

MYCOBACTERIOSIS IN A BULL TROUT FROM HUNGRY HORSE RESERVOIR, MONTANA

Marc M. Terrazas, Montana Fish, Wildlife & Parks, 4801 Giant Springs Road, Great Falls, MT 59405
Leo R. Rosenthal, Montana Fish, Wildlife & Parks, 490 North Meridian Road, Kalispell, MT 59901
Kenneth D. Staigmiller, Montana Fish, Wildlife & Parks, 4801 Giant Springs Road, Great Falls, MT 59405

Kevin R. Snekvik, Washington Animal Disease Diagnostic Laboratory and the Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

Daniel S. Bradway, Washington Animal Disease Diagnostic Laboratory, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

ABSTRACT

An angler-caught bull trout (*Salvelinus confluentus*) from Hungry Horse Reservoir, MT with noticeably poor condition was examined to reveal liver nodules. Further investigation discovered acid-fast bacteria present in these granulomas making this a unique finding requiring further diagnostics. Molecular diagnostics revealed the infectious agent to be *Mycobacterium fortuitum*. This is a range and species expansion for this pathogen in fish. Further examination of additional fish from this water body did not reveal cases similar to this one, allowing for the possibility of this being a lone occurrence.

Key Words: Mycobacteriosis, *Mycobacterium*, bull trout, fish

INTRODUCTION

Mycobacteriosis is a common disease affecting cultured and wild fishes worldwide (Gauthier & Rhodes, 2009). Infected fish exhibit clinical signs including loss of scales, depigmentation, hyperpigmentation, cachexia, abnormal behavior and lethargy (Lansdell *et al.*, 1993). Postmortem examination often reveals gray to white nodules in many organs (Heckert *et al.*, 2001) due to this infection. In fish, kidney, liver and spleen tissue are commonly found with nodules or granulomas present in infected individuals (Heckert *et al.*, 2001). Historically three species have been recognized to be pathogenic to fish; *M. chelonae*, *M. fortuitum*, and *M. marinum* (Inglis *et al.*, 1993, Belas *et al.*, 1995, Heckert *et al.*, 2001).

Originally *Mycobacterium* spp. were identified by a limited number of phenotypic characteristics which led to variability in diagnosis (Gauthier & Rhodes, 2009). With the advancement of diagnostic techniques there has been documentation of an increasing number of *Mycobacterium* spp. infecting fish (Kaattari *et al.*, 2006; Gauthier

and Rhodes, 2009). Mycobacteriosis is not typically associated with large-scale mortality in wild fishes, although significant losses attributed to the disease have occurred in aquaculture (Hedrick *et al.*, 1987; Bruno *et al.* 1998). However, these infections have been detected in an increasing number of wild populations (Abernethy and Lund 1978, Daoust *et al.*, 1989, Lansdell *et al.*, 1993). While our knowledge has changed dramatically, the impacts of mycobacteriosis are still inadequately understood for most wild populations (Heckert *et al.*, 2001).

METHODS

Hungry Horse Reservoir is an impoundment of the South Fork Flathead River in the upper Flathead drainage, Northwest Montana (Figure 1). The dam creating the impoundment is 564 feet tall, and is the 10th tallest dam in the United States. The resulting reservoir is 34 miles long and covers 23,800 surface acres. The dam acts as a large barrier and the associated reservoir and river drainage have largely retained their native fish species assemblage. This system is home to one of the only

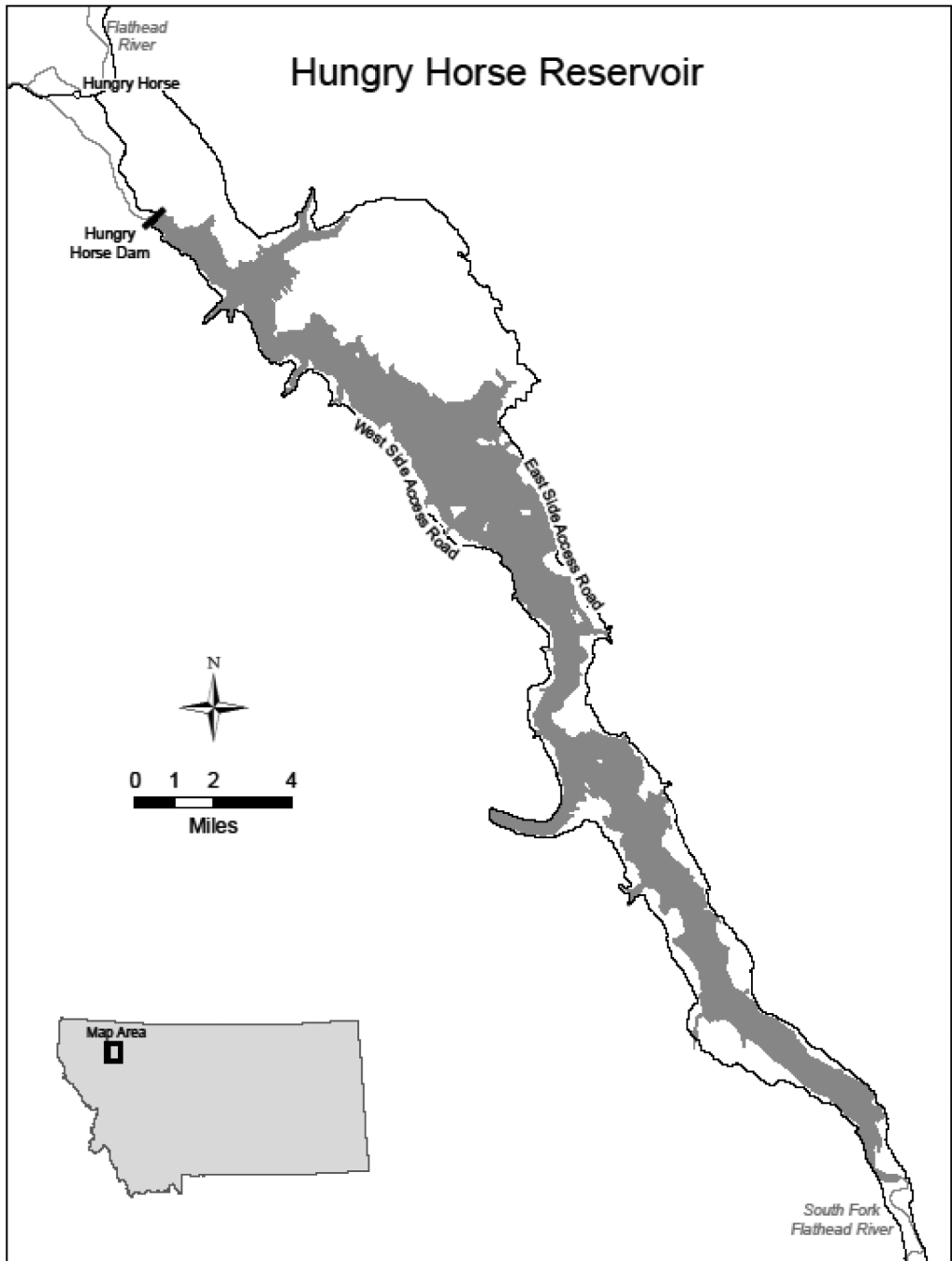


Figure 1. Hungry Horse Reservoir, where the angler caught the infected bull trout

remaining prosperous bull trout populations, a species listed as threatened under the Endangered Species Act, in the contiguous 48 states and is currently the only location bull trout can be legally harvested in Montana.

An angler-caught bull trout was brought in to the Kalispell area office of Montana Fish, Wildlife & Parks (MFWP) in June of 2009. This fish was in noticeably poor condition and was examined internally and externally for gross pathology. Upon

noticing many large nodules in the liver, the fish (Figure 2) was frozen and sent to the MFWP Fish Health Lab for further diagnosis. Upon receipt at the Fish Health Lab the specimen was again examined grossly and tissue smears and imprints were obtained. Portions of kidney, liver and spleen were both Gram stained and acid-fast stained using a TB Quick Stain kit (Becton Dickinson; Franklin Lakes, New Jersey). Upon detection of acid-fast rods, a portion of the liver was preserved using Davidson's fixative and sent to the Washington Animal Disease Diagnostic Laboratory (WADDL) for further identification.

Upon receipt at WADDL, four grossly representative sections of the submitted liver were trimmed, dehydrated through graded ethanol processing and embedded in paraffin wax blocks for histological analysis. Paraffin wax blocks were sectioned at 4 μm and the resulting sections stained with routine hematoxylin and eosin or a Kinyoun's acid-fast stain (Gibson Laboratories; Lexington, KY). After confirmation of acid-fast bacteria consistent with MFWP findings from gross examination and impression smears the imbedded tissue was submitted for molecular diagnostic testing to more specifically identify the bacteria. Samples were screened for the presence of mycobacteria by utilizing conventional PCR and sequencing of the genus specific region of the ribosomal 16S-23S intergenic transcribed spacer (ITS) region. The sequencing and analysis of this region has been validated as suitable targets for mycobacterial identification and the 16S-23S ITS region possesses greater sequence variation than the 16S rDNA (Mohamed, *et al.*, 2005). This provides a greater ability for mycobacterial species differentiation between genotypically similar bacteria (Ngan *et al.*, 2011). DNA was extracted from the paraffin block using a Qiamp DNA Mini-kit (Qiagen; Valencia, CA). The extracted DNA was used as a template for PCR amplification using *Mycobacterium* genus-specific primers to amplify a portion of the 16S-23S rRNA internal transcribed spacer (ITS) region as previously described

(Roth *et al.*, 2000). Primers Sp1 (5'-ACC TCC TTT CTA AGG AGC ACC-3') and Sp2 (5'-GAT GCT CGC AAC CAC TAT CCA-3') were used, yielding a 225 base pair amplicon. The PCR conditions entailed 5 min of denaturing at 94°C, followed by 35 amplification cycles consisting of 1 min of denaturing at 94°C, 1 min of annealing at 58°C, and 1 min of extension at 72°C, with a final extension cycle of 7 minutes at 72°C. Extracted DNA from *M. avium* was used as a positive control. Water was extracted and run as a negative extraction control. The PCR amplicons were visualized on a 1.5% agarose gel containing ethidium bromide, excised using a sterile scalpel blade under UV illumination, and purified using a Freeze 'n' Squeeze kit (BioRAD; Hercules, CA). The amplicon DNA was sequenced directly on both strands by a commercial vendor (Genewiz; South Plainfield, NJ). Forward and reverse sequences were aligned using Clustal W on-line (Thompson *et al.*, 1994). Sequences were compared with previously reported sequences using a BLAST search of GenBank online (NCBI, National Institutes for Health, Bethesda, MD).

RESULTS

Examination of slides at the MFWP Fish Health Lab was conducted at 1000x using oil immersion. Gram positive and acid-fast bacteria were only seen in the liver impression smears. The bacteria can be characterized as numerous brightly staining, red, short rods (Figure 3).

Histological examination of each of the produced slides of liver revealed numerous randomly scattered granulomas separated by abundant dense fibrous connective tissue interspersed with small to moderately sized aggregates of macrophages and remnant clusters of hepatocytes. The granulomas ranged from 100-700 μm in diameter and were characterized by a core of cellular debris and epithelioid macrophages in turn bordered by bands of collagen interspersed with fibroblasts. Serial sections that were stained with a Kinyoun's acid-fast stain revealed numerous bright pink bacilli

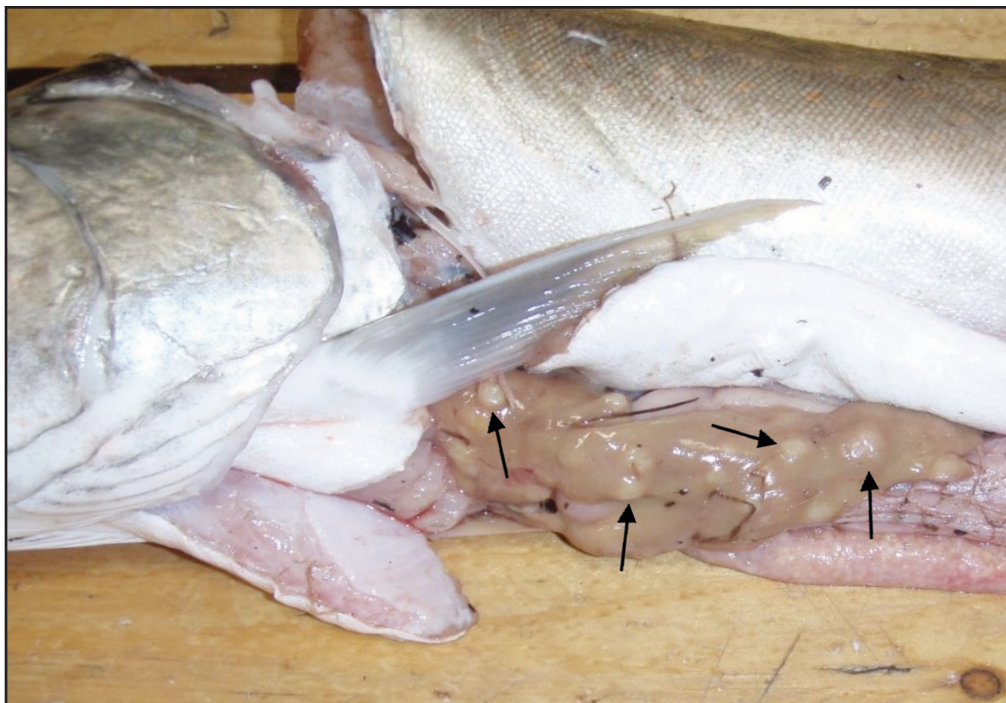


Figure 2. Bull trout with noticeable liver nodules (see →).

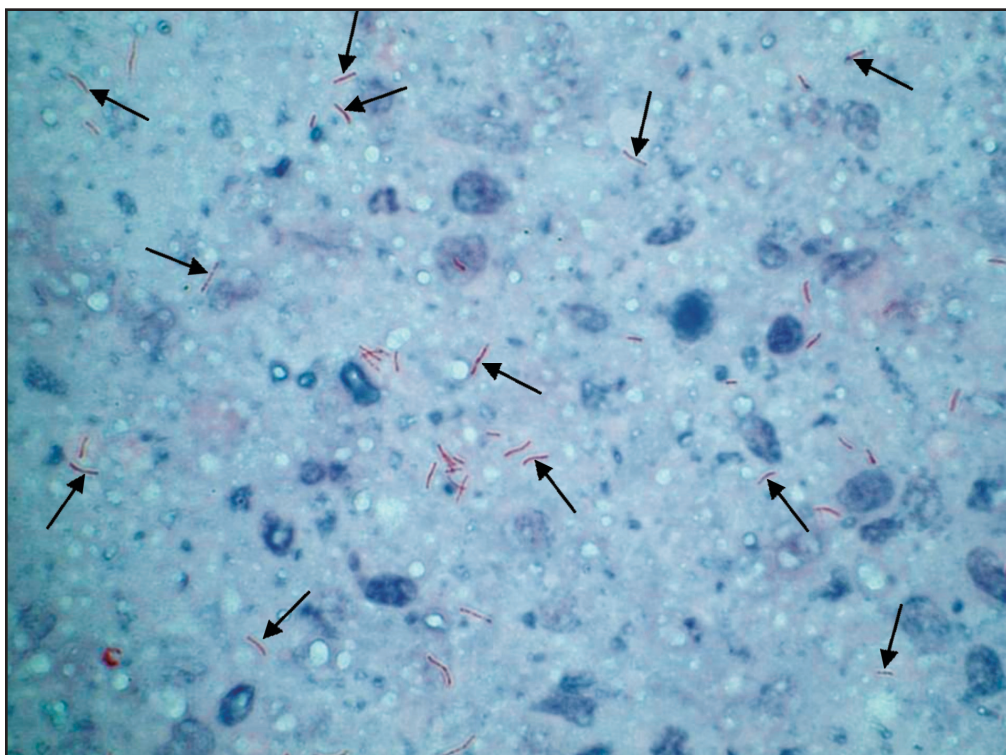


Figure 3. Mycobacterium fortuitum rods (see →) from liver smears, Magnification 1000x (Oil Immersion)

(presumptively *Mycobacterium* spp.) within the cytoplasm of macrophages in the granulomas and scattered within the contiguous connective tissue.

PCR with universal rRNA ITS primers and sequencing of the produced amplicon from DNA extracted from paraffin embedded tissue blocks was performed. The amplified sequence matched *Mycobacterium fortuitum* with 100% sequence identity to GenBank acc #DQ134000.1 when compared to sequences in GenBank and had no other close matches.

DISCUSSION

Mycobacterium fortuitum was first detected from neon tetra (*Paracheirodon innesi*) in 1953 (Ross and Brancato, 1959). It has since been isolated from numerous captive and wild fish (Bragg *et al.*, 1990, Lansdell *et al.*, 1993, Talaat *et al.*, 1999, Beran *et al.*, 2006,) but this detection is an expansion of the current documented extent of this organism. This is a unique finding given the isolation of the fish community from which it came and the uniqueness of the species it was affecting. Hungry Horse Reservoir is the only location in the contiguous U.S. where a bull trout may be legally harvested.

Detection by molecular methods alone wouldn't indicate if disease is present or if the bacteria were viable (Gauthier & Rhodes, 2009). Our diagnosis that acid-fast bacteria were present, along with the presence of DNA and gross clinical signs indicates that this was the pathogenic agent affecting this fish. There have been subsequent efforts to detect mycobacteriosis in Hungry Horse Reservoir. Fish were examined during the fall gill net sets of 2009 ($n = 144$) and 2011 ($n = 98$) with no fish of mixed species composition showing clinical pathology consistent with what was seen in the original bull trout specimen. This supports the conclusion that even if *Mycobacterium* DNA was present, it wouldn't be significant without the accompanying disease signs or observable bacteria present in the tissue.

ACKNOWLEDGEMENTS

We would like to thank the angler, Cody Schneigert, for bringing this bull trout specimen into our offices for further examination. Thank you to the MFWP Region 1 fisheries crews for bringing this unique case to our attention and collecting fish from Hungry Horse Reservoir for further investigation. Thank you to the diagnostic crews at WADDL for a definitive diagnosis on this unique case. In addition, thank you to Dave Stagliano and Phillip Sawatzki for improving this manuscript through the peer review process.

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Received June 15, 2012

Accepted November 19, 2012