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Validation of a single round polymerase chain reaction assay for identification of *Myxobolus cerebralis* myxospores

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ABSTRACT: Validation of a single round PCR-based assay to confirm as *Myxobolus cerebralis* myxospores obtained from pepsin-trypsin digest preparations is described. The assay is a modification of a PCR assay published previously, based on the amplification of a segment of the gene encoding the 18S ribosomal subunit of *M. cerebralis*. The sensitivity, specificity and upper and lower detection limits were determined using known *M. cerebralis* and non-*M. cerebralis* myxospores and *M. cerebralis*-free fish. The sensitivity of PCR confirmation was 100% (95% confidence interval of 83.2–100%). The specificity was 100% (95% confidence interval of 87.2–100%). The upper detection limit was approximately 100 000 myxospores per reaction; the lower detection limit was approximately 50 myxospores per reaction. Given the high sensitivity and specificity of the assay, substitution of this assay for histologic confirmation of *M. cerebralis* infection is encouraged.

KEY WORDS: *Myxobolus cerebralis* · Whirling disease

INTRODUCTION

Myxobolus cerebralis, the agent of whirling disease, replicates in the cartilage of salmonid fish, and can cause skeletal deformities, loss of equilibrium, whirling swimming patterns (from which the disease takes its name) and mortality (Markiw 1991). Recent declines in rainbow trout *Oncorhynchus mykiss* populations in certain Colorado and Montana river systems are at least partially attributed to whirling disease (Mathews 1995, Palmer 1995, Walker & Nehring 1995, Vincent 1996). As a consequence, natural resource agencies in several western states have initiated or intensified *M. cerebralis* surveillance programs. The state of Montana, for example, sampled over 20 000 fish from 230 separate waters from late 1995 through early 1997 (Baldwin et al. 1998). Fundamental to the success of such surveillance programs is the ability of laboratory procedures to detect *M. cerebralis* within sampled fish.

Two laboratory procedures are used commonly to detect *Myxobolus cerebralis*: a continuous plankton centrifuge assay (O'Grodnick 1975) and an enzymatic digestion procedure, commonly known as the pepsin-trypsin digest (Markiw & Wolf 1974). Both methods depend on liberation and collection of *M. cerebralis* myxospores from fish cartilage, and subsequent observation by light microscopy. Unfortunately, both assays provide a presumptive diagnosis only—the presence of myxospores morphologically consistent with *M. cerebralis*. As infections by non-*M. cerebralis* myxosporeans are common in salmonids (Hoffman 1990), presumptive positive cases require confirmation by histological examination of retained portions of sampled fish, or additional fish obtained from the population through further sampling efforts.

Confirmation by histopathology is an expensive, labor-intensive process, which too commonly fails. The

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problem is essentially one of including an infected focus in a 3 to 5 µm thick section (small lesions in large fish are quite problematic). If the number of infected fish is high and multiple cartilages affected, *Myxobolus cerebralis* infection can be confirmed using histopathology. Unfortunately, if disease prevalence is low and corresponding lesions sparse, confirmation by histopathology is difficult to impossible, and the trout population is erroneously classified as *M. cerebralis*-free. Moreover *M. cerebralis* preferentially infects cartilage at different sites in brown trout *Salmo trutta* versus rainbow trout, with gill arches most commonly affected in brown trout and ventral calvarium in rainbow trout (Baldwin et al. 2000). Samples available for histologic examination do not always include cartilage from preferentially infected sites.

An alternative to histologic examination is polymerase chain reaction (PCR) amplification of a DNA sequence unique to *Myxobolus cerebralis*. Andree et al. (1998) described such an assay, designed to produce a 415 base pair amplicon of the gene encoding the 18S ribosomal subunit of *M. cerebralis*. The sequence of the 415 base pair product is not shared by 4 non-*M. cerebralis* myxosporeans (*M. squamalis*, *M. kisutchi*, *M. neurobius*, and *M. arcticus*) that infect salmonids, but it is unknown if other myxosporeans share this sequence. The PCR assay as published is designed to amplify the *M. cerebralis* target sequence out of fish tissue samples rather than from myxospores harvested by pepsin-trypsin digest.

In an effort to circumvent the difficulties inherent in histologic confirmation, a modification of the Andree et al. (1998) PCR assay was developed to confirm as *Myxobolus cerebralis* myxospores harvested from pepsin-trypsin digests. The description and validation of this assay are the subjects of this report. Since PCR-based assays are not foolproof, assay validation is needed before substitution for histopathology occurs. Assay validation includes determination of the lower detection limit, upper detection limit, sensitivity and specificity (Hiney & Smith 1998). Validation also includes testing to ensure that myxospores obtained from geographically diverse areas and identified as *M. cerebralis* by conventional methods are uniformly detected (target species panel), and that non-*M. cerebralis* myxospores are not detected (related species panel).

The lower detection limit as used in this study is the minimum concentration of myxospores that produce a consistently detectable DNA product. Similarly, the upper detection limit is the maximum concentration of myxospores that yield a consistently detectable product. Sensitivity is the percent of true positives classified as positive by the PCR assay (Smith 1991). Specificity is the percent of true negatives classified as negative by the PCR assay (Smith 1991).

MATERIALS AND METHODS

Sample sets. In order to determine the detection parameters of the PCR assay, sample sets of (1) *Myxobolus cerebralis* myxospores, (2) non-*M. cerebralis* myxospores and (3) pepsin-trypsin digest samples from *M. cerebralis*-free trout were created. The *M. cerebralis* sample set consisted of 20 myxospore preparations, each obtained from pooled half-heads subjected to pepsin-trypsin digestion. Corresponding half-heads had histopathologic confirmation of *M. cerebralis* infection, ensuring that myxospores obtained in digest preparations were actually *M. cerebralis*. In an effort to enhance diversity, myxospore samples were chosen from rainbow trout, brown trout, brook trout *Salvelinus fontinalis*, cutthroat trout *Oncorhynchus clarki* and mountain whitefish *Prosopium williamsoni*. Trout samples originated from 5 western states over a period of 4 yr, and were obtained from wild populations, state operated fish hatcheries/rearing facilities and commercial fish farms.

The non-*Myxobolus cerebralis* sample set consisted of 7 myxospore preparations each obtained from pepsin-trypsin digests of pooled half-heads. Corresponding half-heads lacked histopathologic lesions typical of *M. cerebralis*; instead, myxospores present were localized to periosseous connective tissue, skeletal muscle and brain. Moreover, myxospore morphology was frequently different from known *M. cerebralis* myxospores. Myxospores were obtained from cutthroat trout, mountain whitefish, rainbow trout and mottled sculpin *Cottus bairdi*, a non-salmonid fish. The cutthroat trout, mountain whitefish, and mottled sculpin were obtained from wild fish populations in Montana; the rainbow trout were obtained from a state operated fish hatchery/rearing facility in Washington State.

The *Myxobolus cerebralis*-free trout samples consisted of 20 pepsin-trypsin digest preparations of half-head pools, each obtained from trout raised in a commercial aquaculture facility where whirling disease has never been detected (semiannual testing for 15 yr). No myxospores were seen in any preparation.

The target sample size of 20 was chosen because in order to have a 95% confidence level that target DNA sequences, if present, will be detected greater than 99% of the time, 3 independent PCR assays on a minimum of 20 separate digest preparations are necessary (Blyth 1986, adapted by J. Richard Alldredge, Program in Statistics, Washington State University, Pullman, WA). These numbers are applicable since the actual statuses of the samples examined are known. Hence, the known *Myxobolus cerebralis* and *M. cerebralis*-free study sets consist of 20 independent digest preparations. The number (7) of non-*M. cerebralis* myxospore samples was limited by available submissions.

Digest and histopathology. The heads from sampled fish were removed by cross sectioning just caudal to the opercular flaps, and typically pooled into 5 fish units. Each head was cut along the median plane and one-half enzymatically digested and one-half preserved in 10% neutral buffered formalin. A modification of the pepsin-trypsin digest as described by Markiw & Wolf (1974) was used to harvest myxospores from half-heads. Completed digest preparations were stored in 0.01 M phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) at –20°C. If myxospores were seen by light microscopy, heads from suspect groups were processed for histologic sectioning, sectioned, stained by routine methods and examined using light microscopy. Histologic classification of myxospores as *Myxobolus cerebralis* required the presence of typical pathology with intralesional myxospores (Baldwin et al. 2000).

Myxospore enumeration. The number of myxospores in each digest preparation was approximated using a standard hemacytometer. Briefly, myxospores were pelleted by microcentrifugation and re-suspended in 250 µl PBS. Ten µl of the spore suspension was combined with 2 µl of 1% trypan blue, applied to a hemacytometer and the number of myxospores counted in 5 grids. Two replicate counts were performed for each sample and averaged values used to approximate the number of myxospores. In samples with greater than 10 000 myxospores ml^{-1}, PBS was added to dilute the myxospore count down to 10 000. Samples containing approximately 100 myxospores produced the desired PCR product 100% of the time, when processed independently and in triplicate (see below). Hence, the volume used from each digest preparation for the PCR assays varied as necessary to contain approximately 100 myxospores. Samples containing fewer than 100 myxospores were not included in sample sets.

Myxospore lysis. Myxospores were lysed using a modification of the protocol described by Andree et al. (1988). The volume required to contain approximately 100 myxospores was removed from each preparation, placed into a microcentrifuge tube and myxospores pelleted by microcentrifugation (maximum speed, 1 min). The supernatant was removed and the pellet air-dried for 5 min. The microcentrifuge tubes were capped and myxospores exposed to microwaves for 1 min in an 800 W microwave oven at full power. The pellet was re-suspended in 500 µl of Proteinase K lysis buffer (10 mM Tris·CL, pH 8.0, 2 mM EDTA, 0.1% sodium dodecylsulfate, and 0.5 mg ml^{-1} Proteinase K) and incubated at 55°C for 4 h with gentle agitation.

DNA extraction/isolation. A total of 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each lysed myxospore preparation, and each preparation gently inverted several times to form an emulsion. The organic phase was separated from the aqueous phase by centrifugation at 1700 × *g* for 10 min at room temperature. The upper aqueous phase, containing DNA, was transferred to a new microcentrifuge tube and an additional 500 µl of phenol:chloroform:isoamyl alcohol added. The solutions were mixed and centrifuged as before. The aqueous phase was again transferred to a new microcentrifuge tube, 500 µl chloroform added, and the solutions mixed and centrifuged as before. The DNA was precipitated by adding 800 µl of ice-cold ethanol and incubating at –20°C for 1 h, followed by centrifugation at 12 000 × *g* for 10 min at 4°C. The supernatant was discarded and the DNA pellet permitted to air dry. The pellet was re-suspended in 50 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and held overnight at room temperature. Samples were warmed to 65°C for 1 h to ensure solubilization and subsequently allowed to return to room temperature prior to use in the PCR reaction.

Polymerase chain reaction. Primers used were supplied by Gibco-BRL and identified by Andree et al. (1998) as TR 5-16 (5′-GCATTGGTTTACGCTGATG-TAGCGA-3′) and TR 3-17 (5′-GGCACACTACTC-CAACACTGAATTTG-3′). The standard reaction volume was 50 µl (45 µl of master mix and 5 µl of DNA template). The PCR master mix was comprised of PCR buffer (300 mM Tris, 75 mM ammonium sulfate, pH 9.0), 2.5 mM MgCl, 400 µM dNTPs, 20 pmol of each primer, and 2 U $µl^{-1}$ taq DNA polymerase (Fisher Scientific). All reagents were stored at –20°C and kept on ice after thawing. Taq polymerase was the last reagent added.

Amplifications were performed using a Thermolyne Amplitron II thermal cycler (Barnstead/Thermolyne). A denaturation step in which samples were held at 95°C for 5 min took place before amplification cycles began. One complete cycle consisted of 1 min at 95°C, followed by 2.5 min at 65°C, followed by 1.5 min at 72°C. This cycle was repeated 35 times, after which an extended elongation step of 10 min at 72°C concluded the program. When necessary, amplified DNA samples were stored at 4°C.

Controls. Four control samples were included with every PCR assay, 2 to serve as lysis/extraction controls and 2 as PCR reaction controls. *Myxobolus cerebralis* myxospores obtained from trout with lesions pathognomonic for whirling disease were used to ensure that extractions were performed correctly (extraction positive control). Reagents used in myxospore lysis and DNA extraction and isolation were used to ensure that these reagents were free from myxospores or DNA fragments (extraction negative control). *M. cerebralis* DNA, identified by sequence matching (100%) with the reported sequences that encode the 18S ribosomal

subunit, was included to ensure that the PCR reaction occurred properly (PCR-positive control). Master mix reagents, including primers, were included to ensure that master mix reagents were not contaminated with DNA fragments (PCR-negative control).

Gel electrophoresis. PCR products were visualized by horizontal gel electrophoresis, using a 1.5% agarose gel containing 7.5 µl of 1% ethidium bromide in a TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) system. Ten µl of PCR product was diluted with 2 µl loading dye and 10 µl total volume was loaded into each well. A 100 base pair ladder was used to approximate the size of visualized products. Gels were run for approximately 1 h at 140 V. Amplified DNA bands were visualized and photographed under ultraviolet light.

Sequencing. The amplified DNA product from a myxospore preparation from the known *Myxobolus cerebralis* study set was purified using a PCR purification kit (QIAquick, Qiagen). Purified DNA was taken to the Laboratory for Biotechnology and Bioanalysis, Washington State University, Pullman, Washington and sequenced using a Perkin Elmer Applied Biosystems Prism Dye Sequencer. The sequence obtained was compared with sequences published through GenBank, National Center for Biotechnology Information using the BLAST (basic local alignment search tool) program available online (www.ncbi.nlm.nih.gov/BLAST/)

Upper and lower detection limits. The lower detection limit was determined by approximating the number of *Myxobolus cerebralis* myxospores in 3 separate digest preparations, and making from each, sub-preparations containing approximately 1000, 500, 100, 50 and 10 myxospores by dilution with PBS. Each of the sub-preparations was assayed independently in triplicate.

To determine the upper detection limit, *Myxobolus cerebralis* myxospore preparations containing approximately 100 000, 250 000 and 500 000 myxospores were made in triplicate. To obtain sufficient numbers, myxospores from multiple pooled-head submissions were combined, but myxospores used all came from fish with lesions pathognomonic for *M. cerebralis* infection. Each of the preparations was assayed in triplicate.

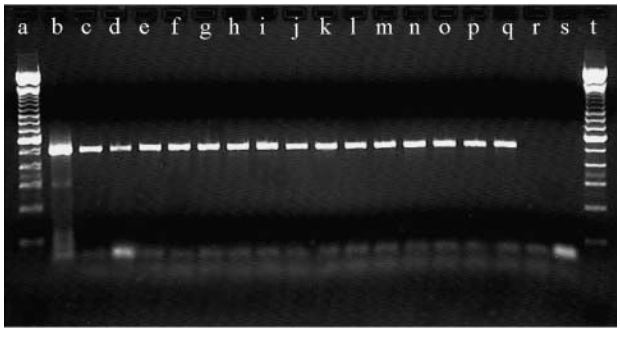

Fig. 1. Polymerase chain amplification products from myxospore preparations from the known *Myxobolus cerebralis* study set. Lanes a and t: 100 base pair molecular weight standards (Gibco-BRL). Lane b: DNA identified as *M. cerebralis* by sequence matching (100%) with reported 18S ribosomal sequences (PCR reaction positive control). Lanes c – e, f – h and i – k: digest preparations (in triplicate) from rainbow trout from New Mexico. Lanes l – n and o – q: digest preparations (in triplicate) from rainbow trout from Utah. Lane r: control for contaminating myxospores or amplicons in extraction reagents. Lane s: control for contaminating DNA in master mix components. Samples themselves constitute the extraction positive control

RESULTS

Sensitivity

A total of 60 PCR assays (20 samples, 3 independent assays each) were performed from the *Myxobolus cerebralis* study set. In 3 of the myxosporean preparations, only 2 of the 3 PCR assays produced the expected product, giving an overall total of 57 of 60 assays (95%). However, since at least 1 of the 3 independent assays produced the expected product, each myxospore preparation was classified as *M. cerebralis*. Hence, the overall sensitivity of PCR confirmation was 100% (95% confidence interval of 83.2–100%). A photograph of a typical gel is provided as Fig. 1.

Specificity

Three independent PCR assays were performed on each of the 20 digest preparations from *Myxobolus cerebralis*-free fish (60 total assays). No PCR product was seen on any assay. In addition, similar assays were performed on each of the 7 myxospore preparations in the non-*M. cerebralis* study set (total of 21 assays). No PCR product was observed on any assay. Similar to the sensitivity, the specificity of the PCR assay is 100% but the 95% confidence interval narrows (87.2–100%) due to the increase in sample numbers.

Detection limits

In determining the lower detection limit, the expected PCR product was seen on all replicates for samples containing approximately 1000, 500, 100 and 50 myxospores. A photograph of a typical gel showing replicates at the 100 myxospore level is provided as Fig. 2. The expected PCR product was seen approxi-

mately 50 % of the time from replicates at the 10 myxospore level (Fig. 2). The decision to run all subsequent assays at the 100 myxospore level was made to ensure sensitivity, while still keeping the number of myxospores required low.

In determining the upper detection limit, the expected PCR product was seen on all replicates for samples containing approximately 100 000 and 250 000 myxospores. The expected PCR product was observed in 1 of 3 samples containing approximately 500 000 myxospores.

DISCUSSION

Results demonstrate the utility of using a PCR-based assay to confirm as *Myxobolus cerebralis* myxospores obtained from pepsin-trypsin digests. Results, however, do not support the use of this assay as a first-screen diagnostic tool. A minimum of 100 myxospores is required, and hence the assay is not useful for digest preparations containing fewer myxospores (or none), or where microscopic examination reveals sparse structures that may, or may not be, actual myxospores. In these circumstances, an assay with greater sensitivity will serve better, such as the original nested procedure described by Andree et al. (1998). As a confirmatory assay, the single round procedure described here is preferred because non-nested procedures minimize the risk of false positives that result from contaminant amplicons, an occurrence more common in nested procedures. In other words, some sensitivity is sacrificed in single round assays to increase specificity (fewer false positives). Even so, in practice the single-round procedure works well since myxospores detected in pepsin-trypsin digest samples almost always number more than 100 (a single myxospore seen on a hemacytometer grid indicates the presence of at least 300 myxospores).

The assay described above is valid only under the conditions tested, which include independent processing of samples in triplicate. If only one test is made from an individual sample, the 95 % confidence interval of detection (based on a sample size of 20) decreases from over 99 to 86 % (Blyth 1986, adapted by J. Richard Alldredge, Program in Statistics, Washington State University, Pullman, WA). This decrease is evident in the data from the *Myxobolus cerebralis* study set where in each of 3 samples, 1 replicate assay failed to produce the expected product. If the laboratory decision were based solely on single assays, the samples may have been classified erroneously as negative.

The assay detected *Myxobolus cerebralis* myxospores from 5 different salmonid species sampled from 5 different states, over a period of 4 yr. Hence the target species panel indicates the assay will work on myxospores obtained from geographically diverse locations and from different salmonid hosts.

Testing on the related species panel (non-*Myxobolus cerebralis* myxospores was compromised since only 7 non-*M. cerebralis* myxospores were available for testing, and the identity of the myxospores used was unknown. In an effort to identify these myxospores, 2 sets of non-specific primers have been designed that amplify segments of the gene encoding 18S ribosomal subunits. The primers were chosen by aligning sequence information available through GenBank, and selecting appropriate areas of commonality. Using these primers, amplified DNA has been obtained and sequenced from myxospores from rainbow trout and sculpin, and the sequence information compared to known sequences using BLAST. The myxospore in rainbow trout has 98 % homology with *Henneguya salminicola*; the myxospore in the sculpin has 97 % homology with *Thelohanellus hovorkai*. Efforts are underway to amplify, sequence and, if possible, identify the remaining myxospores. Moreover, as other non-*M. cerebralis* myxospores become available, testing to ensure the absence of detection by *M. cerebralis* specific primers will be performed.

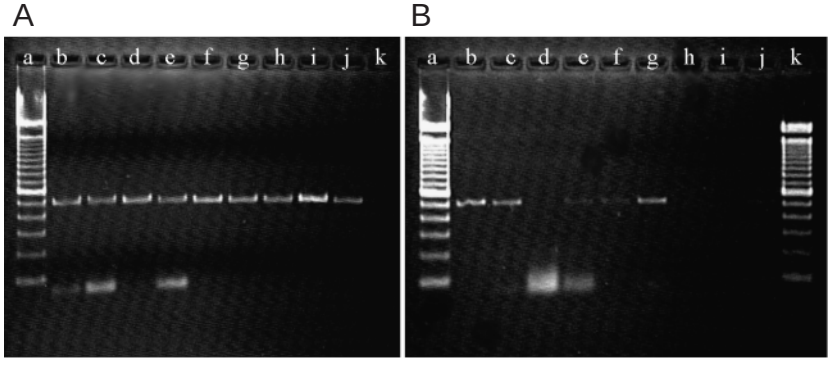

Fig. 2. Polymerase chain reaction products from lower detection limit dilution assay. (A) Lane a: 100 base-pair molecular weight standard (Gibco-BRL). Lanes b – j: digest preparations of 100 spores per reaction. Lane k: control for contaminating DNA in master mix (PCR negative control). (B) Lanes a and k: 100 base-pair molecular weight standard (Gibco-BRL). Lanes b – j: digest preparations of 10 spores per reaction. Samples themselves constitute the extraction positive control. The extraction negative control and PCR-positive control were run with these samples but displayed on separate (not shown) gels

Although the sensitivity of the PCR procedure was not compared directly with that of histopathology, experience argues it is much greater.

Too frequently, myxospores morphologically indistinguishable from *Myxobolus cerebralis* are seen in digest preparations, but their corresponding location in the fish cannot be identified in histologic sections. These fish are often classified (likely erroneously) as *M. cerebralis* negative, in spite of the isolated myxospores. Confirmation using the PCR procedure obviates the need for histologic confirmation, avoids the need to process, section and examine large numbers of sections, and is less expensive and far faster. Its adoption as an accepted confirmatory assay is encouraged.

LITERATURE CITED

Andree KB, MacConnell E, Hedrick RP (1998) A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. J Aquat Anim Health 34:145–154

Baldwin TJ, Peterson JE, McGhee GC, Staigmiller KD, Motteram ES, Downs CC, Stanek DR (1998) Distribution of *Myxobolus cerebralis* in salmonid fishes in Montana. J Aquat Anim Health 10:361–371

Baldwin TJ, Vincent ER, Silflow RM, Stanek D (2000) *Myxobolus cerebralis* infection in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) exposed under natural stream conditions. J Veterin Diagn Invest 12:312–321

Blyth CR (1986) Approximate Binomial Confidence Limits. J Am Stat Assoc 81:843–855

Hiney MP, Smith PR (1988) Validation of polymerase chain reaction-based techniques for proxy detection of bacterial fish pathogens: framework, problems and possible solutions for environmental applications. Aquaculture 162: 41–68

Hoffman GL (1990) *Myxobolus cerebralis*, a worldwide cause of salmonids whirling disease. J Aquat Anim Health 2: 30–37

Markiw ME (1991) Whirling disease: earliest susceptible age of rainbow trout to the triactinomyxid of *Myxobolus cerebralis*. Aquaculture 92:1–6

Markiw ME, Wolf K (1974) *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements—sequential enzymatic digestions and purification by differential centrifugation. J Fish Res Board Can 31:15–20

Mathews J (1995) Chasing our tails: whirling disease, hatcheries and the future of America's wild trout. Trout 36:22–27

O'Grodnick JJ (1975) Whirling disease (*Myxosoma cerebralis*) spore concentration using the continuous plankton centrifuge. J Wildl Dis 11:54–57

Palmer T (1995) Trouble in trout paradise. Mont Outdoors July/August:21–24

Smith RD (1991) Veterinary clinical epidemiology: a problem oriented approach. Butterworth-Heinemann, Boston, p 29–43

Vincent ER (1996) Whirling disease and wild trout: the Montana experience. Fisheries 21:32–33

Walker PG, Nehring RB (1995) An investigation to determine the cause(s) of the disappearance of young wild rainbow trout in the upper Colorado River, in Middle Park, Colorado. Colorado Division of Wildlife, Department of Natural Resources, Denver

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