Vol. 21: 215–220, 1995 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published March 30

Development of a DNA probe for the myxosporean parasite *Ceratomyxa shasta*, using the polymerase chain reaction with arbitrary primers

Jerri L. Bartholomew[1], Rusty J. Rodriguez[2], Cindy K. Arakawa[2]

[1] Center for Salmon Disease Research, Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804, USA
[2] National Biological Survey, Bldg 204 Naval Station, Seattle, Washington 98115, USA

ABSTRACT: The arbitrarily primed polymerase chain reaction (PCR) was used to generate a DNA marker specific for the myxosporean parasite *Ceratomyxa shasta*. The [^{32}P]-labeled marker hybridized to purified *C. shasta* DNA and to parasite DNA combined with salmonid DNA in a dot blot assay, demonstrating its potential as a diagnostic tool. The amplified DNA segment was cloned and sequenced, and primers specific for the marker were designed. When these primers were used in a standard PCR assay, DNA was amplified from *C. shasta* and from infected fish tissues, but not from uninfected fish tissues or from 2 other myxosporean parasites. The sensitivity of the PCR assay will permit detection of low levels of *C. shasta* from infected fish or oligochaetes and will be useful in defining the parasite's life cycle as well as examining its impact on salmonid populations.

KEY WORDS: *Ceratomyxa shasta* · Myxosporean · Arbitrarily primed PCR · DNA probe

INTRODUCTION

Ceratomyxosis, caused by infection with the myxosporean parasite *Ceratomyxa shasta*, has been recognized as a serious impediment to salmonid aquaculture in the Pacific Northwest region of North America since it was first observed in 1948 (Wales & Wolf 1955). The parasite is enzootic in several major water systems in this region (Johnson et al. 1979, Hoffmaster et al. 1988) and is a factor in the survival of the salmonid species migrating through them. Infection by *C. shasta* is first evident in the intestinal tract of the fish, where the parasite multiplies and causes tissue necrosis accompanied by a severe inflammatory reaction and subsequent death of the host (Bartholomew et al. 1989b).

Results of studies on the geographic range of the parasite (Johnson et al. 1979, Hoffmaster et al. 1988) and the susceptibility to infection of different species and strains of salmonids (Zinn et al. 1977, Buchanan et al. 1983) have enabled fishery managers to reduce losses to the disease by using alternative water supplies at the hatcheries and by stocking disease-resistant fish strains in enzootic watersheds. However, studies on the impact of this parasite (Bartholomew et al. 1992a) indicate that these measures have not been completely successful and implementation of control strategies without understanding the parasite's biology and ecology has been ineffective.

Control of ceratomyxosis is complicated because the myxosporean life cycle involves 2 hosts. In the myxosporean life histories described, development of the actinosporean life stages take place in an oligochaete intermediate host (Wolf & Markiw 1984, El-Matbouli & Hoffman 1989, Ruidisch et al. 1991, Styer et al. 1991, Yokoyama et al. 1991, Bartholomew et al. 1992b, El-Matbouli et al. 1992, Großheider & Körting 1992, Kent et al. 1993). The actinosporean completes its own developmental cycle inside the oligochaete and is released as a spore. It is not clear whether the actinosporean spore or the multinucleate plasmodium that it contains is responsible for infecting the fish host, but, once the parasite penetrates, it undergoes proliferative and finally sporogonic cycles to form the characteristic myxosporean spore (Lom & Dyková 1992).

The resulting diversity of life stages which myxosporean parasites undergo in this 2 host life cycle not

only makes them fascinating biologically, but also contributes to the difficulties in laboratory research and diagnostics. Traditional diagnostic methods rely on visual recognition, which requires a great deal of experience with organisms that change so dramatically in their morphology. Development of monoclonal antibodies which react with certain life stages of *Ceratomyxa shasta* has increased the accuracy of detection and has also allowed examination of the pathogenesis of the disease (Bartholomew et al. 1989a, b). However, the specificity of these antibodies also limits their usefulness because major antigens are not conserved between life stages (Bartholomew et al. 1989b). In contrast, the genetic makeup of an organism should not vary throughout its life. Therefore, use of a nucleic acid probe developed using DNA from the readily available myxosporean spore stage will enable identification of morphologically different life stages.

In this study we used a modification of the standard polymerase chain reaction (PCR) to obtain a DNA sequence unique for *Ceratomyxa shasta*, and used this as the basis for a research and diagnostic assay. Conventional PCR amplification requires sequence information to design primers specific for the target organism; however, genetic markers may be obtained without sequence information using the arbitrarily primed PCR (ap-PCR) (Welsh & McClelland 1990, Williams et al. 1990, Bandi et al. 1993, Procunier et al. 1993). The DNA products generated by ap-PCR amplification of *C. shasta* DNA were used directly as DNA probes in a dot blot assay. In addition, by sequencing these markers, specific primers for use in standard PCR were constructed and used to detect the parasite in diseased fish.

MATERIALS AND METHODS

Isolation of DNA. *Ceratomyxa shasta* spores collected from the intestinal tissues of naturally infected rainbow trout were purified by gradient centrifugation (Percoll density 1.13 g ml^{-1}; Sigma Chemical Co., St. Louis, MO, USA) and used to isolate DNA. Spores (10^6 in 200 µl) were lysed in the presence of 40 µl of 10% (w/v) sodium dodecyl sulfate (SDS) and 10 µl of 100 mg ml^{-1} proteinase K for 1 h at 42°C and extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with $^1/_{10}$ volume of 3 M sodium acetate, pH 5.2, and 2 volumes of 100% ethanol (Maizels et al. 1991) and redissolved in 50 mM Tris-HCl, 0.1 M NaCl, pH 7.6, 0.5 mM ethylenediaminetetraacetic acid (EDTA) (TNE). Concentration and purity were determined from absorbance measurements at 260 and 280 nm. The approximate size was determined visually under UV light after electrophoresis in a 3% (w/v) agarose gel in Tris-borate-EDTA (TBE) buffer and staining with ethidium bromide (Sambrook et al. 1989). These procedures were repeated using spores from the myxosporeans *Henneguya salmincola* and *Myxobolus insidiosus* with minor modifications in the length of incubation required for complete spore lysis.

Intestines from *Ceratomyxa shasta*-infected and uninfected fish were incubated in lysis buffer [150 mM EDTA; 50 mM Tris, pH 8.0; 2% (w/v) N-lauroylsarcosine (sarcosyl); 2 µg ml^{-1} proteinase K] at 65°C until the tissue dissolved. Tissue debris was removed by centrifugation and DNA was precipitated at room temperature by the addition of a 20% (v/v) polyethylene glycol (MW 8000) solution containing 2.5 M NaCl, to a final concentration of 8.2%. After centrifugation at 14 000 rpm (16 000 × *g*), the supernatant was discarded and the pellet was resuspended in 0.2 ml TNE. Then 0.1 ml of 7.5 N NH_4OAc was added to the solution on ice, and after 5 min the debris was removed by centrifugation. DNA was precipitated from the supernatant by addition of isopropyl alcohol to 70%, washed with 70% ethanol and resuspended in TNE.

PCR amplification. For ap-PCR, dilutions of DNA (3 and 30 ng) from each of the parasites were amplified in a 20 ml volume containing PCR buffer [10 mM TrisCl, pH 8.5; 50 mM KCl; 2.5 mM $MgCl_2$; 10 mM dithiothreitol (DTT); 0.1% (w/v) gelatin; 0.1% (v/v) Triton X-100], 0.2 mM of each deoxynucleotide triphosphate (dNTP), 2 nM of 1 of the 33 different random oligonucleotide primers tested (15 to 24 base pairs, bp) and 1 unit of Taq I DNA polymerase. Samples were assembled on ice, overlaid with a drop of mineral oil and amplified in a Temptronic II thermocycler (Barnstead/Thermolyne, Dubuque, IA, USA). After an initial denaturation for 4 min at 95°C, 40 amplification cycles were completed. Each cycle consisted of a 30 s denaturation at 95°C, 30 s annealing between 48 and 60°C (optimal temperature was determined for each oligonucleotide primer), and 90 s synthesis at 72°C. A 1 s $°C^{-1}$ ramp time was set between denaturation and annealing temperatures with a 0.5 s $°C^{-1}$ ramp between annealing and synthesis temperatures.

The standard PCR reaction mix was as described for the ap-PCR, but with 20 pmol of each of the *Ceratomyxa shasta*-specific primers under the same conditions as described above. The primers were used to amplify DNA from the plasmid clone, *C. shasta*, *C. shasta*-infected salmonids, uninfected salmonids, *Henneguya salmincola* and *Myxobolus insidiosus*. For analysis of the amplification products, 2 µl aliquots of each reaction mixture were subjected to electrophoresis in a 2% agarose gel in TBE buffer. The DNA was visualized with UV light after ethidium bromide staining.

All of the primers used in these studies were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA, USA).

DNA hybridization. The ap-PCR products generated by amplifying *Ceratomyxa shasta* DNA were extracted from the agarose gel using the GENECLEAN II Kit (BIO 101, Inc., La Jolla, CA). The products were labeled with [^{32}P]dCTP using the Prime-a-Gene labeling system (Promega, Madison, WI, USA) and unincorporated label was removed by centrifugation through a Select-D, G-50 Spin Column (5 Prime-3 Prime, Inc., West Chester, PA, USA).

Blots for hybridization were prepared using dilutions of *Ceratomyxa shasta* genomic DNA (0.08 to 0.02 µg). Salmonid DNA (0.10 µg) and *Henneguya salmincola* and *Myxobolus insidiosus* DNA (0.16 µg each) served as negative controls and tests for cross reactivity. To determine if *C. shasta* DNA could be detected in fish tissues, dilutions of the parasite DNA were added to constant amounts of chinook salmon DNA (0.10 µg). Denatured DNA was applied to the nitrocellulose under suction and fixed to the membrane using UV radiation. The blot was then placed into a prehybridization solution [5 × standard saline citrate (SSC), 5 × Denhardt's solution (Sambrook et al. 1989), 50 µg ml^{-1} salmon sperm DNA, 10% SDS] and incubated overnight at 65°C. Labeled probe was denatured, added to the prehybridization solution and hybridized overnight at 65°C. Unhybridized probe was removed by washing the blots twice at RT in 2 × SSC, 0.1% SDS for 15 min and once at 65°C in 0.25 × SSC, 0.1% SDS for 10 min. The membrane was then dried and autoradiographed by exposure to X-OMAT AR Kodak X-ray film at −70°C.

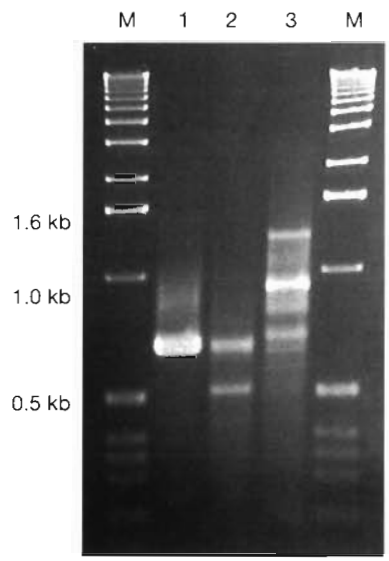

Fig. 1. Ethidium bromide stained agarose gel of PCR products generated by amplification of DNA derived from *Ceratomyxa shasta* (lane 1), *Myxobolus insidiosus* (lane 2), and *Henneguya salmincola* (lane 3) with the 19 bp primer p104 (AGG_5-TCGA). DNA size standards are shown in lanes M with some sizes (kilobases) indicated on the left

Cloning. A *Ceratomyxa shasta*-specific ap-PCR product generated with primer p104 (AGG_5-TCGA) was isolated after electrophoresis and cloned into *Escherichia coli* using the pT7 Blue T-Vector kit (Novagen, Madison, WI). Plasmid DNA was obtained from 36 transformed colonies using a standard procedure (Sambrook et al. 1989) and the cloned fragment sizes were determined by digestion with *EcoRI* and *PstI* followed by agarose gel electrophoresis (Sambrook et al. 1989). To identify sequences homologous with *C. shasta* DNA, the digested plasmid DNA was transferred to nitrocellulose by capillary action (Maizels et al. 1991) and hybridized to the [^{32}P]-labeled p104 PCR product as described above.

Sequencing. Sequencing was performed using the Sequenase Version 2 kit (USB, Cleveland, OH, USA) and the protocol provided by the manufacturer. Sequencing gels [6% (w/v) acrylamide, 7 M urea in TBE] were run at 70 W for 3 h and then fixed with 5% (v/v) methanol and 5% (v/v) acetic acid before transfer to filter paper and exposure to X-ray film.

RESULTS

Analysis of amplified PCR products

A total of 33 random primers were tested for generation of DNA products specific to *Ceratomyxa shasta*. Of those, 7 primers failed to amplify *C. shasta* DNA at the reaction temperatures used and 12 others produced multiple DNA fragments (data not shown). Amplification of *C. shasta* DNA using the remaining 14 primers resulted in 1 or 2 DNA products for *C. shasta*. The DNA products generated using the primers resulted in species-specific DNA fragment patterns. The 19 bp primer p104 (AGG_5-TCGA) reproducibly amplified a single distinct DNA fragment of approximately 700 bp from *C. shasta* and was selected for further work because the primer sequence could be removed by digestion with Taq 1 (Fig. 1). This digestion proved to be necessary for initial screening because the random primer termini hybridized nonspecifically with fish DNA (data not shown).

A [^{32}P]-labeled probe made using the Taq 1 digested amplification product of p104 as a template hybridized to *Ceratomyxa shasta* DNA alone, to the combined samples of parasite and fish DNA, but not to fish DNA alone or to either of the 2 other myxosporean parasites tested (Fig. 2).

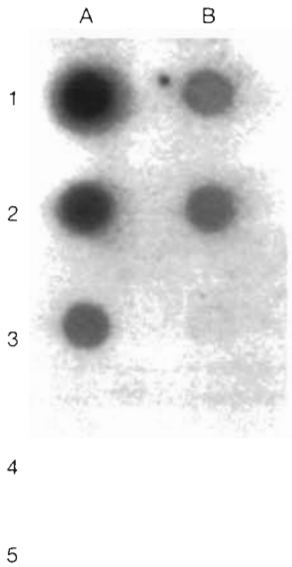

Fig. 2. Dot blot showing hybridization of the Taq 1 digested p104 PCR product with *Ceratomyxa shasta* DNA: (A1) 0.08 µg, (A2) 0.04 µg and (A3) 0.02 µg. With *C. shasta* + salmonid DNA: (B1) 0.08 µg *C. shasta* DNA + 0.1 µg salmonid DNA, (B2) 0.04 µg *C. shasta* DNA + 0.1 µg salmonid DNA, (B3) 0.1 µg salmonid DNA. Dots A4 and B4: 0.16 µg *Henneguya salmincola* DNA; Dots A5 and B5: 0.16 µg *Myxobolus insidiosus* DNA

Cloning of the amplified product

The 700 bp fragment produced by amplification of *Ceratomyxa shasta* DNA with p104 was purified and cloned into the PT7 Blue T-Vector. Insert size, as determined by endonuclease digestion of the plasmid DNA, was approximately 700 bp (Fig. 3a), the same size as the PCR product. Hybridization analysis of the digested plasmid DNA with the [^{32}P]-labeled p104 product indicated that the cloned 700 bp insert was the PCR product (Fig. 3b, c).

Designing *Ceratomyxa shasta*-specific primers for standard PCR

Sequence analysis of cloned p104 products from 3 *Escherichia coli* transformants resulted in identical sequences. Based on sequence information, primers were designed to specifically amplify the p104 product. The forward primer (TGA GCT CTG GAG TAC TTT) is 18 bases long and is located 37 bp downstream from the random primer sequence. The reverse primer (CAC CAC TTC TTG AGT GCT TC) is 20 bases long and located 8 bp upstream from the random primer

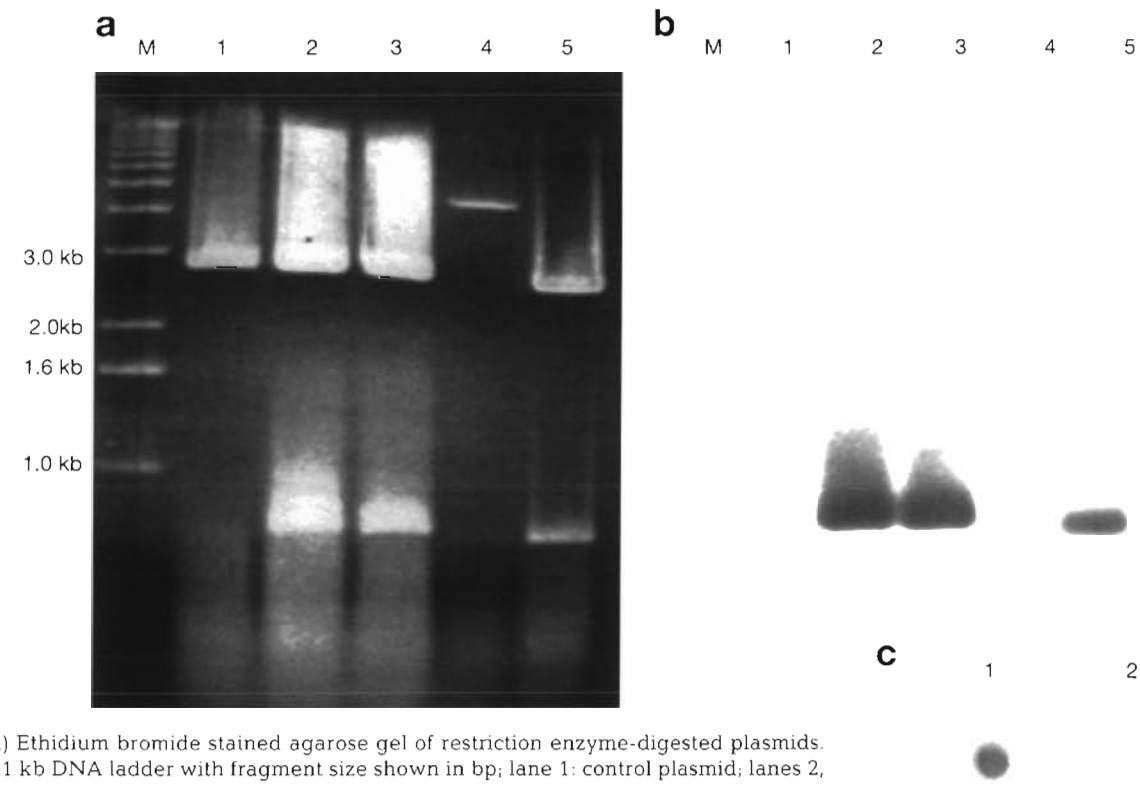

Fig. 3. (a) Ethidium bromide stained agarose gel of restriction enzyme-digested plasmids. Lane M: 1 kb DNA ladder with fragment size shown in bp; lane 1: control plasmid; lanes 2, 3 and 5: plasmid containing DNA insert; lane 4: plasmid with no insert. (b) Southern blot of agarose gel stained in (a) and hybridized with PCR product amplified using p104. Lane designations are identical. (c) Dot blot of (1) *Ceratomyxa shasta* DNA (0.08 mg) and (2) salmonid DNA (0.1 mg) hybridized with cloned PCR product amplified using p104

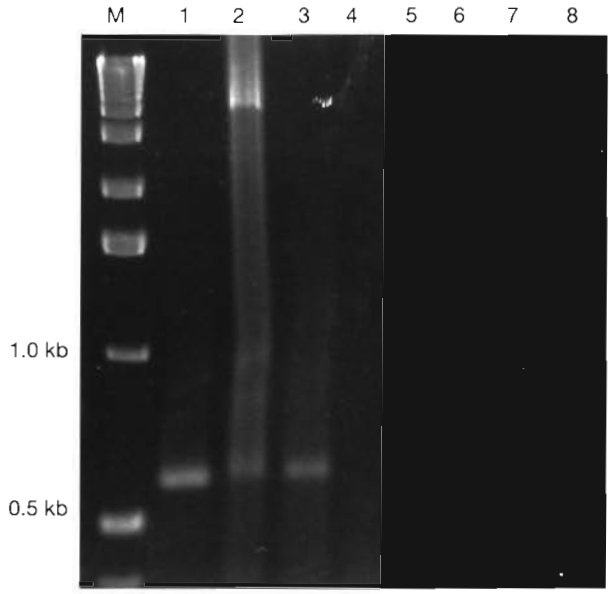

Fig. 4. Ethidium bromide stained agarose gel of PCR products generated by amplification of DNA derived from *Ceratomyxa shasta* (lane 1), the plasmid containing the *C. shasta* marker (lane 2), chinook salmon infected with *C. shasta* (lane 3), uninfected chinook salmon (lane 4), *Henneguya salminicola* (lane 5), and *Myxobolus insidiosus* (lane 6). Lanes 7 and 8 are no-DNA PCR controls.

sequence. Standard PCR using the primers to amplify template DNA from the plasmid, *C. shasta*, *C. shasta*-infected salmonids, uninfected salmonids, *Henneguya salmincola* and *Myxobolus insidiosus* resulted in the amplification of a fragment of approximately 650 bp from the plasmid, *C. shasta* and the infected fish. There was no amplification of DNA from uninfected fish or the other myxosporeans (Fig. 4).

DISCUSSION

Several obstacles have been encountered in studies directed at defining the life cycle of *Ceratomyxa shasta*. First, the changing morphology and antigenic structure of the parasite throughout its life cycle have precluded identification of all life stages even with the development of monoclonal antibodies (Bartholomew et al. 1989a). Additionally, it has been difficult to obtain large numbers of the putative oligochaete host infected with actinosporean spores. In diagnostic programs the obstacles are similar, and there is a reluctance to make a confirmatory identification based on a life stage other than the easily identified myxosporean spore. Because diagnosis relies on identification of mature spores, found only during the terminal stages of infection, it is necessary to maintain fish until mortality occurs or examine only moribund or dead fish. These problems emphasize the need for a specific and sensitive nucleic acid assay which will identify all life stages of the parasite.

In this study, ap-PCR was used to generate DNA markers for *Ceratomyxa shasta*. To increase the probability that the amplified DNA would be useful as a species-specific probe, we selected a primer (p104) which amplified a DNA product of approximately 700 bp which did not hybridize with DNA from the other myxosporean parasites or salmonid DNA in a dot blot procedure. To test the potential of the dot blot as a diagnostic tool, the labeled marker was hybridized with combinations of parasite and salmonid DNA at several concentrations. The probe was capable of detecting 0.04 µg of *C. shasta* DNA in the presence of 0.1 µg of salmonid DNA (the lowest concentration tested) and did not cross-react with the salmonid DNA alone. Further development of the dot blot assay using a biotinylated probe should allow earlier detection of the infection and reduce the need to maintain infected fish for prolonged periods.

Ceratomyxa shasta-specific primers were constructed using the sequence data from the p104 product. When used in standard PCR assays, these primers specifically amplified a 650 bp fragment from purified *C. shasta* DNA and *C. shasta* DNA present in intestinal tissues of an infected salmonid. The increased sensitivity of the standard PCR over the dot blot assay will allow detection of low levels of *C. shasta* DNA from infected fish or oligochaetes. In combination, the probe and primers developed in this study give us the tools necessary to explore the life cycle of *C. shasta* and to answer questions about the true impact of the disease on migrating salmonid populations.

Acknowledgements. We thank Don Stevens, Center for Salmon Disease Research, Oregon State University, Corvallis, for providing the parasite and fish samples. We also thank Dr James Winton, National Biological Survey, Seattle, WA, for advice and support during the course of this project and Bill Batts, National Biological Survey, for the synthesis of oligonucleotide primers. This work was performed at the Pacific Northwest Natural Science Center (National Biological Survey) with the additional support of a USDA-CSRS Animal Health and Disease Special Research Grant, Project No. 94-GRAH-0-6041. This is Oregon Agricultural Experiment Station technical paper no. 10523.

LITERATURE CITED

Bandi C, La Rosa G, Bardin MG, Damiani G, de Carneri I, Pozi E (1993) Arbitrary primed polymerase chain reaction of individual *Trichinella* specimens. J Parasitol 79(3):437–440

Bartholomew JL, Fryer JL, Rohovec JS (1992a) Impact of the myxosporean parasite, *Ceratomyxa shasta*, on survival of migrating Columbia River basin salmonids. In: Svrjcek RS (ed) Control of disease in aquaculture. Proceedings of the 1990 U.S. and Japan Meeting on Aquaculture Ise, Nie Pre-

fecture, Japan, Oct 29–30, 1990. NOAA Tech Rep NMFS 111

Bartholomew JL, Fryer JL, Rohovec JS (1992b) *Ceratomyxa shasta* infections of salmonid fish. In: Proceedings of the OJI International Symposium on Salmonid Diseases. Hokkaido University Press, Sapporo, p 267–275

Bartholomew JL, Rohovec JS, Fryer JL (1989a). Development, characterization, and use of monoclonal and polyclonal antibodies against the myxosporean, *Ceratomyxa shasta*. J Protozool 36:397–401

Bartholomew JL, Smith CE, Rohovec JS, Fryer JL (1989b) Characterization of a host response to the myxosporean parasite, *Ceratomyxa shasta* (Noble), by histology, scanning electron microscopy and immunological techniques. J Fish Dis 12:509–522

Buchanan DV, Sanders JE, Zinn JL, Fryer JL (1983) Relative susceptibility of four strains of summer steelhead to infection by *Ceratomyxa shasta*. Trans Am Fish Soc 112: 541–543

El-Matbouli M, Fischer-Scherl Th, Hoffman RW (1992) Transmission of *Hofferellus carassii* Achmerov, 1960 to goldfish *Carassius auratus* via an aquatic oligochaete. Bull Eur Ass Fish Pathol 12:54–56

El-Matbouli M, Hoffman RW (1989) Experimental transmission of two *Myxobolus* spp. developing bisporogeny via tubificid worms. Parasitol Res 75:461–464

Großheider G, Körting W (1992) First evidence that *Hofferellus cyprini* (Doflein, 1898) is transmitted by *Nais* sp. Bull Eur Ass Fish Pathol 12:17–20

Hoffmaster JL, Sanders JE, Rohovec JS, Fryer JL, Stevens DG (1988) Geographic distribution of the myxosporean parasite, *Ceratomyxa shasta* Noble, 1950, in the Columbia River basin, USA. J Fish Dis 11:97–100

Johnson KA, Sanders JE, Fryer JL (1979) *Ceratomyxa shasta* in salmonids. U.S. Dept Int Fish Wildl Ser Fish Dis Leafl No 58

Kent ML, Whitaker DJ, Margolis L (1993) Transmission of *Myxobolus arcticus* Pugachev and Khokhlov, 1979, a myxosporean parasite of Pacific salmon, via a triactinomyxon from the aquatic oligochaete, *Stylodrilus heringianus* (Lumbriculidae). Can J Zool 71:1207–1211

Lom J, Dyková I (1992) Protozoan parasites of fishes. Developments in aquaculture and fisheries science, Vol 26. Elsevier, Amsterdam

Maizels RM, Baxter ML, Robertson BD, Selkirk ME (1991) Parasite antigens, parasite genes: a laboratory manual for molecular parasitology. Cambridge Univ. Press., Cambridge

Procunier JD, Fernanda MA, Barta JR (1993) Species and strain differentiation of *Eimeria* spp. of the domestic fowl using DNA polymorphisms amplified by arbitrary primers. Parasitol Res 79:98–102

Ruidisch S, El-Matbouli M, Hoffman RW (1991) The role of tubificid worms as an intermediate host in the life cycle of *Myxobolus pavlovskii* (Akhmerov, 1954). Parasitol Res 77: 663–667

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Styer EL, Harrison LR, Burtle GL (1991) Experimental production of proliferative gill disease in channel catfish exposed to a myxozoan-infected oligochaete, *Dero digitata*. J aquat Anim Health 3:288–291

Wales JH, Wolf H (1955) Three protozoan diseases of trout in California. Calif Fish Game 41:183–187

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nuc Acids Res 18:7213–7218

Williams JGK, Kublelik AR, Livak KL, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nuc Acids Res 18: 6531–6535

Wolf K, Markiw ME (1984) Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452

Yokoyama H, Ogawa K, Wakabayashi H (1993) Some biological characteristics of actinosporeans from the oligochaete *Branchiura sowerbyi*. Dis aquat Org 17:223–228

Zinn JL, Johnson KA, Sanders JE, Fryer JL (1977) Susceptibilty of salmonid species and hatchery strains of chinook salmon (*Oncorhynchus tshawytscha*) to infections by *Ceratomyxa shasta*. J Fish Res Bd Can 34:933–936

Responsible Subject Editor: W. Körting, Hannover, Germany

Manuscript first received: July 26, 1994
Revised version accepted: November 21, 1994