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Experimental mycobacteriosis in Atlantic Salmon, *Salmo salar* and Rainbow Trout, *Oncorhynchus mykiss*

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ABSTRACT

Mycobacteriosis is a progressive disease of a wide range of wild and captive marine and freshwater fish species. Mycobacterium marinum, M. fortuitum and M. chelonae are the most frequently reported species to be involved in the disease, although several new species of Mycobacterium have recently been reported to be involved. In the present study, rainbow trout, Oncorhynchus mykiss, and Atlantic salmon, Salmo salar, were inoculated intraperitoneally with 10^7 cells of M. salmoniphilum (NCIMB 13533) and maintained at 15˚C. Infected fish were sampled for histopathology, bacteriology, and polymerase chain reaction (PCR)at weeks 4, 6, 8, 10 and 12 post-infection. While a number of rainbow trout died or were clinically ill by week 3 post-infection, there were no clinical abnormalites in the Atlantic salmon except for anorexia. Pronounced diffuse granulomatous inflammation was seen in the peritoneal cavity of the salmon, comprising large numbers of mainly epithelioid macrophages, and an occasional multinucleated giant cell. By contrast, the response in the trout was minimal. Multifocal granulomas were not observed in the internal organs of either species of fish as previously reported to occur in naturally infected fish. Some acid-fast bacteria were detected in the early stages of infection in rainbow trout and in all cases of Atlantic salmon by Ziehl-Neelsen staining. Unlike Atlantic salmon, from which Mycobacterium was isolated from the anterior kidney at all sample times, no bacteria were detected after 8 weeks in rainbow trout. Using species-specific probes, PCR was used to confirm the presence of the bacterium, especially in samples which were positive by culture. The presence of bacteria in internal organs of Atlantic salmon over the duration of the trial indicated a sub-clinical infection and reflects the chronic nature of mycobacteriosis in this species.

Keywords: *M. salmoniphilum*, Experimental infection, Atlantic salmon, Rainbow trout

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INTRODUCTION

Mycobacteriosis is a progressive disease of a wide range of wild and captive marine and freshwater fish species.Severity ofinfections can range from chronic, associated withlow mortality, to more serious outbreaks in whichthe entire colonies have to be killed [1].Clinical signs of fish mycobacteriosis are non-specific and may include emaciation, anorexia, exophthalmia, ascites, pigmentation changes and dermal ulcerations [2-3]. Miliary granulomatous inflammation throughout the viscera is characteristic of mycobacteriosis, and enlargement of affected organs may occur [4]. Histologically, granulomas resemble those found in mammalian mycobacterial infections, and acid-fast bacilli are usually present [5].

Mycobacterium chelonae, *M. marinum*and*M. fortuitum* are the most frequently reported species to be involved in the disease [6-7]; however, many recentstudies have revealed that an increasing number of mycobacterial species can be responsible for the disease in fish [8-11].

The mycobacterial infection of freshwater fish was first described as early as 1897 [12]. In areas of the temperate zone and in the tropics, mycobacterial infection is among the most common chronic diseases of freshwater and sea fish [13-14]. In the last few years, mycobacteriosis has been described in many species of freshwater and sea fish in the wild and kept in captivity, in which it causes major economic losses. The salmonid fish, rinbow trout,*Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar*, are among the numerous commercially important fish species that could be affected by mycobacteriosis. In this respect, the occurrence of *M. salmoniphilum* among salmonid fish is considered rare, with few examples in the literature. Thus, in this study the ability of *M. salmoniphilum* in producing of infection in these two most important cold-water fish species was experimentally investigated.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Mycobacterium salmoniphilum (NCIMB 13533) was grown on Lowenstein-Jensen slants at 25 °C for 3-5 days. Blood agar plates were used for determining colony forming units (CFUs). After growth of the inoculum, bacterial colonies were washed and resuspended in phosphate buffered saline (PBS), spun at 3000 g for 15 min at 4 °C, and thereafter, concentration of the final mycobacterial suspension was adjusted by using McFarland standards and verified by plating.

Fish

Rainbow trout and salmon (body weight 50±2 g) were obtained from a local farm and acclimatized for 2 weeks at the research facility at Ilam University, Iran. Water temperature was maintained at 12±2 ºC, and ammonia and nitrate concentrations in the aquaria were controlled by monitoring levels with test kits and periodic water changes. Each aquarium was equipped with a biological filter made from a box-type aquarium filter. For each of the above species, 10 fish were anaesthetized in a 0.02% solution of ethyl-3-aminobenzoate methanesulphonate salt (MS-222) prior to infection by intraperitoneal injection (ip) with 50 µl *M. salmoniphilum* suspended in PBS containing 2.2×10^6 CFU/ ml. In addition, 10 fish of both species, were injected ip with 50 µl sterile PBS and transferred to a separate tank with clean water as a sham control.

Sampling and histological examination

Three fish from each group were sacrificed at 4,6, 8, 10 and 12 weeks post-infection. Aseptic necropsies of the fish were conducted within a Biosafety Level 2 laminar flow cabinet. Small portions of spleen, kidney and liver were fixed in 10% neutral buffered formalin for 48 h at room temperature and embedded in paraffin wax after processing using a Tissue Processor (Leica Biosystems, Germany). Five µm sections were prepared using a rotary microtome. Slides were de-waxed, and stained with H & E and ZN. Stained tissue sections were observed for the presence of granulomatous lesions at 40 X and 100 X magnifications. In addition, approximately 0.25 – 0.5 g of spleen tissue from each fish was aseptically collected and added into a 2 ml microcentrifuge tube (Eppendorf, Germany) containing sterile PBS for bacteriological analysis. A second, similar-sized sample of each spleen was placed in an autoclaved microcentrifuge tube for molecular processing.

DNA extraction and PCR

Briefly, a loopful of bacterial colonies was washed twice in PBS and resuspended in 500 ul extraction buffer (EB) (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). In screw-capped microcentrifuge tubes, the suspension was subjected to 3 cycles of 5 min freezing at – 20 °Cand12 min heatingin a boiling water bath. In the case of frozen splenic tissues, approximately 500 µl of the thawed homogenate was spun briefly and the tissue pellet was resuspended in 500 µl EB as defined previously. Additional purification of the genomic DNA was then carried out by phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation as described elsewhere [15]. The genomic DNA content was quantified, using Eppendorf spectophotometry. Approximately 500-1000 ng of purified DNA preparation was utilized as template DNA in the PCR reaction.

Amplification of a portion of the 16S rRNAgene (121 bp) was used for identification of both DNA isolated from spleen and bacteria recovered from spleen homogenates. Three to five µl of each respective DNA sample was amplified with primers SalF (5´-GGGGTACTCGAGTGGCGAACG-3´) and SalR (5´-TGCACCACTCACCATGAAGTGT-3´) in 25 µl PCR reactions using *Taq* DNA polymerase (Fermentas, Ukraine). Each reaction contained 1.5 units of enzyme, 200 mM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, and 10 pmol of each primer and ultra-pure water. Cycling was performed in a gradient thermal cycler (Eppendorf, Germany) as follows: 95 °C for 5 min; 35 cycles of 95 °C, 55 °C and 72 °C each for 1 min followed by final extension at 72 °C for 7 min. Five microliters of each reaction mixture was analyzed on 1.2% agarose gels stained with ethidium bromide for visualization of PCR products. A no-template DNA negative control was included with each run of PCR.

RESULTS

Three out of 10 rainbow trout were clinically ill and 1 fish was dead by week 3 post-infection (pi), whereas there were no clinical abnormalites in the Atlantic salmon except for anorexia. Pronounced diffuse granulomatous inflammation was seen in the peritoneal cavity of the salmon, comprising large numbers of mainly epithelioid macrophages, and an occasional multinucleated giant cell (Fig. 1).By contrast, the response in the trout was minimal.

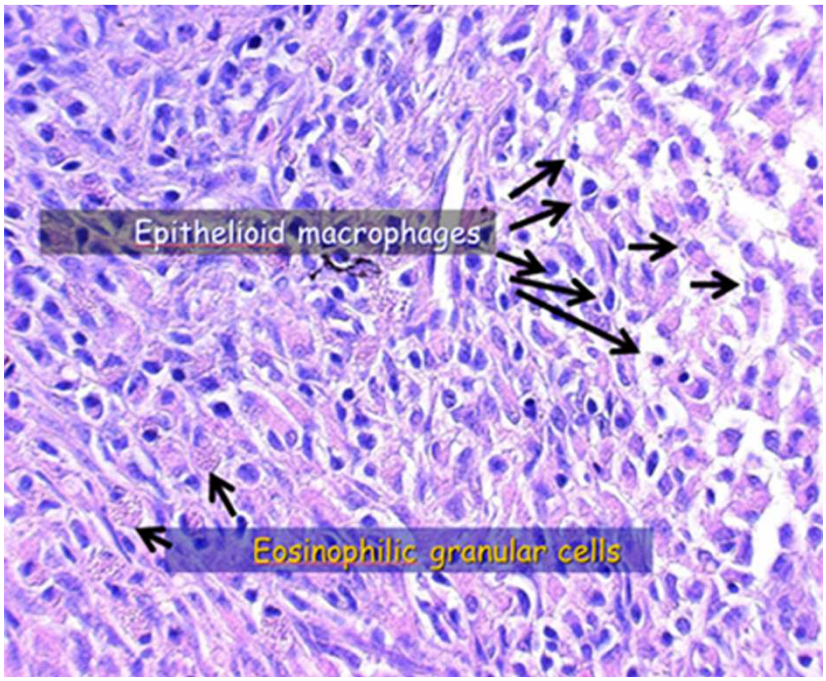

Fig. 1: Granulomatous inflammation in spleen tissue of an Atlanticsalmon experimentally infected with *M. salmoniphilum* (×40 magnification)

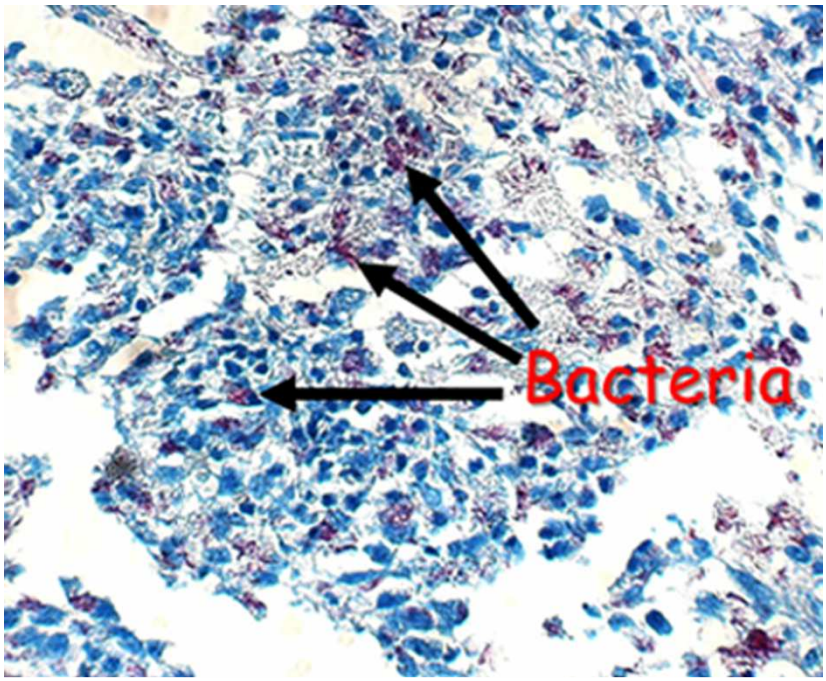

Fig. 2: Ziehl-Neelsen staining of the infected kidney tissue in an Atlantic salmon (×40 magnification)

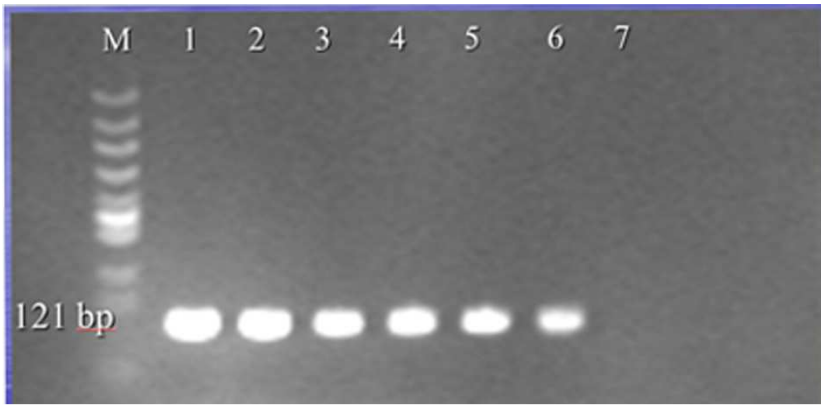

Fig. 3: **Fig. 1. PCR amplification of DNA extracted from Atlantic salmon spleen following experimental infection with *M. salmoniphilum***
Lanes: M= molecular size marker, (1)M. salmoniphilum (NCIMB 13533) (+ve control); (2) week 4 pi; (3) week 6 pi; (4) week 8 pi; (5) week 10 pi; (6) week 12 pi, 7) TE buffer (–ve control)

Some acid-fast bacteria were detected in the early stages of infection in rainbow trout and in all cases of Atlantic salmon by Ziehl-Neelsen staining (Fig. 2). Unlike Atlantic salmon, from which *Mycobacterium* was isolated from the anterior kidney at all sample times, no bacteria were detected after 8 weeks in rainbow trout.

All of the DNA templates either extracted from bacteria isolated from experimentally infected fish or directly obtained from splenic tissues resulted in amplification of 121 bp PCR product, confirming detection of the 16S RNA gene of *M. salmoniphilum* that used in this experiment (Fig. 3).

DISCUSSION

The mycobacteria, comprising the genus *Mycobacterium*, are typical aerobic, Gram-positive, rod shapes and acid-fast staining bacteria.Mycobacteriacause disease in more than 160 species of freshwater and marine fish species [6,13].The infection is very common in aquarium fish [16-17], and is a major concern for zebrafish, *Danio rerio*, in research facilities [1, 18]. In the temperate zone and in the tropical areas, mycobacterial infection is among the most common chronic diseases of freshwater and marine fish [13-14, 19]. *Mycobacterium salmoniphilum*, a species more common in salmonid fish, has been described by Whipps et al., in 2007 [20]. Since then, only few studies have shown the association of this species in salmonid and non-salmonid fish[21-22]. In this research, we have conducted an experimental infection to fulfil Koch's postulates for pathogenicity of this organism in two salmonid fish.

The presence of bacteria in internal organs of Atlantic salmon over the duration of the trial indicated a sub-clinical infection and reflects the chronic nature of mycobacteriosis in this species. Multifocal granulomas were not observed in the internal organs of either species of fish as previously reported to occur in naturally infected fish [2].
In this study we developed an in house PCR.Using this 16S rRNA species-specific primer set, it was possible to confirm the presence of the bacterium, especially in samples which were positive by culture.This gene is highly conserved amongst all *Mycobacterium* spp. and thus represented a reliable type of DNA to serve as an indication of mycobacterial infections in fish species.

The presence of bacteria in internal organs of Atlantic salmon over the duration of the trial indicated a sub-clinical infection and reflects the chronic nature of mycobacteriosis in this species. The differences in the immune system of Atlantic salmon and rainbow trout may contribute to inter-species variation in mycobacteriosis susceptibility and inflammatory response. However, further analysis employing a wide number of fish is warranted to further support the results of this study.

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