in many aspects including specificity, sensitivity, and it is time consuming (Colorni, 1992; Pungkachonboon *et al*, 1992). Many mycobacterial isolates were misidentified in the past and some have been reclassified, mostly by biochemical tests (Collins *et al*, 1984; Dalsgarrd *et al*, 1992). In the last few years, genotypic analysis has played a major role in the identification of *Mycobacterium* spp. (Bottger, 1989; Edwards *et al*, 1989; Böddinghaus *et al*, 1990; Rogall *et al*, 1990a & 1990b). A variety of DNA-based methods have been developed and among these, PCR appears to be the most extensively used, particularly in the study of human tuberculous mycobacteria (Kox *et al*, 1994; 1995a, 1995b; 1996; 1997; Kolk *et al*, 1992; 1994; 1998). The PCR results

presented here provide evidence that aquatic mycobacteria have been mis-classified in the past and highlight the important of a species specific detection method for fish mycobacteria.

Detection of the etiological agent in fixed tissue is practical for analysis of samples fixed on the farm and transported to the laboratory for analysis. IHC and ISH were employed here to detect mycobacteria in fixed fish tissue. Mycobacteria has a thick waxy layer around the cell to protect and isolate it from the host defence mechanisms as described for *M. lepraemurium* (Darper & Rees, 1973). Mab 8F7 was produced by immunising mice with UV-irradiated mycobacteria, so as the preserve this outer layer, and hence develop antibody to the bacterial surface. There is no report on this method of antigen preparation for Mab production in the literature. Previous studies have used lysates of *Mycobacterium* spp to immunise the mice (Adams *et al*, 1996). The production of Mabs against mycobacterial cell wall has previously been reported for *M. avium* isolated from AIDS patients (Wagner *et al*, 1992). There are no reports on the use of IHC with Mabs on human biopsies. Mab 8F7 successfully identified *M. marinum* in infected fish tissue by IHC. Future work could include the detection of *M. marinum* on human biopsies by IHC.

The cell wall properties of other *Mycobacterium* spp. have been studied in detail particularly for the human mycobacterial pathogens. Hasegawa (1971) reported that the coating around *M. lepraemurium* consisted of lipid and protein. Draper & Rees (1973) identified the capsule surrounding *M. lepraemurium* as peptidoglycolipid and suggested this structure may be present in other mycobacterial strains. Ortalo-Magné *et al* (1995) analysed the outermost capsular material of *M. tuberculosis*, and found

polysaccharides and proteins associated with a small amount of lipid to be the main components of the capsule.

Although the species-specific probe, Mab 8F7, was used to identify *Mycobacterium* spp to species level (i.e. *M. marinum*), a wide range of mycobacteria detection is also required for diagnosis of the disease. With the use of available genus-specific probes, ISH was developed in this study. The aim was to establish a method for the detection of *Mycobacterium* spp. in infected fish tissue. Future work should also include the use of the available species-specific probes, following on from genus-specific positive samples. Confirmation of the *Mycobacterium* species present is not yet possible by IHC as only the *M. marinum* probe exists. Other researchers have used Pabs to detect a wide range of *Mycobacterium* in IHC (Gómez *et al*, 1996).

Identification of mycobacteria by both IHC and ISH was mostly focused on the presence of the bacteria in granulomas in the infected tissues. Granuloma formation in internal organs is a typical clinical sign of mycobacteriosis. The granulomas might form with different histopathological figures including necrotizing, nonnecrotizing and caseous, and *Mycobacterium* spp were often found in the centre of the granulomas (Talaat *et al*, 1998).

With respect to the present finding that Thai *M. marinum* strains were different from Israeli ones, new Mabs against Israeli strains should be developed. This would enable the identification of all *M. marinum* isolates regardless of geographical origin. Moreover, Mabs, which are species specific for *M. fortuitum* and *M. chelonae*, should also be developed as for use in ELISA and IHC.

Colorni (1992) and Hatai *et al* (1993) reported isolation of *Mycobacterium* from European sea bass (*Dicentrarchus labrax*) and pejerrey (*Odonthestes bonariensis*), respectively, however the species identification was unsuccessful. Species identification is useful as it may indicate the antibiotic susceptibility of the pathogen, and lead to effective antibiotic treatment.

The antibiotic sensitivity of *M. marinum*, isolated from sea bass (*Dicentrarchus labrax),* has been examined by Colorni *et al* (1998). The authors reported that streptomycin and allicin (a garlic extract), separate or in combination, suppressed the development of granulomas in the spleen of infected fish. However, the pathogen could still be isolated from infected animals at the end of the treatment. Thompson (pers. comm.) reported that *M. marinum* isolated from snakehead fish and Siamese fighting fish in Thailand were susceptible to amikacin, sarafloxacin and kanamycin. Santacana *et al* (1982) also reported that kanamycin sulphate successfully controlled the mycobacteriosis in Siamese fighting fish. A study of the antibiotic sensitivities of the isolates in the collection was outwith the scope of the current study. Antibiotics are not the ideal treatment for the disease, as they can be expensive and treatment is long term. Nevertheless, the effectiveness of different antibiotics should not be overlooked in future studies. If successful in disease treatment, they may be useful for controlling the disease of the high value fish stock or pet fish.

PyMS analysis divided the isolates into three distinctive groups: one group was represented by *M. marinum* isolates from fish in Thailand, another group was represented by *M. fortuitum-M. chelonae*, and the rest, mainly isolated from Israel and

Greece, were not represented by any of the reference *Mycobacterium* species used in the study. It is interesting that *M. fortuitum* (NCIMB 1294) clustered in the same group as *M. chelonae* (NCIMB 1474). It is likely that the *M. chelonae* (NCIMB 1474) strain was originally misidentified. Although Bottger (1989) and Edwards *et al* (1989) found *M. fortuitum* and *M. chelonae* to be closely link by 16S rDNA analysis, the probes used to identify *M. fortuitum* and *M. chelonae* have previously been reported to successfully differentiate between *M. fortuitum* and *M. chelonae* (Kox *et al*, 1995b &1997). This suggests that some isolates may need to be reclassified by DNA-based methods.

M. fortuitum and *M. chelonae* have been referred to as the *M. fortuitum* complex (Silcox *et al*, 1981; Tsang *et al*, 1984). Although these two species are closed related, they are differentiated by several phenotypic features such as in biochemical tests (Kubica *et al*, 1972; David *et al*, 1981; Lévy-Frébault *et al*, 1983), antibiotic susceptibility (Wallace *et al*, 1985), protein profiles on polyacrylamide gel electrophoresis (Haas *et al*, 1974) as well as genomic analysis (Rogall *et al*, 1990b). Bosne & Lévy-Frébault (1992) identified *M. fortuitum* and *M. chelonae* by mycobactin patterns determined by a thin-layer chromatography. Mycobactin is an iron-chelating compound produced by mycobacteria under the iron-limited conditions (Snow, 1970).

Published oligonucleotide primers (Kox *et al*, 1995b) were used to characterise the *Mycobacterium* isolated from fish by PCR-reverse cross blot hybridisation. Many aspects of the assay had to be optimised before the reaction was effective in recognising *Mycobacterium* spp. DNA contamination, a common problem in PCR,

was eliminated by introducing UDG enzyme to the designed PCR mixture (Kox *et al*, 1995b; 1997; Arnauld *et al*, 1998). However, this enzyme can only help to control previously amplified DNA, produced using dUTP in the PCR mixture. DNA amplified using dTTP and natural DNA may still contaminate the samples. The inclusion of positive and negative controls in each PCR reaction is important for interpreting the results (Hiney & Smith, 1997). In the study of DNA extraction prior to testing field samples (Chapter 6), both controls were applied to investigate the presence of any inhibitors which may have occurred in samples or in the amplification reaction by adding known DNA (*M. smegmatis* 1008) as a internal control (Kolk *et al*, 1994).

PCR-reverse cross blot hybridisation appears to be a successful method for identifying *Mycobacterium* spp in infected fish tissue and environmental samples. However, the level of *Mycobacterium* present in the environment was not quantified. The amount of DNA obtained from bacteria in the water samples was related to bacterial concentration, but the environmental samples used in the study were for bacterial identification, and not for quantification. However, establishing the concentration of bacteria presence in the fish environment may help to determine the infection dose needed for fish to succumb to mycobacteriosis. Different authors have found this dose to vary between studies. Colorni *et al* (1998) reported that i.p. injection of sea bass with *M. marinum* at 8.8 x 10^4 cells/ 100-g fish caused the fish to develop granulomas in their spleens within 4-6 weeks post-injection. A similar result was reported by Talaat *et al* (1998) who found that the minimum dose needed for goldfish (*Carassius auratus*) to produced systemic granulomas within 8 weeks was 600 CFU per fish, and at the dose of 10^8-10^9 CFU resulted in death of fish within 4-17

days. The dose required in the field to infect fish is unknown. Development of a quantitative PCR to determine environmental levels is desirable.

The probes used in reverse cross blot hybridisation here focused on only three species of mycobacteria most frequently isolated from fish, *M. marinum*, *M. fortuitum* and *M. chelonae*. However, nine other probes, which identify *M. tuberculosis*, *M. avium* & *M. paratuberculosis*, *M. intracellulare*, *M. kansasii* complex & *M. scrofulaceum* complex, *M. xenopi*, *M. genavense*, *M. leprae*, *M. gordonae* and *M. smegmatis* (Kox *et al*, 1997) were used for optimisation of bacterial DNA extraction from spiked fish (Chapter 6). Among these, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, *M. xenopi*, and *M. gordonae* were found in environment (Viallier & Viallier, 1973; Joynson, 1979; Howard *et al*, 1987; Wayne & Sramek, 1992).

Since Nigrelli & Vogel (1963) described *M. fortuitum* isolated from Neon Tetra (*Hyphessobrycon innesi*) was the same as *M. fortuitum* isolated from a patient in South Africa, there have been few literature reports of *M. fortuitum* from the environment infecting man (Herndon *et al*, 1972; Westmoreland *et al*, 1990). Unlike *M. fortuitum*, *M. marinum* has been more often reported as a human pathogen, particularly in those who have close contact with fish or water (Huminer *et al*, 1986; Gray *et al*, 1990; Suh & Hoffman-Steeger, 1992; Paul & Gulick, 1993; Ryan & Bryant, 1997). *M. marinum* infections are normally reported as superficial lesions (Jolly & Seabury, 1972; Donta *et al*, 1986; Paul & Gulick, 1993), however deeper infections have also been reported in the hand, including tenosynovitis, arthritis, bursitis and osteomyelitis (Chow *et al*, 1983; Beckman *et al*, 1985; Hurst *et al*, 1987; Shih *et al*, 1997). *M. fortuitum* has been reported to infect cutaneous soft tissue

associated with wounds, surgical trauma or injection (Carey *et al*, 1994; Hong *et al*, 1995; Escalonilla *et al*, 1998). Infections of deeper structures have also been reported, including lungs (Lessing & Walker, 1993), bones and joints (Wolinsky, 1979).

M. ulcerans, another *Mycobacterium* species, has been reported to cause a necrotizing skin disease called Buruli ulcer in man (George *et al*, 1998). It was first described in patients in Australia (MacCallum *et al*, 1948). Since then, it has been reported occasionally in different regions including Australia, Congo, Uganda, Mexico and South East Asia (Pettit *et al*, 1966; Veitch *et al*, 1997; Johnson *et al*, 1999). Although there is no report of *M. ulcerans* infection in fish, it should be taken into account since *M. ulcerans* is taxonomically similar to *M. marimun*, and the infection of Buruli ulcer disease was associated with water (Hayman, 1991; Marston *et al*, 1995; George *et al*, 1998). The pMar2 probe used in PCR-reverse cross blot here was specific to both *M. marinum* and *M. ulcerans* (Kox *et al*, 1997). Ross *et al* (1997a; 1997b) reported the new specific *M. ulcerans* DNA target for amplification in PCR, which may possibly detect the pathogen in environment. To develop such a species probe would be useful.

The PCR-reverse cross blot hybridisation clearly identified *M. marinum*, *M. fortuitum* and *M. chelonae* in pure culture, fish tissue and the environmental samples. Some mycobacteria in the samples still remained unspeciated, however, since they did not react to these three probes, but were positive for the *Mycobacterium* genus probe. Further analysis should be performed on the unspeciated samples. Nine more probes are already available. For example, *M. simiae*, a common species in water and which

has been isolated from fish (Lansdell *et al*, 1993), can be identified by the pKan7 probe.

Recently Pacific white shrimp (*Penaeus vannamei*), an important food species, was reported to be infected with *M. peregrinum* (Mohney *et al*, 1998). *Mycobacterium* infections in crustaceans was first reported in the 1980s (Brock *et al*, 1986; Krol *et al*, 1989; Brock & Lightner, 1990). However, this recent paper was the first report of *M. peregrinum* in Pacific white shrimp, an economically important food species. Moreover, this *Mycobacterium* species is also a potential human pathogen (Ishii *et al*, 1998; Escalonilla *et al*, 1998). The authors (Mohney *et al*, 1998) isolated the causative agent by conventional bacteriology, and characterised it using biochemical tests. PCR-reverse cross blot hybridisation analysis would be useful in the confirmatory identification of this species in Pacific White shrimp. The pFor1 probe, which reacts with *M. fortuitum*, also cross reacts slightly with *M. peregrinum* (Kox *et al*, 1995b). However a probe specific for *M. peregrinum* is preferable.

The presence of environmental mycobacteria that are pathogenic for humans was recently reported (Stabel, 1998a, 1998b). It is known that *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*) is an etiologic agent of Johne's disease, a chronic granulomatous enteritis of ruminants (Chiodini *et al*, 1984). *M. paratuberculosis* has recently been detected in cow's milk in the U.K. (Millar *et al*, 1996). *M. paratuberculosis* has also been suggested as a possible cause for Crohn's disease in man, a chronic enteritis with clinical symptoms similar to Johne's disease in animals (McFadden *et al*, 1987). *M. paratuberculosis* is a subspecies of *M. avium*, which is also commonly found in environment (Wayne & Sramek, 1992). PCR-

reverse cross blot hybridisation has been shown to be a useful method for the identification of *Mycobacterium* spp in environmental samples. This method would therefore be useful for establishing the presence of *M. paratuberculosis* in milk or water samples.

Export of ornamental fish is a large and profitable trade for Thailand and other countries. Diseases can easily be spread, however, if there is no effective screening tool. Infected fish act as carriers spreading *Mycobacterium* spp from place to place, and this also provides the opportunity of introducing new pathogenic strains from one place to another. The probes and methods developed at this study appear to be useful for detection and identification of aquatic *Mycobacterium* spp. They are useful tools for screening for *Mycobacterium* spp. that may infect fish or be present in fish environment before trading. Moreover, they can be used in the fish farms for monitoring disease.

For routine screening of mycobacteriosis, Mab 8F7 has been shown to be a useful tool for detection of *M. marinum* in fixed tissue, using IHC and ELISA, respectively. Although the IHC and ELISA technique are simple and relative inexpensive, the use of the Mab 8F7 probe is limited as it does not recognise all *M. marinum* isolates. Clearly more Mabs are required against *M. marinum*, and the development of *M. fortuitum* and *M. chelonae* probes is desirable. ISH appeared to be powerful technique for use on fixed tissue but further studies are require to enhanced the consistency of the method and develop species-specific protocols. At present, the PCR-reverse cross blot hybridisation appears to be the most suitable screening method for fish and environment samples, as both sensitivity and specificity are high.

Nevertheless future studies should include the development of additional probes to broaden the number of aquatic *Mycobacterium* species identified.

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