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Development of a polymerase chain reaction diagnostic assay for *Ceratomyxa shasta*, a myxosporean parasite of salmonid fish

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ABSTRACT: A diagnostic procedure based on the polymerase chain reaction (PCR) was developed for the myxosporean parasite *Ceratomyxa shasta*. Three sets of oligonucleotide primers were designed to specifically amplify *C. shasta* ribosomal RNA genes and several parameters of the assay were tested and optimised. A simple protocol for the processing of fish tissue samples was also developed. In a single round, 20 µl volume reaction the optimised procedure allows the detection of 50 fg of purified *C. shasta* genomic DNA, or 0.01 spore from a seeded fish intestine sample. This protocol is considerably faster, cheaper and more reliable than any previous diagnostic procedure for a myxosporean parasite, and can be an invaluable tool for the monitoring of early and/or subclinical *C. shasta* infections in wild and cultured salmon populations.

KEY WORDS: Myxozoa · Diagnostics · *Ceratomyxa* · Polymerase chain reaction · Ribosomal RNA gene · rDNA

INTRODUCTION

Myxosporeans (Myxozoa: Myxosporea) constitute a major group of fish parasites. More than 1250 species infect fresh and saltwater fishes (Lom & Dyková 1995) and their impact on wild and cultured animals can be significant. For a number of freshwater species, it has been demonstrated that myxozoans undergo 2 developmental cycles, one of them (myxosporean) in the fish and the other (actinosporean) in an aquatic oligochaete or polychaete worm (El-Matbouli et al. 1992, Bartholomew et al. 1997). Each cycle terminates in a spore stage, which is infective for the other host. However, the routes of infection, life cycle and pathogenesis are still unknown for the vast majority of species, making their control very difficult. Although a similar model of heteroxenous life cycle is assumed for most members of the phylum Myxozoa, the identification of each putative life stage on the basis of their morphological characteristics poses important difficulties. Specific and sensitive diagnostic tools for myxozoans are thus highly desirable, and those based on conserved attributes of the parasites during this complex life cycle are currently the best choice.

Infection with *Ceratomyxa shasta* has been recognised as a serious impediment to salmonid aquaculture in the Pacific Northwest of North America since it was first observed in 1948 (Wales & Wolf 1955). The parasite invades the intestinal tract of the fish, causing tissue necrosis accompanied by a severe inflammatory reaction and subsequent death of the host (Bartholomew et al. 1989a). Presumptive diagnosis of ceratomyxosis relies on the detection of mature *C. shasta* spores, which are found only during the terminal stage of infection. The first non-morphological approach for the diagnosis of *C. shasta* and other myxosporean species was the development of immunological or lectin-based histochemical assays (Bartholomew et al. 1989b, Adams et al. 1992, Marín de Mateo et al. 1993). However, antibodies and lectins have limited diagnostic usefulness because major antigens and lectin-binding sites can change dramatically during parasite ontogeny and they can be shared by different host cell types and by other parasite species (Lumsden 1986, Jacobson & Doyle

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1996). Moreover, although rapid assays using tissue imprints have been developed, as reported for the diagnosis of PKX (Hedrick et al. 1992), the execution of these techniques is in general time-consuming (at least 2 to 3 d from sampling to results) and often requires histological techniques and equipment.

Diagnostic methods based on the detection of parasite DNA may circumvent some of these problems. Initial efforts to use DNA-based probes for the diagnosis of a myxosporean (Bartholomew et al. 1995) included the generation of *Ceratomyxa shasta* genomic fragments by the arbitrarily primed-polymerase chain reaction (AP-PCR). These fragments were labelled and used as probes in a dot-blot assay. A pair of oligonucleotides was also designed based on the sequence of one of these fragments, allowing the detection of *C. shasta* in infected fish tissues by PCR. Although these probes did not amplify DNA from salmon or from 2 different myxosporean species tested, diagnostic PCR probes should be preferably targeted to known genomic fragments unique to the parasite species, thus insuring the specificity of the assays. Recently, the *C. shasta* small subunit ribosomal RNA gene (SSUrDNA) was sequenced (Bartholomew et al. 1997). The availability of homologous sequences of this gene from other related and unrelated organisms allows the development of specific diagnostic probes based on divergent sequence fragments. This approach has been successfully used for the detection of PKX (Saulnier & de Kinkelin 1997) and *Myxobolus cerebralis* (Andree et al. 1998) by PCR. In this work we describe the design and use of a diagnostic PCR assay for *C. shasta*. A simple tissue preparation method was also developed, which allows a fast and cost-effective processing of large numbers of samples.

MATERIALS AND METHODS

PCR primer selection. The *Ceratomyxa shasta* SSUrDNA sequence (GenBank accession no. AF001579) was manually aligned with homologous sequences of the following: 3 teleosts, *Oncorhynchus mykiss* (R. P. Hedrick pers. comm.), *Fundulus heteroclitus* (M91180) and *Sebastolobus altivelis* (M91182); a polychaete worm, *Lanice conchilega* (X79873); a cnidarian, *Anemonia sulcata* (X53498); and 3 myxosporean parasites, *Myxobolus cerebralis* (U96492), *Myxobolus squamalis* (U96495) and *Henneguya doori* (HDU37549) using the Genetic Data Environment software (GDE, v 2.2) included in the GCG Wiscosin package v 9.0 (Genetics Computer Group, Madison, WI, USA). Primers were designed from regions within the *C. shasta* sequence where correct alignment was unambiguous and considerable divergence from the other sequences was found. In addition to the primers designed to be specific for *C. shasta*, and in order to control false-negative results caused by the absence or poor quality of template in the reactions, a set of primers designated 18SUNI were combined to amplify a conserved region of the SSUrDNA from all the species used in this study. Primers were chosen based on the considerations of Innis & Gelfand (1990), and their sequences were analysed with Gene Runner software (v 3.02, Hastings Software Inc. 1994) for strong secondary structure and dimer formation with themselves or with their corresponding reverse PCR primer. In addition, potential oligonucleotide sequences were sent to the BLAST electronic mail server (blast@ncbi.nlm.nih.gov) as queries to discard primers that had non-target gene homologies.

DNA extraction. All the reagents and materials used were nuclease-free. Special care was used to avoid false-positives and contamination of samples during the extraction and PCRs as proposed by Yap et al. (1994). Appropriate controls were included in every experiment, and dissecting tools were either disposable or contaminating DNA was destroyed between samples by immersion in 10% bleach for 1 min (Prince & Andrus 1992).

For the initial development of the assay, template DNA was generated from spores of *Ceratomyxa shasta*, 4 other myxosporeans (*Henneguya salminicola*, *Myxobolus arcticus*, *M. squamalis* and *Kudoa* sp.) and from rainbow trout tissues, by digestion with proteinase K and phenol/chloroform extraction. Briefly, samples were digested in DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 1% w/v SDS pH 8) with 200 µg ml^{-1} proteinase K, overnight at 37°C on a rocking platform. Samples were further digested with 100 µg ml^{-1} RNase A for 30 min at 37°C, and the DNA was purified by standard phenol/chloroform extraction and ethanol precipitation (Sambrook et al. 1989). Eventually, to ensure the release of DNA from resistant spores, 4 freeze-thaw cycles were added to this protocol after the initial digestion, followed by a second proteinase K treatment (2 h at 37°C).

To optimise the procedure, 4 different DNA extraction methods were tested using intestinal tissues with varying intensities of *Ceratomyxa shasta* infection, as diagnosed by light microscopy (LM), in fresh smears of the tissue. Two small pieces of intestinal tissue (25 to 100 mg each) were removed from each fish and placed in 1.5 ml microtubes. The content of 1 microtube was homogenised in 10 µl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) per mg of tissue, using a disposable plastic pestle. An aliquot of this homogenate was used as a 'crude sample' (C) for the PCR and the remainder was boiled for 5 min, after which it was designated as

'boiled-crude sample' (BC). Tissue in the second microtube was digested with DNA extraction buffer (10 µl mg^{-1} tissue), proteinase K and RNase as described above. An aliquot of this digested tissue was boiled for 5 min to inactivate the proteinase K and used as 'boiled-digested sample' (BD). Finally, the DNA of the remaining digested tissue was purified by phenol/chloroform extraction and ethanol precipitation, after which it was resuspended in 50 µl TE and labelled as 'extracted-digested sample' (ED).

To determine the potential sensitivity of the optimised extraction method and PCR assay for infected fish, a *Ceratomyxa shasta* infection was simulated by adding dilutions of Percoll® (Pharmacia Biotech, Uppsala, Sweden) purified spores (Bartholomew et al. 1989b) to uninfected rainbow trout tissue. Fish gut pieces (50 to 90 mg each) in 500 µl of DNA extraction buffer were seeded with *C. shasta* spores, for final 10-fold decreasing concentrations of 10^6 to 0 spores per ml of sample. Two replicate pieces of tissue were seeded with each dose of spores, and then digested and processed as for the BD samples (see above).

The DNA content of all the samples was determined with a fluorometer (Hoefer, San Francisco, CA, USA). Samples were stored at –20°C until used, and diluted with HPLC-grade H_2O (Sigma, St Louis, MO, USA) as needed.

Polymerase chain reaction. All reactions were performed in 20 µl final volumes, with Promega (Madison, WI, USA) Taq polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% v/v triton X-100) containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 µM of each primer and 1 U of Taq DNA polymerase (Promega). One microlitre of template DNA at variable concentrations in H_2O was used in each reaction. The components were overlaid with mineral oil, and the amplification reactions were carried out in an Amplitron II thermal cycler (Barnstead-Thermolyne, IA, USA). The mixtures were denatured for 3 min at 95°C and amplification was performed with 35 cycles consisting of: 1 min for denaturation at 94°C, then 30 s for primers annealing at 55, 58, 60 or 65°C and finally 1 min for Taq extension at 72°C. Ten microlitres of each reaction product were electrophoresed in 1 to 1.5% (w/v) 1 × TAE (40 mM Tris acetate, 1 mM EDTA pH 8.3) agarose gels containing ethidium bromide and visualised under UV transillumination.

RESULTS

Three sets of primers (Table 1) were designed to specifically amplify *Ceratomyxa shasta* SSUrDNA. Most possible combinations of these primers were tested for specificity, product size and unique conspicuous bands in the PCR products. All the primer sets resulted in specific detection of *C. shasta* DNA, yielding amplicons of the predicted size (Figs. 1 & 2). The identity of the products amplified with primers Cs1 & Cs3, Cs1 & Cs4 and Cs2 & Cs4 was confirmed as *C. shasta* SSUrRNA gene fragments by direct sequencing (data not shown). Reactions using 'universal' 18SUNI

Table 1. Oligonucleotide primers designed

Primer name	5'-3' Sequence
Cs1	gggccttaaaacccagtag
Cs2	attacaagggtcaatactttgc
R	ttgtatagcgtgccttgaat
Cs3	ccgtttcaggttagttacttg
Cs4	tctcagtagaacgacaaatgc
Cs5	ggagagaccaacttggctc
18SUNI(f)	ttaatttgactcaacacggg
18SUNI(r)	gggcatcacagacctgtta

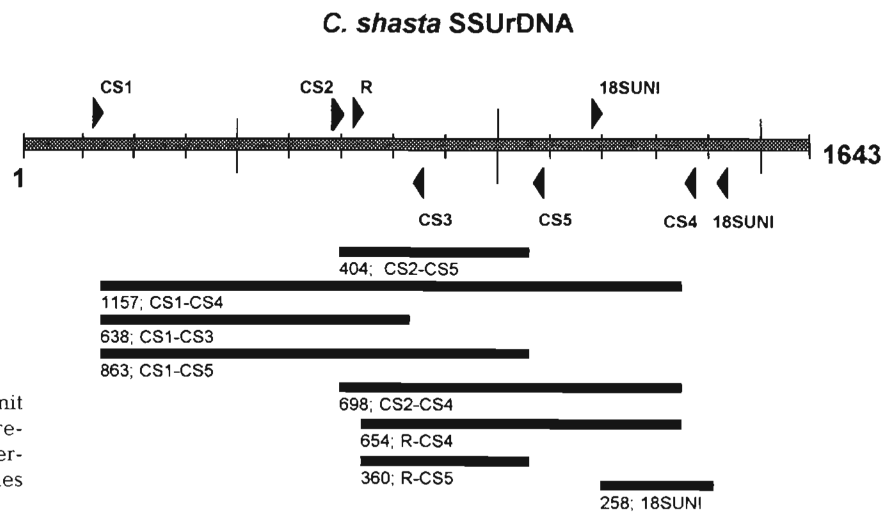

Fig. 1. *Ceratomyxa shasta* small subunit ribosomal RNA gene primer map. Predicted amplicon sizes obtained with different combinations of the oligonucleotides are given in base pairs

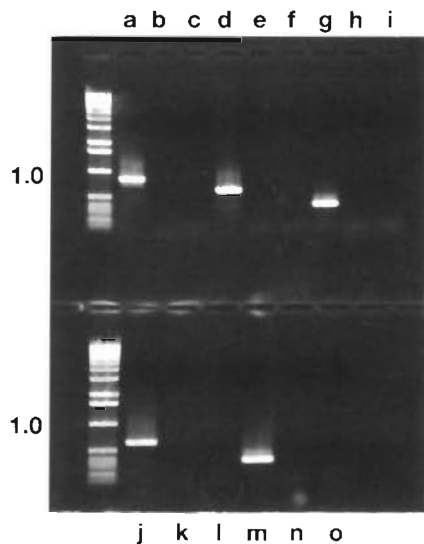

Fig. 2. Specificity of some combinations of the *Ceratomyxa shasta* primers with purified DNA from: *C. shasta* spores (lanes a, d, g, j, m), rainbow trout (lanes b, e, h, k, n) and *Henneguya salminicola* (lanes c, f, i, l, o). Primer sets used are Cs1 & Cs5 (lanes a–c); Cs1 & Cs3 (lanes d–f); Cs2 & Cs5 (lanes g–i); R & Cs4 (lanes j–l) and R & Cs5 (lanes m–o). PCRs were carried out as described in the text, with temperature = 55°C and template DNA at 50 pg per reaction. One percent agarose gel, ethidium-bromide stained

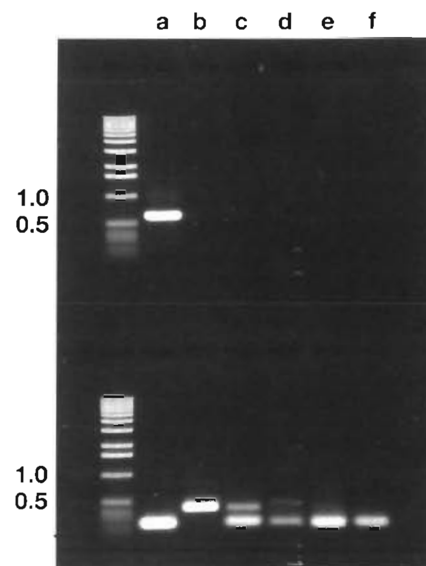

Fig. 3. Specificity of primers Cs1 & Cs3 (top) and 18SUNI(f) & 18SUNI(r) (bottom) with purified DNA from: *Ceratomyxa shasta* (lane a), *Myxobolus squamalis* (b), *Myxobolus arcticus* (c), *Henneguya salminicola* (d), *Kudoa* sp. (e) and rainbow trout (f). PCRs were carried out with template DNA at 500 pg (a, b, c, f), 10 ng (d) and 30 ng (e) per reaction, and temperature = 58°C. One percent agarose gel, ethidium-bromide stained

oligonucleotides (Table 1) yielded variable size amplicons with all the different templates used in this study (258 to 442 base pairs, bp), consistent with the size of the rDNA of each organism in the target region. The 18SUNI primers were subsequently used as controls for the presence of template DNA in the reactions, thus demonstrating suitability for PCR amplification. Primers Cs1 & Cs3 were chosen for further tests on sensitivity, specificity and optimisation of the assay.

Specificity

The specificity of primer set Cs1 & Cs3 was evaluated with genomic DNA from 4 different myxosporean species and from rainbow trout (Fig. 3). These oligonucleotides specifically primed *Ceratomyxa shasta* rRNA genes, but none of the other templates tested. When primers 18SUNI(f) & 18SUNI(r) were used in the reactions, it was clear that all the myxosporean and rainbow trout samples tested contained amplifiable SSUrDNA (Fig. 3). Primer sets Cs1 & Cs3 and Cs1 & Cs4 were also used in PCRs with samples of *C. shasta*-infected tissues from different sites throughout the geographic range of this parasite. Infected samples from Idaho, California, Oregon (USA) and British Columbia (Canada) were positive by PCR, yielding amplicons of the expected size (data not shown).

DNA extraction method

'Crude' and 'boiled-crude' fish tissue-preparation methods produced inconsistent amplifications including false-negatives and contradictory results at different dilutions of the samples (not shown). In contrast, template DNA generated by digestion of the tissues followed by either phenol/chloroform extraction or a 5 min boiling was equally suitable for PCR as determined by the presence of consistently amplified *Ceratomyxa shasta* DNA fragments on ethidium bromide-stained agarose gels. Comparison of the sensitivity of the PCR assay using template DNA generated by both techniques is illustrated in Fig. 4. It must be noted that BD-generated samples had to be diluted at least 1:50 in water to produce repeatable PCR results, while the fully extracted samples were suitable undiluted.

Sensitivity

The sensitivity of the PCR with primers Cs1 & Cs3 was evaluated using phenol/chloroform-extracted *Ceratomyxa shasta* DNA from Percoll® purified spores. Under the optimum conditions for the assay (58°C annealing temperature and reaction components as described in 'Materials and methods'), as low as 50 fg

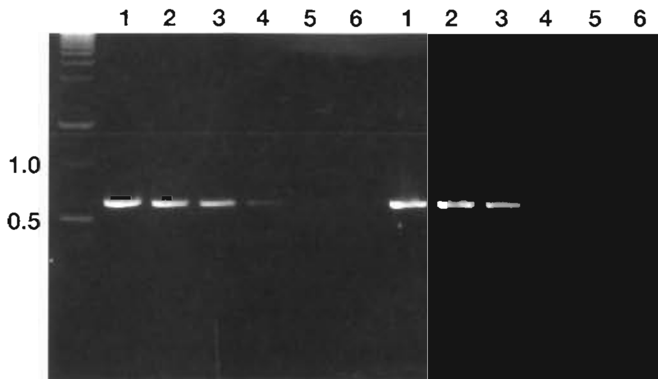

Fig. 4. Comparison of 'boiled-digested' and 'extracted-digested' sample preparation methods. Genomic DNA from Percoll® purified *Ceratomyxa shasta* spores was obtained by proteinase K digestion, followed by phenol/chloroform extraction (left) or 5 min incubation at 100°C (right). PCRs were carried out with primers Cs1 & Cs3 and temperature = 58°C. Template DNA was used in 10-fold decreasing concentrations, from 50 pg (lane 1) to 0.5 fg (lane 6) per reaction. One percent agarose gel, ethidium-bromide stained

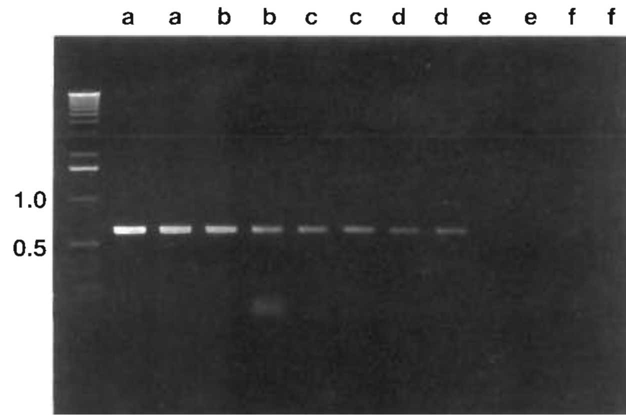

Fig. 5. Sensitivity of *Ceratomyxa shasta* primers Cs1 & Cs3, with DNA from uninfected gut tissue seeded with known numbers of spores and processed by the simplified, boiling-digesting preparation method. Two replicate samples containing 10-fold decreasing concentrations of 10^6 to 0 spores ml^{-1} were processed, diluted 1:100 in water and used as templates for the reactions. The corresponding number of *C. shasta* spores used per reaction ranges from 10 spores (lanes a) to 0.001 spores (lanes e). Lanes f do not contain *C. shasta* spores (negative controls). PCRs were carried out with temperature = 58°C. One percent agarose gel, ethidium-bromide stained

of DNA were detected (Fig. 4). When template DNA was obtained from naturally infected fish intestines with different intensities of infection by *C. shasta*, all infected samples, regardless of their intensities (evaluated by light microscopy), were positive by PCR. Furthermore, several samples diagnosed as uninfected by the visual examination were found positive by PCR. No visually positive samples were found uninfected by PCR.

In uninfected tissue samples seeded with purified *Ceratomyxa shasta* spores, the limit of sensitivity was achieved with those samples containing 10^3 spores ml^{-1} (Fig. 5). Given that these samples were diluted 1:100 and 1 µl of sample was used for each amplification reaction, the assay detection limit was 0.01 spores per 20 µl PCR mixture.

DISCUSSION

An increasing number of PCR-based protocols have been developed for the detection and identification of fish and shellfish pathogens (Leong 1995, Kent et al. 1996). These assays are particularly useful for the diagnosis of fastidious organisms which cannot be grown in culture or for those whose morphology does not permit confident visual identification (Weiss 1995, McManus & Bowles 1996), as is the case for myxosporeans. When compared to morphological, immunological or chemical methods, PCR is in general much more specific, sensitive and reliable. However, many pathology laboratories are still reticent in the use of these new technologies (Leong 1995). Most published PCR protocols involve time-consuming and expensive steps, like protein extraction with organic solvents, and ambiguous results can be obtained when these assays are performed by non-experienced personnel. Our purpose was to produce a sensitive and simple assay for the diagnosis of *Ceratomyxa shasta*, based on the SSUrDNA of this parasite. In addition to the increased sensitivity achieved with oligonucleotide primers targeted to a multiple copy gene, the availability of a large and continuously growing number of related and unrelated sequences allows the design of highly specific probes. Furthermore, the SSUrDNA displays significant inter- and intrageneric variability in the Myxozoa (Andree et al. 1999, authors' unpubl. data); thus, it is relatively simple to design probes for the discrimination of closely related myxosporean species.

Probably the main constraint of PCR diagnostic methods is the time-consuming, relatively expensive and hazardous traditional DNA extraction methods. Alternative protocols, based on commercial DNA-binding resins and spin-columns are an easy and safe alternative, but their cost and the need for centrifugation are limiting factors. For the *Ceratomyxa shasta* PCR, we found the extraction and precipitation steps which typically follow digestion of tissues (Sambrook et al. 1989) to be unnecessary. Comparable results were obtained with 5 min incubation in boiling water, allowing the procedure to be completed in one tube and without centrifugation. This simple procedure not

only reduces dramatically the chances of cross-contamination between samples and the time and expense of the procedure, but it also allows simultaneous processing of a large number of samples.

Using primers Cs1&Cs3 and the simplified tissue preparation procedure, as low as 50 fg of phenol/chloroform-extracted DNA was detected from purified *Ceratomyxa shasta* spores, or 1/100th of a spore from *C. shasta*-seeded, uninfected fish tissue. A similar sensitivity limit has previously been reported in a diagnostic assay for the microsporidian *Loma salmonae* (Docker et al. 1997). Although a gross estimation, these data suggest the existence of a minimum of 100 rDNA gene copies per *C. shasta* spore. To produce consistent results boiled samples had to be diluted at least 1:50, and a 1:100 dilution is routinely used in our laboratory. Thus, the overall sensitivity achieved with these samples is lower than the potential detection limit obtained with fully extracted samples. However, this level is sufficient for most purposes and the simplification of the procedure is worth the loss in sensitivity. The inconsistency of the PCR with more concentrated BD samples is probably caused by the presence of Taq inhibitors (Rossen et al. 1992), or by residual activity of proteinase K.

Only 2 PCR-based assays for the detection of myxozoans have been reported to date. The procedure for *Myxobolus cerebralis* can detect 1 parasitic cell in a single, 50 µl volume reaction, though results may become equivocal at a very low parasite to host DNA ratio (Andree et al. 1998). These authors also developed a nested approach, more sensitive and robust under all levels of infection examined. The sensitivity reported for the PKX-specific PCR is lower, about 5 cells per 100 µl reaction (Saulnier & de Kinkelin 1997). However, the latter presents the advantage of an extremely simple tissue-processing method, a single boiling step without any enzymatic digestion. Nevertheless, in our hands this procedure was inconsistent for the detection of *Ceratomyxa shasta* at low infection levels.

By PCR, *Ceratomyxa shasta* infections in gut tissue were diagnosed from experimentally infected trout, as soon as 5 d following exposure (data not shown). Although the procedure has been extensively tested on intestinal tissue, it can be easily adapted for different biological samples. Parasite DNA was detected from whole gill arches and from pieces of skin and muscle, as early as 2 d after exposure to *C. shasta* actinospore stages. *C. shasta* stages have also been detected by PCR in water samples and from tissues of the polychaete host, *Manayunkia speciosa* (Bartholomew et al. 1997). The PCR for *C. shasta* has been demonstrated a powerful tool for detecting all the stages of this parasite in the fish and in a variety of biological samples. The increased sensitivity afforded by the PCR is invaluable for monitoring early or subclinical infections, allowing the development of prophylactic policies for the introduction of fish to parasite-free areas. The development of similar assays for other myxozoans can contribute to a better understanding of their biology and epidemiology, and could contribute to strategies for reducing the impact of these parasites on wild and cultured fish populations.

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