JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Polymerase Chain Reaction Assay for the Detection of *Kudoa paniformis* and *Kudoa thyrsites* in Pacific Hake (*Merluccius productus*)

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Myoliquefaction of Pacific hake has been attributed to proteolytic action associated with parasitic infection. Among the two infecting species of *Kudoa*, *Kudoa paniformis* and *Kudoa thyrsites*, the former is reported to be more virulent for the "soft flesh" phenomenon in Pacific hake. The objective of this research was to develop a sensitive and specific polymerase chain reaction (PCR) assay to detect infection of hake by *K. paniformis*. Primers based on specific regions (~1562 bp) of the small subunit ribosomal DNA of *K. paniformis* successfully amplified the target DNA segments from both spore and muscle extracted DNA templates. DNA sequencing confirmed the veracity of this method to distinguish parasitic infection by *K. paniformis* versus *K. thyrsites*. The established PCR method was applied to investigate *Kudoa* infection in 44 Pacific hake samples using DNA extracted from muscle and/or spores, and the results were compared to infection evaluated by microscopic examination of extracted spores.

KEYWORDS: Pacific hake; *K. paniformis; K. thyrsites*; Polymerase Chain Reaction (PCR)

INTRODUCTION

Pacific hake (*Merluccius productus*), also known as North Pacific hake in Canada, or as Pacific whiting in the United States, is found off the Pacific coast of North America from Baja California to Vancouver Island, British Columbia (*1*). It has been regarded as the most abundant fishery resource of the northwest coast of the North American continent, with average annual harvests in the time periods 1990−1999 and 2000−2002 of 43×10^6 and 85×10^6 metric tons, respectively, in Canada and 173×10^6 and 209×10^6 metric tons, respectively, in the United States (*2*).

Despite its abundance, however, Pacific hake has gained an infamous reputation in the international market place over the past few decades, due to the susceptibility to "soft flesh" phenomenon. The "soft flesh" or postmortem myoliquefaction results from the action of proteolytic enzymes released into the muscle, which are activated rapidly after the fish is harvested (*3*), and is generally attributed to parasites of the genus *Kudoa* (Myxozoa: Multivalvulida) that infect the Pacific hake muscle fibers (*4*−*6*). Two species of the *Kudoa* genus, namely *Kudoa paniformis* and *Kudoa thyrsites*, have been found in the fish fillets of Pacific hake (*7, 8*). Previous studies have reported that of these two *Kudoa* species, *K. paniformis* is more virulent with regard to the postmortem myoliquefaction of Pacific hake (*9, 10*), while *K. thyrsites* infection has been positively correlated with severity of soft flesh in Atlantic salmon (*Salmo salar*) (*8*).

The life cycle of *Kudoa* species has not been completely elucidated, although early development of myxozoans including *Kudoa* has been documented (*9*). The sporogenic process of *K. paniformis* may be summarized as follows. The earliest stage of the parasite is the generative cell that can be detected within the muscle cells. The generative cells mature into spores, which are concentrated in the center of the infected muscle and, as their numbers increase, they expand into the fiber. As this occurs, the muscle is gradually replaced by the parasite. The parasite growth causes a breakdown of the fiber content without immunological host reaction (*11*). The muscle fibers look white at this stage. As a result of the increase in spore numbers within the muscle fiber, the parasite comes into contact with the inner surface of the fiber sarcolemma, and eventually the host immunological response is triggered (*12*). The host response consists of encapsulation of the pseudocysts, which causes parasite degradation and gradual destruction of the parasite. At this point, pseudocysts darken and gradually shrink. The final stage at which the parasite can be observed occurs when the pseudocysts are reduced to strings of small black beads (*13*). This suggests that the signs of infection may eventually disappear.

Microscopic examination of extracted spores or plasmodia in muscle tissue (*14*, *15*) is the most direct and commonly used method to describe the prevalence or intensity of parasitic infection. However, as mentioned above, not every stage of *Kudoa* infection on fish is visible. Furthermore, the ability of the microscopic method to detect the spores is a function of the sampling size, dilution factor, and the hemocytometer used.

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10.1021/jf062927+ CCC: $37.00
Published on Web 04/10/2007

Serological detection of soluble *K. thyrsites* antigen using an antigen capture enzyme linked immunosorbent assay (ELISA) method with polyclonal antibodies was developed for detection of *K. thyrsites* in Atlantic salmon (*16*). The results suggested that ELISA could detect the parasite in histologically undetectable extrasporogonic stages. *K. thyrsites* spores have also been analyzed using monoclonal antibodies (mAbs) produced by immunization of inbred BALB/c mice with highly purified spores (*17*). Some of the mAbs were found to be specific for antigens on the surface of *K. thyrsites* spores, whereas other mAbs reacted with polar capsules or with polar filaments of spores of *K. thyrsites*, *K. paniformis*, and *Kudoa crumena*. As few as 100 spores were detected by immunoblotting, indicating that these mAbs may have potential for use in developing a field-based diagnostic test. Nevertheless, a disadvantage of serological methods is the requirement for immunization of animals to raise specific antibodies.

Application of the Polymerase Chain Reaction (PCR) for detecting *Kudoa* infection based on specific DNA sequences has been reported for *Kudoa* in general and also specifically for *K. thyrsites* infection in Atlantic salmon (*18*). The PCR method has several potential advantages, including the capability to detect infection by *Kudoa* at stages when no or low levels of spores are present, or when spore counts are below the limit of detection of the microscopic method.

Partial sequences of the 18S small subunit (SSU) ribosomal DNA (rDNA) have been published for many *Kudoa* species, including AF031412 (GenBank accession number) for *K. thyrsites* from Atlantic salmon and AF034640 for *K. paniformis* from Pacific hake (*18*, *19*). Sensitive PCR assays based on the SSU rDNA sequences have been reported to provide the means for detecting all life stages of the organism and have become useful diagnostic tools for identification of myxozoans, including *Myxobolus cerebralis*, *Ceratomyxa shasta*, *Tetracapsula bryosalmonae*, as well as various *Kudoa* spp. (*18*−*20*).

Partial sequences of the 28S large subunit (LSU) rDNA have also been reported for 12 myxozoan species, including *K. paniformis* (AY302732) and *K. thyrsites* (AY302734) (*21*). However, the LSU partial sequences are only about half the length (710 and 689 bp for *K. paniformis* and *K. thyrsites*, respectively) of the SSU sequences (∼1600 bp). Whipps et al. (*21*) reported that the phylogenetic analyses of LUS sequence produced trees that were similar to those derived from SSU sequence analyses, but with poor resolution regarding interrelationships of *K. thyrsites*, *K. quadricornis*, *K. miniauriculata*, and *K. paniformis*, probably due to the limited sequence length and number of sequences completed for LSU.

Of the *Kudoa* spp., *K. thyrsites* in particular has been the target for PCR assays due to the severe myoliquefaction that results in infected Atlantic salmon as well as many other marine species of commercial importance over a wide geographical region (*22*). To our knowledge, development for a specific PCR assay has not yet been reported for *K. paniformis*, which is associated with myoliquefaction of Pacific hake.

The objective of this research was therefore to design *K. paniformis* specific primers for detection of *K. paniformis* infection in Pacific hake by PCR assay. The developed primers were used in conjunction with previously published *K. thyrsites* specific primers (*18*) to distinguish between infections by *K. thyrsites* and *K. paniformis*, and results of infection in field samples as assessed by the PCR method compared to spore counts evaluated concomitantly by wet-mount observations of the same samples.

MATERIALS AND METHODS

Fish. Pacific hake (*M. productus*) used for this research were harvested from October 2004 to June 2005 from waters close to Vancouver Island and the Strait of Georgia, ranging from longitude 124° 30′ W to 125° 15′ W and latitude 48° 26′ N to 48° 32′ N. During this period, 10−30 Pacific hake fish were obtained weekly, and spore enumeration was performed by microscopic observation. From these samples, 44 Pacific hake fish from 19 separate fishing trips were used for the evaluation of parasitic infection by PCR and microscopic evaluation. Alaska pollock (*Theragra chalcogramma*) used as a negative control in this study were obtained as by-catch from the hake fishery in the Juan de Fuca Strait in the fall of 2004.

Spore Isolation. Spores of *K. paniformis* were isolated from the skeletal muscle of infected Pacific hakes by discontinuous gradient centrifugation using Percoll (Sigma, St. Louis, MO) as described by Chase et al. (*17*). Spores of *K. thyrsites* purified from infected salmon were a gift from Dr. S. Jones of the Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, BC.

Spore Enumeration by Microscopic Observation. Preparation of extracts of fish tissue for spore counting was done according to the method of Dawson-Coates et al. (*15*) with minor modifications, as described by Samaranayaka et al. (*23*). Fish muscle sample (∼1−2 g) was taken from the nape area. Approximately a 100 mg sample of this tissue was accurately weighed into 15 mL vials, and 10 mL 0.04% (w/v) of trypsin (Sigma, EC 3.4.21.4 Type II-S from porcine pancreas; activity of 1800 BAEE units/mg solid) in phosphate buffer solution at pH. 7.4 (PB) was added to the sample. After being mixed with a vortex, the samples were incubated at 37 °C in a shaking water bath for 30 min. The samples were vortexed again for 5 to 10 s and then incubated at 37 °C for another 30 min. The samples were then centrifuged at 3000 rpm with a Beckman GS-6 centrifuge (Fullerton, CA) for 15 min at 4 °C. After the supernatants were discarded, the pellets containing spores were suspended in 1 mL of PB. The resultant slurry was diluted 2, 10, or 100 times as needed, and the spores were counted using a hemocytometer (Hausser Scientific, Horshan, PA) under an Axioskop Routine microscope (Zeiss Germany) using 200× magnification. The hemocytometer contains eight equal square areas with a volume of 0.1 μL (10^{-4} mL) each. Duplicates were performed for the spore counting on each sample. Reproducibility of the spore counting was confirmed by independent analyses of a subset of the samples by two or three analysts, yielding four or six replicate data for each of those samples.

The infection was expressed as number of spores per gram of fish muscle, calculated as shown in eq 1:

$$\text{infection intensity} = NVF \times 10^4/W \quad (1)$$

where N is the average number of spores counted per area of hemocytometer, V is the final volume of spore suspension (1 mL in this research), F is the dilution factor, and W is the weight of sample (100 mg). On the basis of this equation, if a single spore was counted from any of the 16 areas of the hemocytometer (8 areas per trial with duplication), an infection of 6.25×10^3 spores per gram would be reported. Any infection lower than this limit (no spore counted in any of the 16 areas) was reported as "not detected". Therefore, the detection limit using this method is 6.25×10^3 spores per gram of fish muscle.

DNA Extraction. DNA samples were extracted from the isolated spores or directly from fish muscle tissue using the Qiagen DNeasy Tissue Kits (Qiagen Inc., Ontario, Canada). Approximately 25−50 mg of fish muscle from the nape (taken from the 1−2 g tissue previously described in the sampling for spore enumeration) was used. The extracted DNA solutions were diluted, and the absorption was measured at 260 nm using a UV−visible spectrophotometer (Shimadzu UV-1700). The concentration of extracted DNA was calculated based on A_{260} value of 1 (with a 1 cm detection path) corresponding to 50 μg DNA per milliliter water (DNeasy Tissue Handbook). The extracted DNA concentration ranged from 25 to 100 μg/mL.

Primers for PCR. The *K. paniformis* SSU rDNA partial sequence (AF034640 GenBank accession number) published in the NCBI database was aligned with other available *Kudoa* sequences in this databank, including those of the SSU rDNA of *K. thyrsites*, to identify

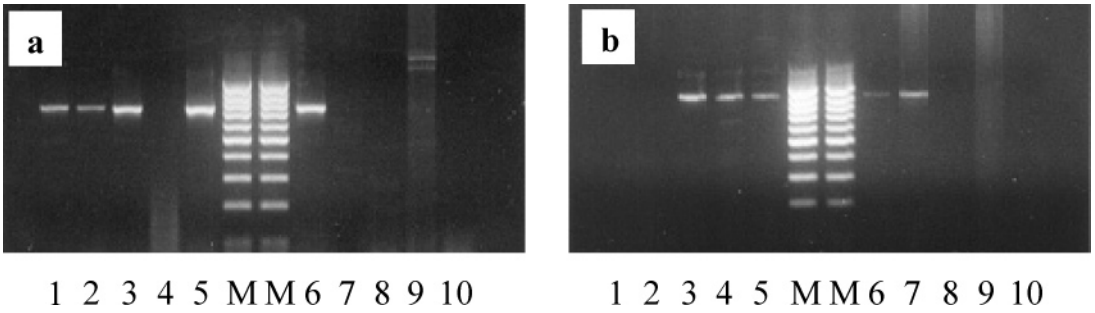

Figure 1. PCR amplification with *K. paniformis* primers (**a**) and *K. thyrsites* primers (**b**) of the SSU rDNA extracted from muscle or isolated spores from four Pacific hake fish samples. DNA from Pollock muscle and water were analyzed as negative controls. Lanes: 1, hake 1 (muscle); 2, hake 1 (spore); 3, hake 2 (muscle); 4, hake 3 (muscle); 5, hake 4 (muscle); 6, hake 4 (spore); 7, isolated *K. thyrsites* (spore); 8, pollock (muscle); 9, H_2O; 10, pollock (muscle); M, DNA ladder from high to low molecular weight (1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp).

unique sequences that could be suitable candidates for *K. paniformis* specific primers. Two *K. paniformis* specific primers were designed: KP18S4f (GCTCAAAGCAGGCGTTACGTC) and KP18S4r (CAAGATTCCCCATCCCTCTCG). Two primers specific for *K. thyrsites* were also identified based on the published literature (*18*): KT18S6f (CTCAACCAACTGGCCTCG) and KT18S1r (CGTCAATTTCTTTAAATTTGG). The four primers were synthesized by Qiagen (Alameda, CA).

DNA Amplification, Identification, and Sequencing. PCR experiments were performed in 25 μL volumes using PCR buffer, 1.5 mM Mg^{2+}, 0.2 mM dNTP, 0.5 μM of each primer, and 0.025 U/μL *Taq* DNA polymerase (Invitrogen Canada Inc., Burlington, ON). Amplifications were run in a Perkin-Elmer GeneAmp 4800 Thermocycler (Perkin-Elmer Cetus, Norwalk, CT). For PCR with *K. thyrsites* primers, DNA was denatured at 95 °C for 3 min, followed by 35 cycles of amplification (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min). For PCR using the *K. paniformis* primers, an annealing temperature of 56 °C was used, rather than 53 °C; all other conditions remained the same. The amplifications were terminated by a 10 min extension at 72 °C. The amplified DNA segments were applied to a 1.5% agarose gel for electrophoresis. A low-range MassRuler DNA ladder (Fermentas Life Sciences) was used as a molecular weight marker.

The PCR products were submitted to the Nucleic Acid Protein Service Unit (Michael Smith Laboratories, University of British Columbia) for DNA sequencing using a Perkin-Elmer GeneAmp 4800 Thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The resulting sequences were compared with sequences reported on the NCBI database using the Basic Local Alignment Search Tool (BLAST).

Sensitivity of the PCR Tests for the Detection of *K. paniformis* Using Spore and Muscle Extracted DNA. The DNA samples extracted from purified *K. paniformis* spores and *K. paniformis* infected fish muscle were diluted to different concentrations. PCR was performed using the diluted DNA templates to determine the lowest concentration of DNA that could be amplified by this method. PCR tests were also applied to field Pacific hake samples with different infection levels based on spore counts evaluated by the wet-mount microscopic method as described above.

RESULTS AND DISCUSSION

Moran et al. (*9*) described *K. paniformis* as "Myxospores, quadrate in apical view, with valve tips rounded and no ornamentation, polar capsules pyriform, subequal", while *K. thyrsites* was described as "Myxospores stellate in apical view, no ornamentation, polar capsules pysiform, unequal". On the basis of these descriptions, microscopic observation of the hundreds of Pacific hake sampled between October 2004 and June 2005 revealed that over 90% of the fish were infected either by both *Kudoa* species, or by *K. paniformis* only, or *K. thyrsites* only. Spore counts ranged from the limit of detection of the microscopic enumeration method (6.25×10^3 spores per gram of tissue) to $\sim 10^6$ spores per gram for *K. thyrsites* and $> 10^8$ spores per gram for *K. paniformis*. A small percentage of the fish samples were not infected by either of these two parasites. Although the microscopic method for spore enumeration has some limitations with regard to both sensitivity and precision, the estimates of both spore type and intensity of infection obtained by this method provided useful information for the selection of samples to be investigated using the PCR assay.

Parts **a** and **b** of **Figure 1** show the PCR products of *K. paniformis* and *K. thyrsites* primers, respectively, on DNA extracted from fish muscle tissue or from spores. DNA extracted from isolated *K. paniformis* spores was used as the positive control. Two negative controls were used in this study. One was the DNA from Pollock fish muscle, which was used to distinguish host (fish muscle) DNA from parasite DNA. The other negative control was DNA from isolated *K. thyrsites* spores, which was used to test the specificity of the *K. paniformis* primers.

Bands appeared at the 700−800 bp region in **Figure 1a** and at about 900 bp in **Figure 1b**, which correspond to the designed target DNA segments (728 bp for *K. paniformis* and 908 bp for *K. thyrsites*). Both the blank (water) and Pollock fish DNA showed negative results using *K. paniformis* and *K. thyrsites* primers. DNA extracted from isolated *K. thyrsites* spores showed a positive band when *K. thyrsites* primers were used (lane 7, **Figure 1b**), while the result was negative when *K. paniformis* primers were used (lane 7, **Figure 1a**). These results indicated that the primers were able to distinguish spore DNA from the host fish DNA. Meanwhile, the designed *K. paniformis* primers were found to be specific for *K. paniformis* and could be used to distinguish *K. paniformis* infection from *K. thyrsites* infection (lane 7 in both parts **a** and **b** of **Figure 1**). Comparing parts **a** and **b** of **Figure 1**, hake 1 (lanes 1 and 2) was infected by *K. paniformis*, hake 3 (lane 4) was infected by *K. thyrsites*, while hake 2 (lane 3) and hake 4 (lanes 5 and 6) were infected by both species.

In order to further characterize the PCR products, DNA sequencing experiments were conducted. Since the PCR products were considered to contain the major target DNA segments with little contamination, they were used without further purification to perform the DNA sequencing. Three PCR products using *K. paniformis* primers were submitted for sequence analysis. Six DNA segments (three from KP18S4f and three from KP18S4r primers) were achieved with molecular weight of 685 to 709 bp, close to the target DNA of 728 bp that should be amplified by the designed primers. The results were then compared to published nucleotide sequences using the "BLAST" tool on the NCBI website. The results showed highly homologous identities (>99%) to AF034640 (Genbank accession number), the *K. paniformis* small subunit ribosomal RNA gene partial sequence. Similarly, results obtained by

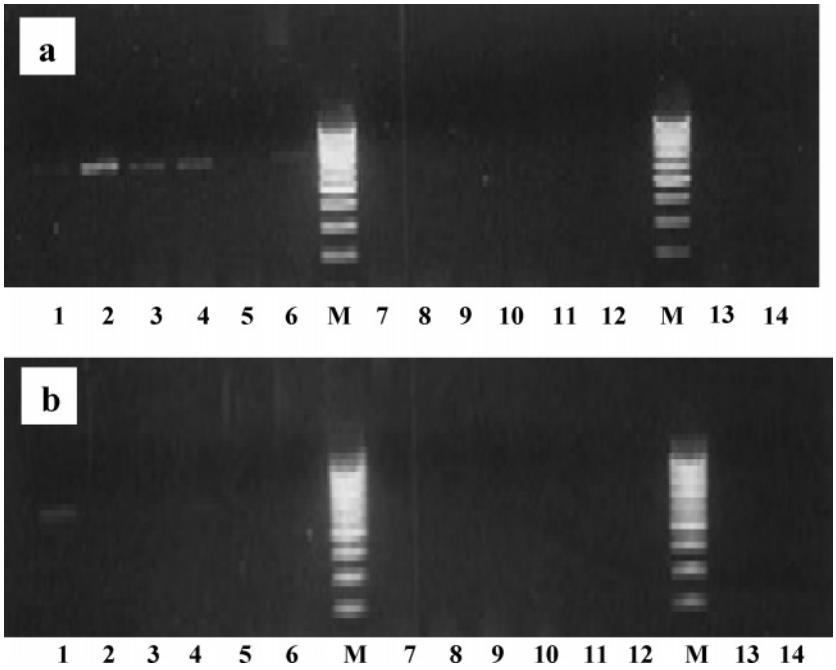

Figure 2. PCR amplification of the SSU rDNA from spore (**a**) and Pacific hake muscle (**b**) at different concentrations. The amount of DNA in 25 μL reaction volumes are as follows: lanes 1 and 2, 3.75 ng; lanes 3 and 4, 0.375 ng; lanes 5 and 6, 0.075 ng; lanes 7 and 8, 0.0375 ng; lanes 9 and 10, 0.0075 ng; lanes 11 and 12, 0.00375 ng; lane 13, pollock DNA; lane 14, H_2O; lane M, DNA ladder of molecular weights from 1000 to 100 bp.

sequencing of the PCR products using *K. thyrsites* primers and comparison to the NCBI databank showed over 99% identity of the sequenced DNA to the published *K. thyrsites* small subunit ribosome RNA partial sequences. These results, in conjunction with the agarose gel electrophoresis results, indicated that the PCR method established with the designed primers could be used for specific identification of infection by *K. paniformis* and/or *K. thyrsites*.

The results shown in **Figure 1** demonstrate that DNA extracted from both infected fish muscle and isolated spores could be amplified by the PCR. The ability to perform PCR with DNA extracted from infected fish may confer advantages of speed and simplification of the procedure resulting from elimination of the need to perform tedious spore isolation procedures. However, these advantages may be attained through a compromise in the sensitivity of the method, since the extracted DNA used in the PCR assay would include both fish and spore DNA, but only the latter would serve as template DNA.

To further study the sensitivity of this method, the PCR tests using the designed KP18S4f and KP18S4r primers were performed on DNA extracts diluted to different concentrations. Parts **a** and **b** of **Figure 2** show the agarose gel results for PCR amplification from different dilutions of DNA extracted from spore and muscle, respectively. The spore extracted DNA showed visible bands until lane 6 (**Figure 2a**), corresponding to a detection limit of 0.075 ng of DNA. As for the muscle extracted DNA, only lane 1 showed a positive result (**Figure 2b**), corresponding to a detection limit of 3.75 ng. Since the DNA extracted from the muscle would be a mixture of fish DNA and spore DNA, it is not surprising that the detection limit for the target (*Kudoa* spore) DNA from muscle is inferior to that from the isolated spores.

PCR assay using both *K. paniformis* and *K. thyrsites* primers was applied to 44 Pacific hake samples indicated by the microscopic spore counting method to be infected by *K. paniformis* only (infection intensity from 10^8 to 10^3 spores per gram of fish muscle), *K. thyrsites* only (infection intensity from 10^5 to 10^3 spores per gram of fish muscle), both *K. paniformis* and *K. thyrsites*, or that had no detectable infection (**Table 1**). Initially, DNA extracted from muscle was used for the PCR assay. Since negative results were found by PCR for some samples that had been identified as positive based on the microscopic spore counting method, PCR assays were repeated on these samples using the DNA extracted from isolated spores rather than directly from muscle. The results for PCR assay using isolated spore DNA are listed in parentheses next to the results using DNA extracted from muscle tissue (**Table 1**).

Good agreements between PCR and spore counting were found for highly infected samples. PCR with *K. paniformis* primers showed positive results for samples 1−12, which had infection levels ranging from 10^8 to 10^6 per gram of fish based on the microscopic spore counts. One sample (#10) showed a positive result by PCR using isolated spore DNA but not when DNA extracted from muscle was assayed. Interestingly, PCR with *K. thyrsites* primers showed that samples 1−7 were also infected by *K. thyrsites*, although these were not detected under the microscope. The low number of *K. thyrsites* spores among high numbers of *K. paniformis* spores may have made their detection difficult under the microscope. For the samples infected with lower levels of *K. paniformis* (samples 13−24), five showed negative results by PCR using both muscle and spore extracted DNA, while three were found by PCR to be infected by *K. thyrsites* rather than by *K. paniformis* as implied by microscopic observation.

Samples that were only infected by *K. thyrsites* presented lower spore counts (samples 25−34) compared with the *K. paniformis* infections (sample 1−24). This result agrees with previous research reporting that *K. paniformis* is the major infection in Pacific hake (*10*). Samples 35−37 which were indicated by microscopic spore enumeration to be infected by relatively high levels of both *K. paniformis* and *K. thyrsites* were confirmed by PCR to contain both types of *Kudoa*, whereas samples identified microscopically as positive for both types at infection levels near the limit of detection (6.25×10^3 spores per gram) were shown by PCR to be negative (sample 39) or

Table 1. Comparison of Microscopic Spore Counting Method and PCR Assay for Detecting *Kudoa* Infection in Pacific Hake

	microscopic spore count[a]		PCR analysis muscle DNA (spore DNA)[b]	
sample no.	*K. paniformis*	*K. thyrsites*	*K. paniformis*	*K. thyrsites*
1	1.89E+08	ND	+	+
2	1.02E+08	ND	+	+
3	2.87E+08	ND	+	+
4	1.54E+08	ND	+	+
5	1.85E+07	ND	+	+
6	3.19E+07	ND	+	+
7	1.45E+07	ND	+	+
8	1.61E+07	ND	+	−
9	9.31E+06	ND	+	−
10	5.19E+06	ND	− (+)	− (−)
11	1.35E+06	ND	+	−
12	3.17E+06	ND	+	−
13	7.00E+05	ND	+	−
14	1.30E+05	ND	− (−)	− (−)
15	2.56E+05	ND	−	+
16	2.50E+05	ND	+	−
17	1.87E+04	ND	− (−)	− (+)
18	3.75E+04	ND	− (−)	− (−)
19	1.25E+04	ND	− (−)	− (−)
20	4.37E+04	ND	− (+)	− (−)
21	6.25E+03	ND	− (−)	− (−)
22	6.25E+03	ND	+	−
23	6.25E+03	ND	−	+
24	6.25E+03	ND	− (−)	− (−)
25	ND	1.87E+05	− (−)	− (+)
26	ND	1.37E+05	− (−)	− (−)
27	ND	6.37E+04	− (−)	− (−)
28	ND	6.87E+04	−	+
29	ND	6.88E+04	−	+
30	ND	2.50E+04	− (−)	− (−)
31	ND	6.25E+03	− (−)	− (+)
32	ND	6.25E+03	− (−)	− (−)
33	ND	6.25E+03	− (−)	− (+)
34	ND	6.25E+03	− (−)	− (+)
35	1.02E+08	1.25E+06	+	+
36	3.43E+07	1.25E+05	+	+
37	4.14E+06	3.40E+05	− (+)	− (+)
38	6.87E+04	1.25E+04	+	−
39	6.25E+03	5.00E+04	− (−)	− (−)
40q	3.12E+04	6.25E+03	−	+
41	ND	ND	−	+
42	ND	ND	−	+
43	ND	ND	− (−)	− (−)
44	ND	ND	+	+
K. paniformis[c]	4.90E+08	ND	+	−
K. thyrsites[c]	ND	NA	−	+
pollock[d]	ND	ND	−	−
water	ND	ND	−	−

[a] Spore counts per gram of muscle; ND = not detected (below detection limit of 6.25E+03 spores per gram); NA = not applicable. [b] PCR was conducted using DNA extracted from muscle for all samples and also from isolated spores for some samples (values in parentheses). [c] DNA was extracted from isolated *K. paniformis* and *K. thyrsites* spores. [d] DNA was extracted from pollock fish muscle.

to be infected by only either *K. paniformis* (sample 38) or only *K. thyrsites* (sample 40). In contrast, three out of four samples classified as "non-infected" by microscopic inspection (samples 41−44) showed positive results on PCR assay; sample 44 was infected by *K. paniformis* while samples 41 and 42 were infected by *K. thyrsites*.

The present results suggest that the PCR method is more sensitive and specific in detecting no or lower level of infections, as well as capable of detecting low levels of *K. thyrsites* in the presence of high infection of *K. paniformis*. Due to the overall morphological similarity of the two *Kudoa* spore species when observed microscopically at 200-fold or lower magnification, it is sometimes difficult to confirm visually if the spores observed are *K. paniformis* or *K. thyrsites*. The PCR method showed its advantage over the microscopic observation in this regard, since as presented above, the two sets of primers used were found to be specific for *K. paniformis* and *K. thyrsites*.

However, the results of this study also showed that PCR using DNA extracted from muscle failed to show the existence of infection for some samples that had been identified as positive by microscopic observation, even though DNA extracted from isolated spores from those same samples did show positive PCR results. Furthermore, a few samples showed negative results even when DNA supposedly extracted from "spores" was used for PCR analysis. There are two possible explanations for these discrepancies, which were observed primarily for samples with low spore counts determined microscopically. First, the "spores" observed under the microscope may not in fact actually have been spores but may have been small tissue particles released upon trypsin digestion of the fish muscle. As seen from eq 1, even if only a single "spore" was counted in one area on the hemocytometer, a final infection level of 10^4 spores per gram of fish would be reported. On the other hand, the discrepancy may also be attributed to non-representative sampling in the PCR method; in other words, due to the small sample size (25−50 mg of fish muscle) used for DNA extraction for the PCR assay, spores may have been missed during sampling from fish muscle having low infection levels. For example, even if the spores were ideally evenly distributed, 50 mg of muscle with an infection level of 10^4 per gram of fish may contain less than 500 spores. In this case, the target spore DNA concentration might be too low to be detected. Moreover, considering that the small amount of spore DNA would be mixed with a large amount of fish DNA, it would be even more difficult to be amplified by PCR.

Anomalies between PCR and wet mount microscopic observations for *K. thyrsites* that were observed by Moran et al. (*20*) were also suggested to reflect problems inherent in small sample sizes. These authors reported that, with respect to screening for *K thyrsites* infections, the PCR assay may be more effective than wet mount preparations as it not only detects all stages of the parasite but does so by utilizing a greater amount of the tissue (*20*). False negative ELISA and PCR scores for two samples that were weakly positive by histological analysis were reported by Taylor and Jones (*16*), who suggested that this could be the result of over-dispersed distribution of the parasite in samples with less severe infections.

One approach to minimize these problems in the PCR assay developed in the present study therefore could be to increase the sample size of muscle used for DNA extraction. Preliminary trypsin digestion and/or Percoll extraction to remove fish muscle and to concentrate the spores could also be beneficial to minimize false negatives attributed to sampling in the PCR assay. However, it should also be noted that discrepancies between PCR and microscopic detection were observed primarily in cases with lower spore counts and may be considered to be of less importance in terms of practical significance to the soft flesh problem, which is associated with more severe infections (*16*).

As mentioned previously, the purpose for *Kudoa* detection is to estimate the impact of these parasites on soft flesh in infected fish and to determine the threshold infection level that leads to postmortem myoliquefaction. St-Hilaire et al. (*14*) reported that an infection level of 2×10^4 g^{-1} could cause soft flesh of Atlantic salmon, while Dawson-Coates et al. (*15*) reported that a much higher threshold of 4.0×10^5 g^{-1} of *K.*

thyrsites was required to result in severe texture degradation of Atlantic salmon. Samaranayaka et al. (*23*) found that Pacific hake infected by *K. paniformis* at a level of 3×10^6 had very soft texture with maximum hardness force of compression <165 g. However, the relationship between the texture of Pacific hake and *Kudoa* infection by either or both *K. paniformis* and/or *K. thyrsites* is not clear, and it is the subject of research currently underway in our laboratory. Based on the results presented here, it is suggested that PCR assay combined with microscopic spore counting could be used to elucidate the relative contribution and threshold levels of *K. paniformis* and *K. thyrsites* leading to soft flesh in infected fish.

ACKNOWLEDGMENT

The fish used in this study were provided by Nicholas Knott of Steveston Seafood Direct Ltd. and Ron de Silva of Pacific Fisheries Technologies Inc. Thanks are extended to Dr. Christopher Whipps (Department of Microbiology, Oregon State University) for helpful advice on primer design, Dr. Simon Jones (Fisheries and Oceans Canada, Pacific Biological Station) for technical training, and our summer and work study students (I. Cheung, T. Ho, E. Leung, C. Tam, and L. S. Zhou) for their assistance in spore counting.

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Received for review October 12, 2006. Revised manuscript received February 21, 2007. Accepted March 7, 2007. This research was funded by the strategic project program of the Natural Sciences and Engineering Research Council (NSERC) of Canada.

JF062927+