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Pathology attributed to *Mycobacterium chelonae* infection among farmed and laboratory-infected Atlantic salmon *Salmo salar*

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ABSTRACT: This study was promoted following concern over increasing mortality on 2 farms rearing Atlantic salmon *Salmo salar* in the Shetland Isles, Scotland. A *Mycobacterium* sp. was isolated from moribund, market-sized Atlantic salmon. Biochemical tests, lipid analysis and PCR (polymerase chain reaction) techniques confirmed the bacterium to be *Mycobacterium chelonae*. Multiple greyish-white miliary granuloma-like nodules were observed in several tissues. Dense hard-packed nodules contained abundant acid-fast bacteria. Atlantic salmon injected with *M. chelonae* remained sub-clinically infected, demonstrating the chronic nature of this disease. The source of the pathogen was not identified.

KEY WORDS: Mycobacteriosis · *Mycobacterium chelonae* · Atlantic salmon · Pathology · PCR

INTRODUCTION

Mycobacteria were first described from salmonids by Earp et al. (1953). The genus *Mycobacterium* is now recognised as being widely distributed amongst many fish groups. All fish species should therefore be considered susceptible. Outbreaks predominate, however, in tropical aquarium fish, such as the three-spot gourami *Trichogaster trichopterus* (Santacana et al. 1982), but also in cultured fish, including sea bass *Dicentrarchus labrax* (Colorni 1992), striped bass *Morone saxatilis* (Hedrick et al. 1987), yellowtail *Seriola quinqueradiata* (Kusuda et al. 1987) and farmed Pacific salmon *Oncorhynchus* spp. (Ashburner 1977, Arakawa & Fryer 1984). In European waters, mackerel *Scomber scombrus* have been the subject of a presumptive mycobacteriosis (MacKenzie 1988). The literature indicates that mycobacteriosis in Atlantic salmon *Salmo salar* is uncommon (Humphrey et al. 1987). During the late 1950s a high prevalence within hatchery Pacific salmonids promoted an increase in research activity (Parisot & Wood 1960, Ross 1963). However, when the practice of using unpasteurised fish meal in diets ceased, infection in these fish and the subsequent prevalence in returning wild fish was reduced (Parisot & Wood 1960). Ingestion is probably a major source of infection, although ovarian transmission has been suggested as a possible route in some fish groups (Ross & Johnson 1962, Ashburner 1977). Mycobacteriosis continues periodically to contribute to significant chronic mortality in farmed fish, principally from freshwater (Ashburner 1977), but also within the marine environment (Hedrick et al. 1987, Colorni 1992). The mycobacteria, comprising the genus *Mycobacterium*, are typical aerobic, Gram-positive, acid-fast staining, rod-shaped bacteria. There are 3 species of *Mycobacterium* recognised as potential agents of fish disease, namely *M. chelonae*, *M. fortuitum* and *M. marinum* (Inglis et al. 1993, Belas et al. 1995). Typical gross pathology including emaciation, exophthalmos, keratitis and skin ulcers; and abnormal swimming behaviour has been observed (Beckwith & Malsberger 1980, van Duijn 1981, Gómez et al. 1993). Involvement of the

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visceral organs consists of creamy-coloured, variable-sized, tumour-like nodules with possible ulceration (Ashburner 1977).

This study reports on the occurrence and identification of *Mycobacterium chelonae* from farmed Atlantic salmon in Scotland during December 1996. The disease was identified at 2 marine fish farms in waters off the Shetland Isles, Scotland. Gross and light microscopical lesions are described. Data on the fulfilment of Koch's postulates are presented.

MATERIALS AND METHODS

The 2 marine farms studied here were designated I and II; they operate under independent management with no fish movement between the sites and are located approximately 27 km apart. Both farms held fish with a common freshwater origin, and an additional stock was present on 1 farm only.

Fish from natural outbreaks. Moribund farmed Atlantic salmon *Salmo salar* (weight 2 to 3 kg) were selected from sea cages with the help of a diver or the use of a hand net at farms I and II (Shetland Isles). The water temperature was 4 to 5°C. The fish were sampled immediately following terminal anaesthesia with MS-222 (Sigma) or held in crushed ice and examined within 1 h of their removal from the cages. In total, 45 moribund fish were removed for examination between mid-November–December from farm I and 5 fish were examined in mid-December from farm II. Examination of the fish consisted of recording visual gross lesions as well as sampling tissues for bacteriology, histology and virology. No recent or ongoing anti-microbial treatment was being carried out at either farm. The freshwater origin of the 2 stocks examined at each farm was established. Thirty dead and 30 apparently healthy fish from farm I were also dissected and examined for gross lesions. Atlantic salmon from 2 sites (n = 10) located near to farm I were also examined for gross abnormalities.

Experimental infection. Eighty Atlantic salmon post smolts (~150 g) were transported from the FRS Marine Laboratory's site at Aultbea, Ross-shire to the Aberdeen facility, where they were allowed to acclimatise for 3 wk. All stock held at this unit are subject to regular health monitoring and no infective agent has been identified. Each fish was anaesthetised with MS-222 and injected intraperitoneally with a single dose of 0.1 ml containing a cell suspension of OD_{540}, 0.2 or 0.7 *Mycobacterium chelonae* (MT1890; isolate from farm outbreak) in phosphate-buffered saline (PBS). Following injection, fish were placed according to dose into 1 m circular tanks to recover. Five fish were marked ventrally with Alcian blue (injected with 0.1 ml PBS) and placed with each challenge group. A further 10 fish were injected with PBS and held in an identical tank. Sea water was supplied to each tank at 320 to 350 l h^{-1} at ambient temperature (6 to 8°C). Fish were fed a commercial diet throughout the study.

Injected fish were netted, anaesthetised (MS-222) and examined, in groups of 2 to 3 per treatment over 120 d at approximately 7 to 10 d intervals. The uninfected (cohabiting and control) fish were sampled on completion of the study. Fish were sampled for histology and bacteriology according to the protocol outlined for farmed fish. Tissues from laboratory-injected fish were not sampled for virus isolation.

Bacterial isolation. Bacterial isolation was attempted from the kidney, spleen and liver of moribund farmed Atlantic salmon. Samples were inoculated onto tryptone soya agar with 2% sodium chloride (TSA), Anacker and Ordal's agar (A&O) (using 0.5% tryptone, as modified by Lorenzen 1993), brain heart infusion agar (BHI), and Mueller Hinton (MH). Plates were incubated at 22°C (TSA, BHI, A&O) and 15°C (MH), and examined on alternate days for up to 5 wk. The powdered media were purchased from Unipath (TSA, BHI, A&O) or Difco (MH) and prepared in the laboratory. Kidney tissue only was sampled from the experimentally infected fish for bacteriology. This was inoculated onto TSA and MH and similarly incubated.

Morphology and biochemical characteristics. Colony morphology was recorded and cultures were subjected to Gram and Ziehl-Neelsen staining and examined microscopically. Oxidase and catalase tests were conducted and the ability of the organism to grow at 37°C was tested. Four isolates (MT1890, MT1892, MT1900 and MT1901) from naturally infected fish recovered on TSA were selected for additional tests. The laboratory isolates were subjected to all biochemical tests. Each isolate was inoculated onto plates of the following media: urea agar, MacConkey agar, Simmons citrate medium, pyruvic acid egg medium and Lowenstein Jensen (LJ) medium. These plates were incubated at 15°C for up to 10 d and checked on alternate days. The tests for urease, nitrate reduction, citrate utilisation and iron uptake were validated with +ve and –ve cultures from the FRS Marine Laboratory culture collection. The reduction of nitrate to nitrite required the use of a heavy inoculum taken from each isolate on TSA and suspended in 10 ml nitrate broth (Merck) and incubated at 15°C for 96 h. Equal volumes of API test reagents (Bio Mérieux) NIT1 and NIT2 were mixed, and 7 drops of the resulting solution added to each sample. Oxidative/fermentation (O/F) medium (Bio Mérieux) was stabbed with a heavy inoculum and incubated at 15°C for up to 1 mo. Iron uptake by production of siderophores was examined using chrome azurol S (CAS) agar (Schwyn & Neilands 1987).

Light microscopy. Portions of gill, kidney, heart, liver, spleen, brain, gut and pyloric caeca were dissected from farmed and laboratory-infected fish and fixed in 10% buffered formalin. These were then processed for light microscopy by sectioning and staining with haematoxylin and eosin (H&E), Gram and Ziehl-Neelsen (methods given by Bruno & Poppe 1996). Additional sections were stained with auramine-rhodamine (Bancroft & Stevens 1990) and examined by fluorescence microscopy. The farmed fish were reported as infected when typical acid-fast bacilli were observed in tissue sections.

Virology. Kidney tissue was tested for the presence of infectious pancreatic necrosis virus (IPNV) using CHSE (chinook salmon embryo) cells, as described by Smail et al. (1995) from individual naturally infected fish. Dilutions of kidney, spleen and heart and brain homogenate were also tested for the presence of infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) using BF-2 (blue gill fry) and EPC (epithelioma papillosum cyprini) cells according to the protocol described in European Community Commission Decision 92/532/EEC (Official Journal of the European Communities 1992). Appropriate positive and negative controls were included.

Mortality. Mortality data were collected weekly by staff at farms I and II and collated at the FRS Marine Laboratory, Aberdeen. The information was summarised as monthly mortality from all causes between August and January inclusive.

Lipid analysis by thin layer chromatography. Two isolates, MT1890 and MT1892, from naturally infected Atlantic salmon were subjected to lipid analysis by thin layer chromatography (TLC). For TLC, isolates were subcultured onto LJ medium slopes. A heavy growth was removed, dried over phosphorus pentoxide in a vacuum desiccator and the lipids extracted using a mixture of diethyl ether:ethanol:water (17:17:6 v/v), as described by Collins et al. (1997). Aliquots (10 µl) were spotted onto TLC plates (Whatman KG Silica Plates, 250 µm thick; Kodak, USA) and separated using n-propanol:water:ammonia (75:22:3 v/v). The plates were dried and the chromatogram developed by treating it with concentrated sulphuric acid.

Polymerase chain reaction. The polymerase chain reaction-restriction enzyme analysis (PCR-REA) was performed essentially as described by Telenti et al. (1993). In brief, primers were used to amplify a region of the heat shock gene, *hsp65*, producing a 439 base pair amplification product that was subjected to electrophoresis on a 1% (w/v) agarose gel (NuSieve; FMC Bioproducts, USA) and visualised by staining with ethidium bromide. The total reaction volume was 50 µl and reaction conditions were 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by an extension time of 10 min at 72°C. The total number of cycles was 30. Aliquots of PCR product (10 µl) were added to each of 2 tubes containing 5 U of *Bst EII* restriction endonuclease with buffer and sterile water or 5U of *Hae III*, buffer and water. One tube was incubated at 60°C, and the other at 37°C for 90 min. All buffers and enzymes were purchased from Boehringer Mannheim, Germany. The resulting restriction digests were subjected to electrophoresis on 4% (w/v) agarose gels and visualised as above.

RESULTS

Bacterial growth and cultural characteristics

Pure cultures were obtained from kidney, spleen and liver of fish sampled at both farms on standard media (TSA, BHI, A&O, MH) within 7 d incubation at 15°C. Confluent growth from laboratory-infected fish occurred over periods of up to 3 wk at 15°C. Biochemical properties revealed the identity of isolates from both farmed and laboratory-infected fish to be *Mycobacterium chelonae*. Circular, smooth, pale-cream colonies were isolated and the bacteria stained Gram-positive and acid-alcohol fast. The cultures were composed of pleomorphic rods, which were non-motile and non-sporulating and measured 2–7 µm × 0.2–0.5 µm. Biochemical characteristics are given in Table 1. *M. che-*

Table 1 Characteristics of *Mycobacterium* isolates MT1890, MT1892, MT1900, and MT1901 isolated from marine reared Atlantic salmon *Salmo salar*, and MT1915, MT1917 and MT1918 isolated from laboratory-infected Atlantic salmon

Test	Result
Colony morphology	Creamy smooth circular colonies
Motility	–ve
Pigmentation	–ve
Gram stain	Weak, Gram-positive rods
Ziehl-Neelsen stain	+ve
Oxidase	–ve
Catalase	+ve
Oxidative/fermentation	Fermentative
Growth at 37°C	–ve
Growth on:	
MacConkey agar	+ve
Pyruvic acid egg	+ve
Lowenstein Jensen	+ve
Nitrate reduction	–ve
Iron uptake by production of sideophores using chrome azurol S agar	–ve
Simmons citrate utilisation	–ve
Urease	+ve

lonae appeared golden in colour when stained with auramine-rhodamine and examined by fluorescence microscopy. A *Vibrio* sp. was recovered in pure culture from some fish at farm I. No bacteria were isolated from the cohabiting or control fish in the laboratory trial.

Gross pathology

Moribund farmed fish moved slowly near the water surface and appeared dark with slight abdominal distention, but no skeletal deformities were observed. Skin ulcers were noted in 2 fish (farm I). Multiple, grouped or single, greyish-white miliary granuloma-like nodules (1 to 3 mm in diameter) were observed throughout the kidney. The tissue was enlarged but normal in colour (Fig. 1). Similar, but smaller (0.1 to 1 mm), granuloma-like lesions were also seen within the spleen and liver. Splenomegaly and ascites were noted in some fish. The gills were pale and focal haemorrhaging occurred in the musculature. The prevalence of typical gross mycobacterial lesions varied between 20 and 40% in the 2 stocks and within the dead fish examined at farm I. No gross lesions were recorded in the apparently healthy fish examined or in fish examined in December at 2 farms adjacent to farm I.

During the infection experiment, 3 fish appeared lethargic and darker than their cohorts. Ascites was apparent in 2 fish from the high dose group. One fish sampled at the end of the experiment from this group had greyish-white miliary granuloma-like nodules in the anterior kidney. As with the farmed fish, there was no evidence of skeletal deformity in the laboratory-infected fish. At necropsy, other tissues appeared normal. No gross pathological changes were seen in the control groups.

Mortality

At farm I during August, mortality from all causes was low, but rapidly increased to 1425 during November. By the end of January losses had decreased to 318 for the month, representing a total loss of 4.1% during August to January. Farm II also recorded high mortality of 2208 in November, but the mortality had declined to 106 in January.

There was no mortality during the 120 d laboratory trial in injected, cohabiting or control fish. Isolates of *Mycobacterium chelonae* were recovered on TSA from the 7/40 (high dose) and 2/40 (low dose) groups of laboratory-infected Atlantic salmon.

Histopathology

Fish from the natural outbreak at farm I had numerous acid-fast bacteria in large circular (~400 µm) densely packed nodules in the kidney, liver and spleen. These nodules comprised epithelioid cells and had no surrounding capsule or fibrin deposition or central necrosis (Fig. 2). No other cellular inflamma-

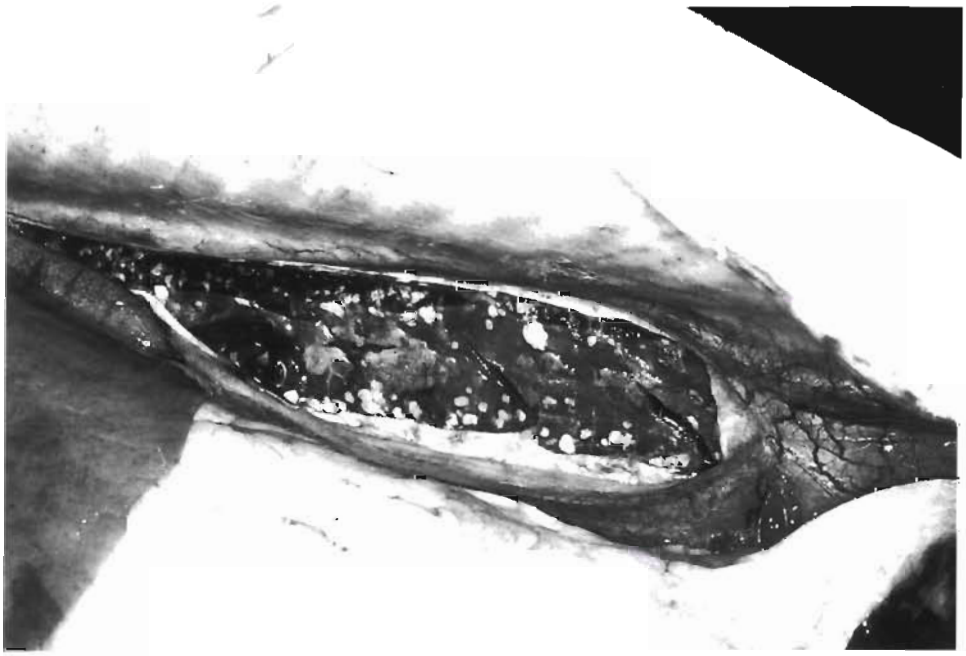

Fig. 1. Multiple, greyish-white miliary granuloma-like nodules observed in naturally infected Atlantic salmon *Salmo salar*

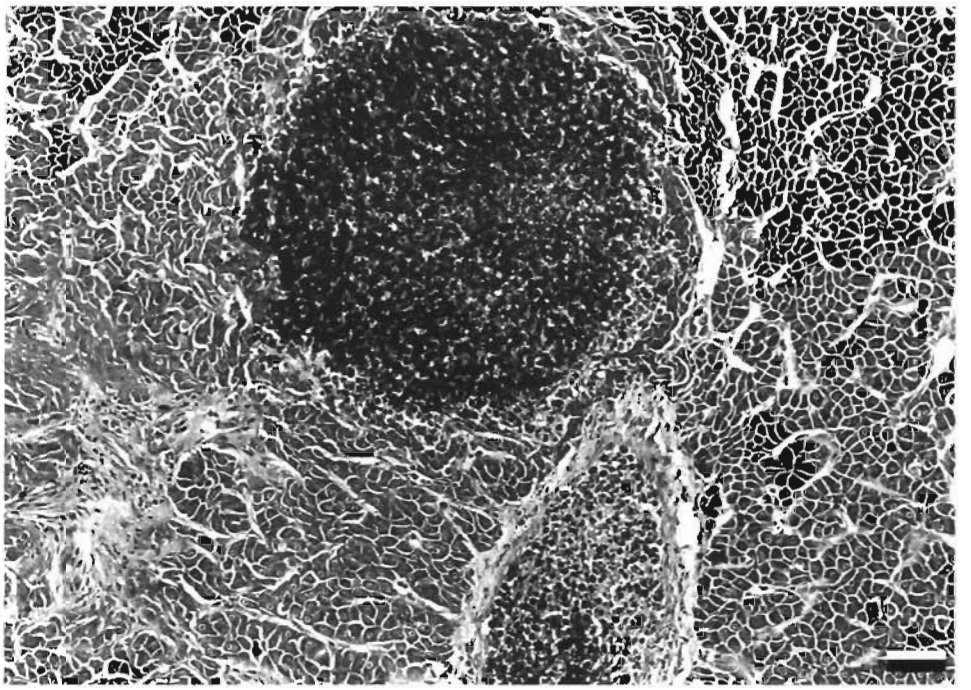

Fig. 2. *Salmo salar*. Abundant *Mycobacteria chelonae* in large, circular, dense, hard-packed nodules in the liver from naturally infected Atlantic salmon. These nodules comprise epitheloid cells with no surrounding capsule or fibrin deposition or central necrosis. Ziehl-Neelsen stain. Scale bar = 80 µm

tory response to the bacteria was noted. Mycobacteria were present in large numbers within phagocytic cells in the liver and kidney (Fig. 3) with displacement of the tubules. In the liver, bacteria were present within the portal veins and there was slight degeneration of the endothelial tract. Similar lesions occurred in the spleen with concurrent congestion. Soft nodules were generally associated with a graded necrosis from the bacterial focus interspersed with melanin particles, but there was no calcification. Multinucleate giant cells were not evident in any tissue. The mycobacteria stained bright red by Ziehl-Neelsen, were weakly positive using Gram staining and only a light pink using H&E.

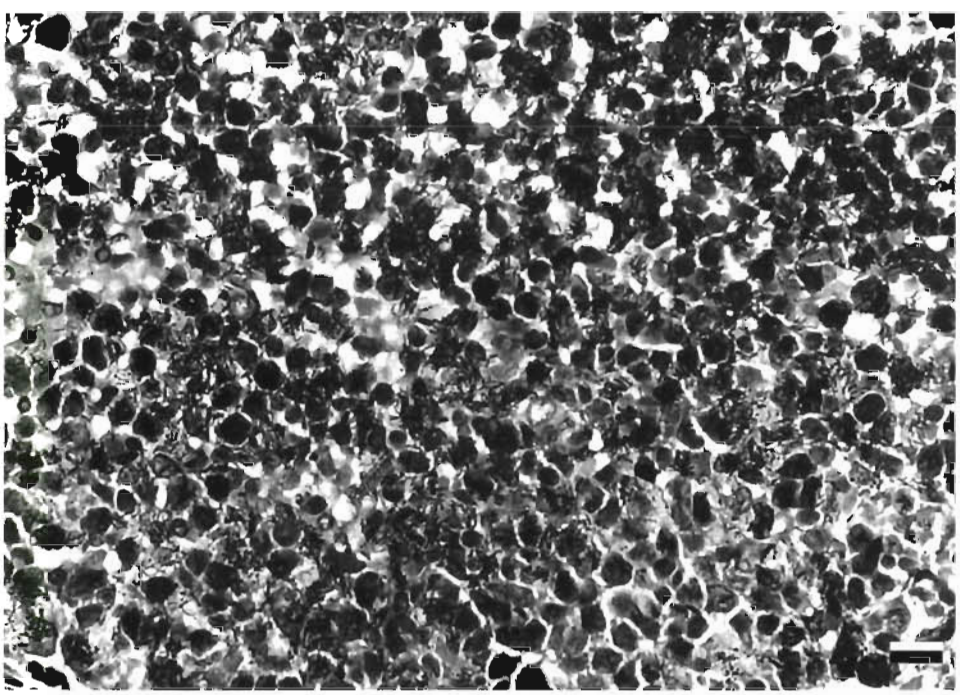

Fig. 3. *Salmo salar*. *Mycobacteria chelonae* proliferation in the kidney of naturally infected Atlantic salmon. Ziehl-Neelsen stain. Scale bar = 20 µm

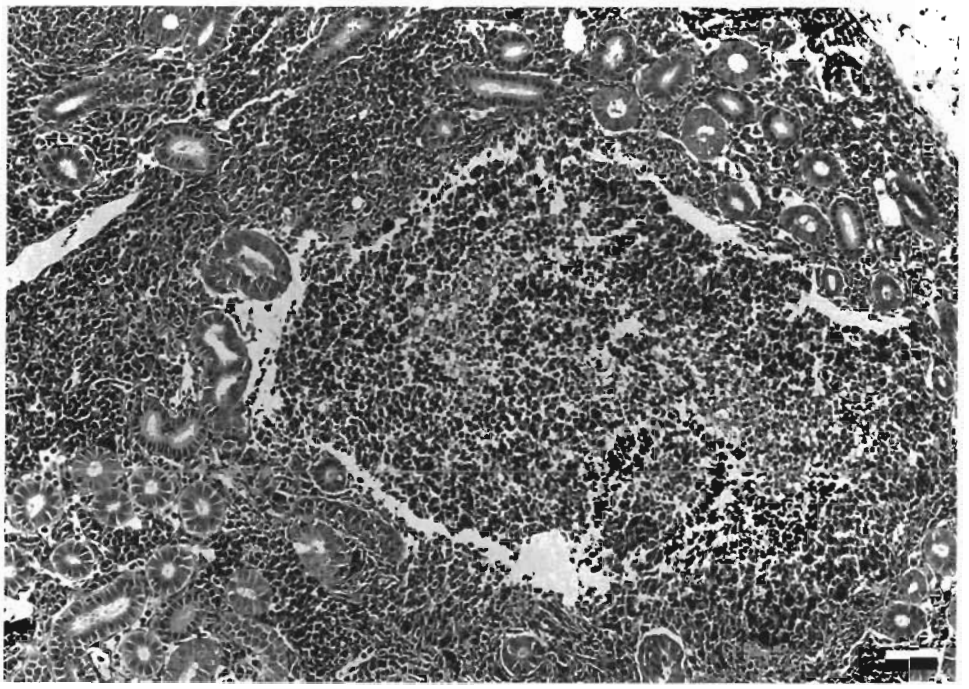

Fig. 4. *Salmo salar*. *Mycobacteria chelonae* present in kidney of naturally infected Atlantic salmon. The section shows compression of adjacent structures. Ziehl-Neelsen stain. Scale bar = 100 µm

Subsequent tissue sampling at farms I and II 21 d later revealed fewer nodules containing acid-fast bacteria. Fish examined at farm II had many extracellular acid-fast bacteria and adjacent caseous necrosis in the kidney with a sparse fibrotic reaction (Fig. 4). Renal elements were similarly compressed and the tubules and glomeruli destroyed. An increase in granular cells in the intestinal granulosum was noted. Throughout the laboratory study, condensed deposits of slightly darker staining, acid-fast debris were noted within the lining of the kidney tubules, or within the splenic pulp with localised necrosis (Fig. 5). This feature was typical of the farmed fish from farm II and similarly the laboratory-infected fish. The distinct granuloma-like

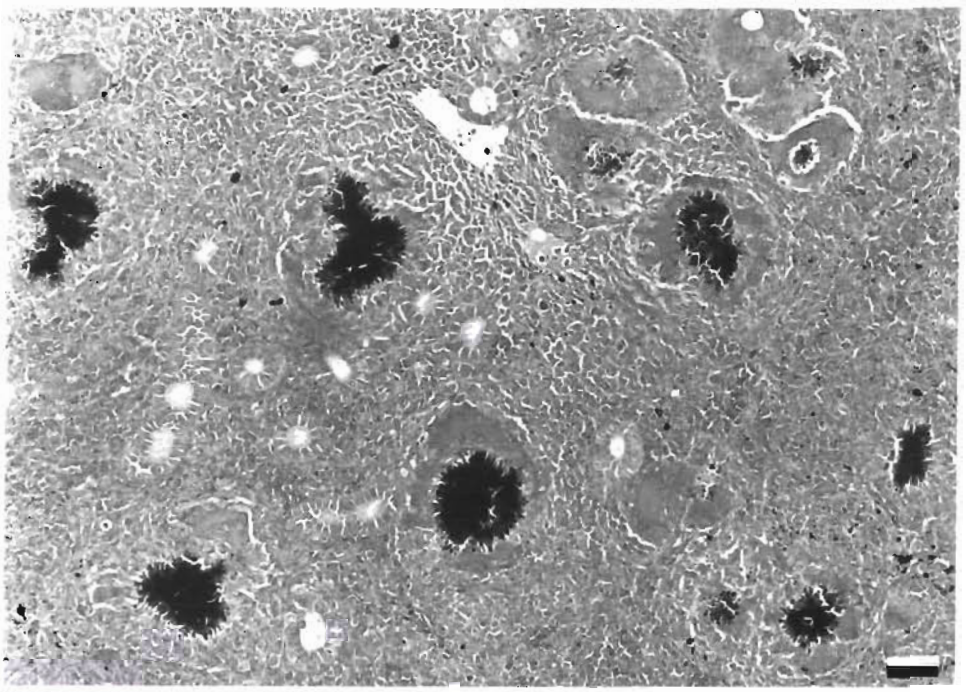

Fig. 5. *Salmo salar*. Condensed deposits of acid-fast debris present within the kidney tubule lumen of experimentally infected Atlantic salmon. Ziehl-Neelsen stain. Scale bar = 100 µm

nodules packed with acid-fast bacteria were only recorded in 1 laboratory-infected fish. An increase in granular cells in the intestinal granulosum was noted.

Virology

Tissue samples collected from the field were tested and found negative for IPNV, IHNV and VHSV.

Lipid chromatographic profile and PCR

Following the biochemical identification of the isolated bacteria as *Mycobacterium chelonae*, additional confirmatory tests were carried out. A lipid chromatographic profile and the presence of digested PCR-product bands of 325 and 140 base pairs using the *Bst EII* enzyme and 210 base pairs for *Hae III* indicated that the cultures isolated were *M. chelonae*.

DISCUSSION

The above investigation was carried out following reports of increasing mortality among Atlantic salmon *Salmo salar* on 2 farms in the Shetland Isles. A *Mycobacterium* sp. was isolated in pure culture from fish at both sites and identified as *M. chelonae* using standard cultural and biochemical characteristics (Heifets & Good 1994, Collins et al. 1997). The identification of *M. chelonae* was strengthened by the correlation between the biochemical properties and subsequent TLC of cell lipids and molecular DNA analysis of the *hsp65* gene (Telenti et al. 1993). In some studies, identification of infections involving fish mycobacteria has not always been practical (Lund & Abernethy 1978, Colorni 1992). However, Arakawa & Fryer (1984) characterised non-chromogenic mycobacteria isolated from fish, which has helped with classification of this group.

The occurrence of *Mycobacterium chelonae* and its association with gross lesions in farmed Atlantic salmon is considered rare, with few examples evident in the literature. Furthermore, there are no apparent records of the occurrence of this bacterium in farmed salmon in the United Kingdom. Currently, mycobacterial infections are infrequent among farmed Pacific salmonids *Oncorhynchus* spp., although during the 1950s infection was widespread (Parisot & Wood 1960, Ross 1960). The withdrawal of unpasteurised fish meal from diets has reduced the prevalence of this disease. Currently, mycobacteriosis occurs predominantly in fish species cultured in warm waters, including the European sea bass *Dicentrarchus labrax* (Colorni 1992), striped bass *Morone saxatilis* (Hedrick et al. 1987) and the cichlid *Oreochromis mossambicus* (Noga et al. 1990).

The mortality recorded from 2 commercial farms in this study was significant, both in terms of the numbers involved and the commercial value of these affected market-sized fish. In addition to *Mycobacterium chelonae*, some of the mortality amongst these fish could be attributed to infection by *Vibrio* sp., which was isolated in pure culture from fish showing clinical signs of vibriosis. In addition to this, *Aeromonas* sp. had previously been recovered from 1 site. Gross examination of moribund fish allowed a provisional distinction to be made between infection by *Vibrio* sp. and that caused by *M. chelonae*. Subsequent culture and bacteriological analysis established that these fish were infected with a single rather than a dual infection. Gross examination of the moribund fish showed lesions consistent with mycobacteriosis in up to 40% of the fish. It was concluded that a significant proportion of the overall mortality could therefore be attributed directly to the mycobacteria. Previous reports have highlighted the significant losses that occur as a result of mycobacterial infections. In particular, the work of Hedrick et al. (1987), who attributed losses of 50% to *Mycobacterium marinum* in cultured striped bass, and Ashburner (1977), who reported losses of between 4 and 42% over 4 yr from infection with *M. chelonae* in chinook salmon *Oncorhynchus tshawytscha* in hatcheries.

Viable *Mycobacterium chelonae* were isolated from fish throughout the laboratory study, therefore confirming Koch's postulates, but in these laboratory experiments no mortality occurred. Gross changes typical of those noted in naturally infected fish were seen in only 1 fish over the 120 d experimental period. This observation confirms the chronic nature of naturally occurring fish mycobacteriosis as recognised by Ashburner (1977) and Noga et al. (1990).

Abundant acid-fast bacteria contained within large nodules in the kidney, liver and spleen were located in naturally infected fish by light microscopy. Epithelioid cells were the dominant cell type and the nodules were enclosed in a thin capsule. The absence of any other inflammatory response or giant cell formation is consistent with the observations for *Mycobacterium anabanti* in the three spot gourami (Santacana et al. 1982), with the case of mycobacteriosis reported in mountain whitefish *Prosopium williamsoni* (Lund & Abernethy 1978) and with the infection in juvenile chinook salmon (Parisot & Wood 1960). Mycobacteria occurred intracellularly and were scattered throughout tissues or present at the peripheral portion of lesions, as described by Lund & Abernethy (1978). Nodules were observed and 2 distinct stages of development recorded. The compact nodules had no epithelioid sheath or central necrosis, as reported by Hedrick et

al. (1987), and the soft nodules were generally associated with a graded necrosis from the bacterial focus. Similarly, Majeed et al. (1981) also noted the development of soft nodules in goldfish *Carassius auratus*. In the laboratory-infected fish, acid-fast staining debris occurred within the kidney tubule lumen. Intact bacteria were not observed in these tissues, suggesting that the bacteria were dying or were killed by the host immune response. This response was clearly limited as viable cells of *M. chelonae* were recovered on TSA throughout the experimental period.

The distance separating farms I and II is not unusual for many fish farms operating within waters around the Shetland Isles. These farms operate as independent companies with no movement of fish between the installations. The only connection in common was a freshwater stock origin. The purchase of smolts from the same supplier might suggest a freshwater origin for this outbreak, although the health status of these fish would have been regularly monitored and no prior evidence of mycobacteriosis was recorded. High infection levels in hatchery salmonids have been reported in Oregon, USA (Arakawa & Fryer 1984) and a prevalence of 15% in some wild fish populations noted (Parisot & Wood 1960). Furthermore, some reports of *Mycobacterium chelonae* infection have specifically linked outbreaks with the freshwater hatchery environment (Daoust et al. 1989, Inglis et al. 1993). This, however, would not explain the presence of *M. chelonae*-infected fish in the unrelated stock held by one farm, suggesting that horizontal transmission may have occurred in sea water. Several farmed fish from the study reported here were recorded with skin lesions and these may be areas from where bacterial shedding could occur. Further study on the source of this infection was not carried out. Interestingly, Inglis et al. (1993) reported that infection with *M. chelonae* had only been identified in cold water fish, which is consistent with the outbreak reported here.

Mycobacterium chelonae outbreaks involving farmed Atlantic salmon are rare, but in this study the organism contributed, probably directly to a significant mortality in valuable farmed stock of market size.

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