Vol. 90: 143–151, 2010
doi: 10.3354/dao02194

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published June 11

Comparison of diagnostic techniques to detect the clam pathogen *Perkinsus olseni*

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ABSTRACT: Perkinsosis in clams in Galicia (NW Spain) is caused by the protozoan parasite *Perkinsus olseni* Lester & Davis, 1981. We used 5 clam species of commercial interest cultured in Galicia (*Ruditapes decussatus*, *R. philippinarum*, *Venerupis pullastra*, *V. rhomboides*, and *Donax trunculus*) to compare various *P. olseni* diagnostic techniques. Results of a nested PCR assay for the diagnosis of *P. olseni* were compared to those obtained using 2 classical methods of diagnosis proposed by the World Organisation for Animal Health (OIE), viz. histology and incubation in Ray's fluid thioglycollate medium (RFTM). Moreover, the same samples were analyzed by 2 separate research groups. The results obtained by PCR showed high sensitivity and good correlation between research groups. In addition, this method is faster than histopathology and incubation on RFTM and less expensive than histopathology. Moreover, nested PCR requires less specialized training for technicians than histology. Histopathology also showed high specificity and a good correlation between research groups. Results from incubation on RFTM suggest that this method could give divergent results between research groups, particularly in the case of low levels of infection, but it is nevertheless useful for disease-monitoring purposes. PCR is appropriate for rapidly screening large numbers of clams.

KEY WORDS: *Perkinsus* · Diagnosis · PCR · Histology · Ray's fluid thioglycollate medium · Clams · Aquaculture

INTRODUCTION

Perkinsus olseni has been associated with mortalities in populations of various groups of mollusks around the world (Lester & Davis 1981, Lauckner 1983, Park & Choi 2001, Bower & McGladdery 2003) and has been catalogued as a notifiable disease by the World Organisation for Animal Health (OIE). *P. marinus* was the first species of the genus to be described and was reported as the causative agent of mortalities of *Crassostrea virginica* in the Gulf of Mexico (Mackin et al. 1950). Since the synonymy between *P. olseni* and *P. atlanticus* (Goggin 1994, Robledo et al. 1999a, Murrell et al. 2002, Cremonte et al. 2005) and between *P. chesapeaki* and *P. andrewsi* (Dungan et al. 2002, Burreson et al. 2005) has been described, the remaining species of the genus are *P. qugwadi* in *Patinopecten yessoensis* (Blackbourn et al. 1998), *Perkinsus mediterraneus* in *Ostrea edulis* (Casas et al. 2004), and *P. honshuensis* in *Ruditapes philippinarum* (Dungan et al. 2006); recently, *P. behaensis* has been described in oysters from China (Moss et al. 2008). However, *Perkinsus* spp. can infect several alternate hosts in addition to the type host, and this is especially true for *P. olseni* (Kotob et al. 1999, Dungan et al. 2002, Bower & McGladdery 2003).

Perkinsosis is produced by both *Perkinsus marinus* and *P. olseni* and is included as a notifiable disease on List B of the OIE. Screening methods recommended by the OIE are histopathology and incubation on Ray's fluid thioglycollate medium (RFTM) (OIE 2006). In addition, other methods have been developed, especially for the oyster parasite *P. marinus*, including mol-

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ecular methods based on PCR (Robledo et al. 1998, Elandalloussi et al. 2004, Russell et al. 2004) and immunological detection (Choi et al. 1991, Dungan & Roberson 1993). Internal transcribed spacers (ITS-1 and ITS-2) are non-coding regions of the RNA gene cluster that are less conserved than the ribosomal RNA coding genes and are suitable to distinguish among proximal species. They have been used in several studies to distinguish different species of the genus *Perkinsus* (Kotob et al. 1999, Robledo et al. 1999b, Casas et al. 2002, Dungan et al. 2002). Here we compared histopathology, RFTM, and PCR of the rDNA ITS region for the diagnosis of *P. olseni* in the principal clam species cultured in Galicia (NW Spain). We intended to compare different diagnostic techniques usually used in the diagnosis of *P. olseni*, focusing on limitations related to these techniques.

Table 1. Host clam species, origin, and analyses carried out for comparing different diagnostic techniques. RFTM: Ray's fluid thioglycollate medium

Species	Origin	Histo-pathology	RFTM	PCR assay
Venerupis rhomboides	Ría de Arousa	X		X
Venerupis pullastra	Ría de Arousa	X		X
Ruditapes philippinarum	Ría de Arousa	X		X
Ruditapes decussatus	Ría de Muros e Noia	X	X	X
Ruditapes decussatus	Ría de Pontevedra	X	X	X
Ruditapes decussatus	Ría de Vigo	X	X	X
Donax trunculus	Ría de Corcubión	X	X	X
Venerupis pullastra	Ría de Vigo	X	X	X
Ruditapes decussatus	Ría de Ferrol	X		X

MATERIALS AND METHODS

Clam samples. Nine batches of 30 clams were screened for the detection of *Perkinsus olseni*, covering 5 different clam species cultured in Galicia (NW Spain). Table 1 and Fig. 1 show analyses conducted with each clam species and the sampling locations. Each animal was divided in half, and each half was simultaneously processed for histopathology and molecular biology under identical conditions by 2 separate research groups (hereafter Groups A and B) from 2 participating laboratories (Laboratories A and B) using the facilities of Laboratory A. In addition, 5 samples of 30 clams each were subjected to culture in RFTM. All 3 diagnostic techniques were carried out on the same animals by the 2 participating research groups.

Fig. 1. Origin of the samples used in this study

For histopathological analysis, the soft parts of the mollusks were fixed in Davidson's fixative for 24 h (Shaw & Battle 1957). Oblique transverse sections, approximately 5 mm thick, were taken from each specimen, including mantle, gills, gonad, digestive gland, nephridia, and foot. Sections were embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin.

For RFTM, 1 entire demibranch for each research group was placed in thioglycollate supplemented with mycostatin and incubated in the dark at room temperature for 72 h. Tissue was stained with lugol iodine and observed under light microscopy for the presence of hypnospores. Both groups applied Mackin's infection intensity scale for diagnosis (Ray 1954).

DNA isolation and specific PCR analysis. One fragment of gill (~20 mg) was excised with a clean scalpel over a clean slide for DNA extraction. DNA from gills was extracted with a DNAzol kit (Invitrogen) following the manufacturer's instructions. A nested PCR was then performed. The first reaction was done according to Kotob et al. (1999) with 10× buffer in a volume of 25 µl containing 10 mM of Tris, 50 mM KCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1 µl (10 to 50 ng) of DNA, and 1.25 U of *Taq* polymerase. Buffer, $MgCl_2$, and *Taq* were obtained from Roche, and primers and dNTPs from Invitrogen. The primers used were Perk-ITS S (5'-CTT AGA GGA AGG AGA AGT CGT AAC-3') and Perk-ITS As (5'-GCT TAC TTA TAT GCT TAA ATT CAG-3'). The reaction was placed in an Applied Biosystems Gene Amp® PCR system 9700 thermocycler, and the following amplification conditions were used: 1 cycle at 94°C for 4 min; 42 cycles at 94°C for 71 s, 50°C for 82 s, and 72°C for 71 s; and a final

extension step of 10 min. Since we observed amplification of host DNA, a second round of PCR amplification was carried out with internal primers. Sequences of *Perkinsus olseni* deposited in GenBank under accession numbers U07697, U07698, U07699, U07701, AF140295, AF369969, AF369970, AF369974, AF369975, AF369977, AF369978, AF369979, AF509333, and AF522321 were aligned using ClustalX (Thompson et al. 1997), and the specific *P. olseni* primers Pk-ITS1 S (5′-TCT GCG AAA CTA GCG GTC TT-3′) and Pk-ITS2 As (5′-ACC GAC AAG CGT GCT ATG AT-3′) were designed. The reaction occurred in 10× buffer containing 10 mM of Tris, 50 mM KCl (pH 8.3), 2 mM $MgCl_2$, 0.16 mM of each dNTP, 0.16 µm of each primer, 1 µl of DNA from the previous PCR reaction, and 1.25 U of *Taq* polymerase. The following program was used: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 90 s, 50°C for 90 s, and 72°C for 90 s; and a final extension for 10 min. All PCR reactions included DNA from a clam heavily infected with *P. olseni* as positive control and water as a negative control. PCR products were resolved in 1% agarose in Tris-acetic acid-EDTA buffer gel (w/v) stained with ethidium bromide and including a 100 bp ladder size standard (Invitrogen).

DNA sequencing. PCR products were purified by enzymatic treatment with 10 U of the enzyme exonuclease I (Exo I) and 1 U of shrimp alkaline phosphatase (SAP; USB) during 1 h at 37°C and 15 min at 80°C and sequenced in an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequences obtained were compared to those previously published in GenBank using BLASTn (Altschul et al. 1990) and ClustalX alignment (Thompson et al. 1997).

***In situ* hybridization.** To confirm that the amplified region corresponded to *Perkinsus olseni* detected by histology, *in situ* hybridization was carried out by labeling the nested PCR product (672 bp) with digoxigenin, using a PCR Dig Labeling Mix (Roche). The probe was made in a volume of 50 µl with 10× buffer containing 10 mM of Tris, 50 mM KCl (pH 8.3), 4 mM of $MgCl_2$, 0.2 mM of dATP, dCTP, and dGTP, 0.19 mM of dTTP and 0.01 mM of digoxigenin-11-dUTP, 0.4 µM of each primer (Pk-ITS1 S and Pk-ITS2 As), and 2.5 U of *Taq* polymerase. The temperature profile was the same as previously described for PCR with this primer pair.

Paraffin blocks of a clam that was highly parasitized by *Perkinsus olseni* were cut in sections of about 7 µm and placed on Silane-prep slides (Sigma) and dried for 24 h at 37°C. Sections were then dewaxed with xylene and treated with 75 µg ml^{-1} of Proteinase K for 30 min at 37°C. Subsequently, they were immersed in inactivation buffer with 0.1 M Tris, 0.1 M NaCl for 3 min at room temperature. They were then dehydrated for 1 min in 95% ethanol and 1 min in absolute ethanol and then air dried. Sections were prehybridized in 500 µl of hybridization buffer (50% formamide, 4× saline sodium citrate [SSC], 20% dextran-sulfate, 2.5 mg ml^{-1} yeast tRNA, 1× Denhardt's solution) for 30 min at 42°C. Afterwards, 3 sections of the parasitized clam were incubated with the probe at a concentration of 50 ng $µl^{-1}$. Sections were covered with Gene Frame® (AB Gene) and were denatured at 95°C for 5 min, cooled on ice for 3 min, and incubated overnight at 42°C for hybridization. Finally, the sections were washed twice for 5 min each in 2× SSC at room temperature and once in 0.4× SSC at 42°C for 10 min, and the detection was carried out with the DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions.

Epidemiological parameters. The diagnostic sensitivity of each analysis was calculated as the proportion of samples found to be positive for *Perkinsus olseni* by the evaluated assay relative to those found to be positive by the 'gold standard' method. In the same way, diagnostic specificity was calculated as the proportion of clams found to be negative for *P. olseni* by the evaluated assay relative to those assessed as negative by the standard. The positive predictive value (PPV) was calculated as the proportion of unequivocal positives divided by the number of positives found by each evaluated assay. This value represents the probability that an animal that tests positive actually has the infection. Similarly, the negative predictive value (NPV) was calculated as the proportion of unequivocal negatives divided by the number of negatives found by each evaluated assay. It represents the probability that an animal that tests negative actually does not have the infection. The κ-value statistic (Fegan 2000) was used to measure the level of agreement of the PCR assay and the combination of the classical diagnostic methods for both research groups, and the result was evaluated by comparison to arbitrary benchmarks (Table 2). For the comparison between Groups A and B and among methods, we used the simple matching coefficient that gives a measure of similarity (s) considering that a negative result gives the same information as a positive one (Dunn & Everitt 1982):

$$s = (a + d)/p$$

Table 2. Arbitrary benchmarks for the evaluation of the κ-value following Fegan (2000)

κ-value	Evaluation
>0.81	Almost perfect agreement
0.61–0.80	Substantial agreement
0.41–0.60	Moderate agreement
0.21–0.40	Fair agreement
0.01–0.20	Slight agreement
0.00	Poor agreement

where *a* is the number of animals positive and *d* is the number of animals negative by the 2 methods under comparison, and *p* is the total number of animals analyzed.

RESULTS

Prevalences of *Perkinsus olseni* observed by the 3 techniques in different clam species are summarized in Table 3. The pathogen was detected by at least 1 technique in each species except *Donax trunculus*.

RFTM culture results were highly discordant between Groups A and B. Rare infections according to Mackin's scale were discarded and not considered for subsequent analysis. Considering only readjusted data, 12.7% and 10% of the samples analyzed by Groups A and B, respectively, showed the presence of dark-blue spherical hypnospores of *Perkinsus olseni* (Fig. 2). Combining histopathological analysis and RFTM, *P. olseni* was detected in 14 and 10% of the clams by Groups A and B, respectively (Table 4). Histopathological analysis showed that *P. olseni* was associated with inflammatory responses (infiltrations; Fig. 2). Considering only the 150 clams for which all techniques were compared, *P. olseni* was detected in 2 and 1.3% of the animals analyzed by Groups A and B, respectively, using all 3 diagnostic methods (Table 4). Considering all samples, *P. olseni* was detected by histological analysis in 6.7 and 5.9% of the 270 clams tested by Groups A and B (Table 5). A PCR product of 673 bp (Fig. 3), the sequence of which corresponded to *P. olseni* as retrieved from BLASTn, was detected in 8.5% of all samples processed by both groups (Table 5). The affiliation of the PCR products to *P. olseni* was also corroborated by ClustalX alignment (data not shown). *In situ* hybridization showed that the digoxigenin-labeled probe with primers Pk-ITS1 S and Pk-ITS2 As hybridized well with *P. olseni* cells (Fig. 4).

Results of sensitivity and specificity are summarized in Tables 6 & 7. Sensitivity of PCR and histology assays decreased dramatically when RFTM was considered in the calculations. Taking only histology as the standard, the sensitivity of PCR was 94.4 and 87.5% for Groups A and B. Using the combination of histology and RFTM as the standard, sensitivity decreased to 14.3 and 13.3%, respectively. In a similar way, using only RFTM as the standard, sensitivity values were 10.5% and 13.3% for Groups A and B. The sensitivity of histology was 73.9 and 60.9% (with PCR as the standard) and 5.3 and 13.3% (with RFTM as the standard) for Groups A and B. RFTM sensitivity ranged from 33.3 to 100% (with histology as the standard) and 28.6 and 33.3% (with PCR as the standard). The sensitivity of histology and RFTM combined (with PCR as the standard) was 42.9 and 33.3% for Groups A and B, respectively.

The specificity of PCR was 97.6 and 96.5 (with histology as the standard), 96.2 and 97.0 (with RFTM as the standard), and 96.9 and 97.0 (with the combination of histology and RFTM as the standard) for Groups A and

Table 3. *Perkinsus olseni.* Prevalence in Galicia in different clam species as assessed by 3 diagnostic techniques. NA: not analyzed; RFTM: Ray's fluid thioglycollate medium

Species	Prevalence (%)		
	Histopathology	RFTM	PCR
Ruditapes decussatus	14.17	23.33	20.83
Venerupis rhomboides	0	NA	3.33
Venerupis pullastra	1.67	20	3.33
Ruditapes philippinarum	0	NA	10
Donax trunculus	0	0	0

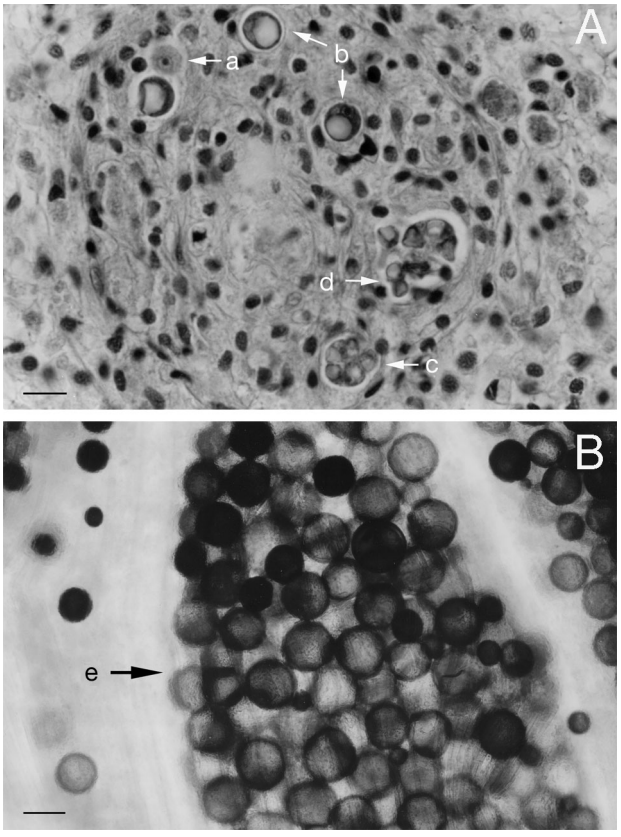

Fig. 2. *Perkinsus olseni* infecting *Ruditapes decussatus.* (A) Histological section showing various developmental stages of *P. olseni* in connective tissue of *R. decussatus*. Immature trophozoites (a), mature trophozoites (b), aggregations formed by mature trophozoite division (c), and rupture of these aggregations (d) are surrounded by an important inflammatory response (infiltration). Stained with hematoxylin and eosin. Scale bar = 10 µm. (B) Enlarged hypnospores (e) of *P. olseni* present in clam gill on Ray's fluid thioglycollate medium. Stained with lugol iodine. Scale bar = 100 µm

Table 4. Comparison among the different techniques used in this study, i.e. PCR, histology, culture in Ray's fluid thioglycollate medium (RFTM), and the combination of histology and culture in RFTM (n = 150). Positive/negative: detection/non-detection of *Perkinsus olensi* by the respective technique on each sample. Table shows numbers of clams investigated (with percentages in parentheses) by Groups A and B

	PCR						RFTM					
	Positive		Negative		Total		Positive		Negative		Total	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
Histology												
Positive	2 (1.3)	2 (1.3)	1 (0.7)	0 (0)	3 (2)	2 (1.3)	1 (0.7)	2 (1.3)	2 (1.3)	0 (0)	3 (2)	2 (1.3)
Negative	5 (3.3)	4 (2.7)	142 (94.7)	144 (96)	147 (98)	148 (98.7)	18 (12)	13 (8.7)	129 (86)	135 (90)	147 (98)	148 (98.7)
Total	7 (4.7)	6 (4)	143 (95.3)	144 (96)	150 (100)	150 (100)	19 (12.7)	15 (10)	131 (87.3)	135 (90)	150 (100)	150 (100)
RFTM												
Positive	2 (1.3)	2 (1.3)	17 (11.3)	13 (8.7)	19 (12.7)	15 (10)						
Negative	5 (3.3)	4 (2.7)	126 (84)	131 (87.3)	131 (87.3)	135 (90)						
Total	7 (4.7)	6 (4)	143 (95.3)	144 (96)	150 (100)	150 (100)						
Combination of histology and RFTM												
Positive	3 (2)	2 (1.3)	18 (12)	13 (8.7)	21 (14)	15 (10)						
Negative	4 (2.7)	4 (2.7)	125 (83.3)	131 (87.3)	129 (86)	135 (90)						
Total	7 (4.7)	6 (4)	143 (95.3)	144 (96)	150 (100)	150 (100)						

B, respectively. Specificity of histology was 99.6 and 99.2% (with PCR as the standard) and 98.5 and 100% (with RFTM as the standard). Specificity of RFTM was 87.8 and 91.2% (with histology as the standard) and 88.1 and 91.0% (with PCR as the standard). The speci-

Table 5. Comparison between PCR and histology (n = 270), showing number of clams investigated (percentages in parentheses) by Groups A and B

Histology	PCR					
	Positive		Negative		Total	
	Group A	Group B	Group A	Group B	Group A	Group B
Positive	17 (6.3)	14 (5.2)	1 (0.4)	2 (0.7)	18 (6.7)	16 (5.9)
Negative	6 (2.2)	9 (3.3)	246 (91.1)	245 (90.7)	252 (93.3)	254 (94.1)
Total	23 (8.5)	23 (8.5)	247 (91.5)	247 (91.5)	270 (100)	270 (100)

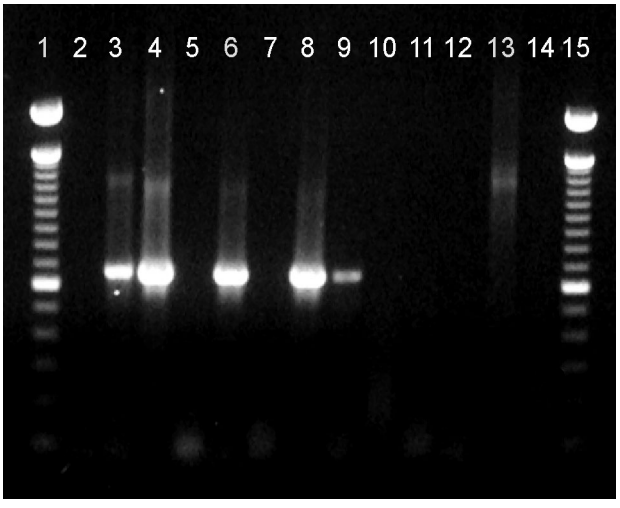

Fig. 3. Agarose electrophoresis gel of PCR products with specific *Perkinsus olseni* PCR. Lanes 1 and 15: 100 bp DNA ladder (brighter bands from top to bottom correspond to 2072, 1500, and 600 bp). Lane 2: Negative control. Lane 3: Positive control. Lanes 4, 6, 8, and 9: clams infected with *P. olseni*. Lanes 5, 7, 10, 11, 12, 13, and 14: non-infected clams

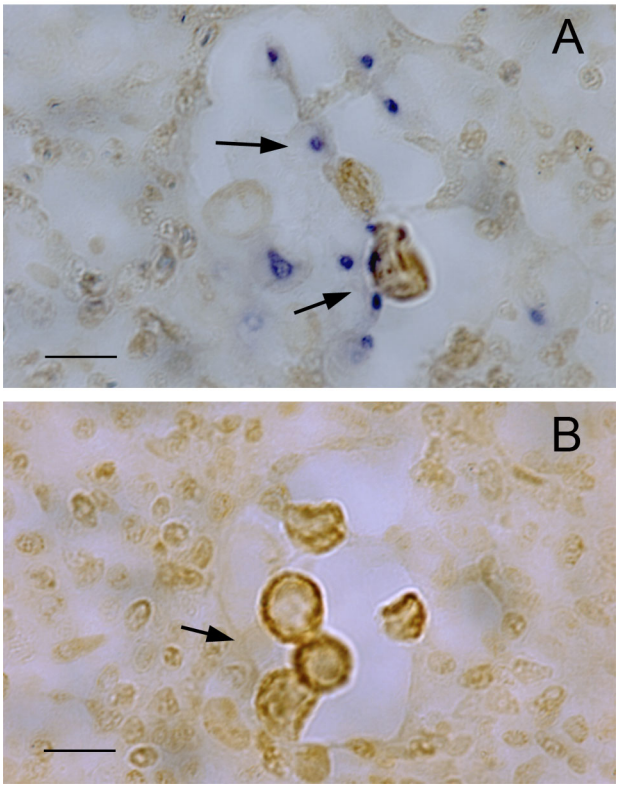

Fig. 4. *In situ* hybridization of *Perkinsus olseni* cells in clam tissues with digoxigenin-labeled probe Pk-ITS1 S/ Pk-ITS2 As. *Perkinsus* cells are indicated by arrows. (A) Positive hybridization with digoxigenin-labeled probe. (B) Negative control with no labeled probe. Scale bar = 10 µm

ficity of histology and RFTM combined (with PCR as the standard) was 87.4 and 91.0%.

PPV and NPV values are summarized in Table 8A for 150 clams comparing PCR to histology and in Table 8B for 270 clams comparing PCR to a combination of the other 2 techniques. The PPV of the PCR analysis was 73.9 and 60.9% for Groups A and B compared to histology and 42.9 and 33.3% compared to the combination of histology and RFTM. NPV from PCR ranged from 99.6 to 99.2% (compared to histology) to 87.4 to 91% compared to a combination of histology and RFTM. PPV from histological analysis was 94.4 and 87.5% and NPV was 97.6 and 96.5% for Groups A and B, respectively. PPV of the combination of techniques was 14.3 and 13.3% and NPV was 96.9 and 97% for Groups A and B, respectively.

The agreement among techniques is summarized in Table 9. Values ranged from 0.99 for the similarity of histology between the 2 research groups to 0.85 when comparing PCR by Groups A and B to RFTM by Group A. The κ-values used to measure the level of agreement of the PCR assay and the combination of the classical diagnostic methods were 0.82 and 0.70, showing almost perfect agreement and substantial agreement (Fegan 2000) for Groups A and B, respectively.

DISCUSSION

In globalized world trade, there is a growing need for more sensitive and more rapid diagnostic techniques in order to minimize the spread of shellfish diseases (Farley 1988, Bower & Figueras 1989, Alderman 1996, Bartley & Subasinghe 1996, Figueras & Novoa 2004). Here we compared nested PCR, culture in RFTM, and histopathological analysis as diagnostic techniques for perkinsosis in clams.

RFTM culture has been used widely as a diagnostic method for perkinsosis (Ray 1952, Gauthier & Fisher 1990, Bushek et al. 1994, Almeida et al. 1999). A major advantage of this method is its low cost and simplicity. The mesomycetozoan *Pseudoperkinsus tapetis* is present in Galicia in the clam *Ruditapes decussatus* and can develop hypnospores in RFTM culture. Sequencing of cells resembling *Perkinsus olseni* resulted in molecular identification of *P. tapetis* (Figueras et al. 2000, Novoa et al. 2002). RFTM cultures of a phytoplankton sample also developed hypnospores (Almeida et al. 1999), and although the real conclusions of these results remain unclear, they indicate that false positives could be obtained by this technique.

Due to this apparent lack of analytical specificity, RFTM diagnosis could overestimate the number of positives in a sample. In our data, we observed diver-

Table 6. Percentages of sensitivity, specificity and false negatives for 150 clams analyzed in parallel by the 2 participating research groups for PCR versus the combination of two other techniques (Ray's fluid thioglycollate medium [RFTM] and histology). The standard for the PCR test was the combination of techniques and vice versa

Group	Sensitivity		Specificity		False negatives		
	PCR	Combination of methods	PCR	Combination of methods	PCR	Histology	RFTM
A	14.3	42.9	96.9	87.4	12.0	3.3	3.3
B	13.3	33.3	97.0	91.0	8.7	2.7	4.0

Table 7. Percentages of sensitivity, specificity, and false negatives for 270 clams analyzed in parallel by the 2 participating research groups for PCR versus histology. The standard for the PCR test was histology and vice versa

Group	Sensitivity		Specificity		False negatives	
	PCR	Histology	PCR	Histology	PCR	Histology
A	94.4	73.9	97.6	99.6	0.4	1.9
B	87.5	60.9	96.5	99.2	0.7	4.1

Table 8. Positive predictive value (PPV) and negative predictive value (NPV) of PCR and (A) histology calculated for 150 clams and (B) a combination of 2 other techniques (Ray's fluid thioglycollate medium [RFTM] and histology) calculated for 270 clams processed in parallel by Groups A and B

Technique	Group	PPV	NPV
(A)			
PCR	A	73.9	99.6
	B	60.9	99.2
Histology	A	94.4	97.6
	B	87.5	96.5
(B)			
PCR	A	42.9	87.4
	B	33.3	91.0
Combination of techniques	A	14.3	96.9
	B	13.3	97.0

gence in results between the 2 participating research groups, especially in samples with low levels of infection. This could indicate difficulties in the correct diagnosis of samples with low levels of infection, which could be due to particles that could be confused with black-stained hypnospores, particularly by poorly trained observers (Moore et al. 2002). Given that RFTM samples are not fixed and that it is therefore not possible to exchange samples and re-evaluate discordant samples as can be done with histopathological or molecular samples, the lack of repeatability was reduced by considering the samples with the lowest level of infection on Mackin's scale to be negative. However, further research is necessary in order to determine whether the high sensitivity of RFTM culture in samples with low levels of infection is due to false positives or actually to a higher sensitivity. Nevertheless, RFTM culture is a useful tool, since it can detect environmental parasites at a low cost.

Table 9. Comparison of techniques between Groups A and B. Similarity is given by the simple matching coefficient (Sokal & Michener 1958 in Dunn & Everitt 1982)

	PCR A	PCR B	Histology A	Histology B	RFTM A
PCR B	0.95	1			
Histology A	0.96	0.97	1		
Histology B	0.97	0.97	0.99	1	
RFTM A	0.85	0.85	0.87	0.87	1
RFTM B	0.91	0.89	0.91	0.91	0.87

Nested PCR was the most sensitive method to detect *Perkinsus olseni* considering all the samples. Sequencing and *in situ* hybridization confirmed that the PCR product was in fact *P. olseni*. If we consider only the subsample for which all diagnostic methods were tested, RFTM was the most sensitive technique. Using RFTM as the standard in the calculations caused a decrease in the PCR sensitivity values due to its high sensitivity. Because the sensitivity of a technique could be influenced by the number of false positives of the standard, whereas specificity could be influenced by the number of false negatives of the standard (Griner et al. 1981), the specificity values did not decrease as much as the sensitivity values did. In addition, nested PCR resulted in the lowest number of false negatives. However, the limits of PCR detection must be considered, since positive detection does not necessarily mean an infection, as the environmental nucleic acids or pathogens could be amplified. Moreover, there is a tendency to produce false negatives if the parasites are focally concentrated, and improper sampling techniques could lead to false positives through sample contamination.

PPV and NPV are dependent on the prevalence of the disease and give a measure of the probability that a sample diagnosed as positive or negative actually is positive or negative, respectively. Since PPV is highly influenced by specificity and NPV by sensitivity, PPV was higher for histopathology and NPV was higher for nested PCR for both research groups. In contrast, PPV of the combination of techniques was low and NPV was high due to the discrepancy between RFTM and PCR data. Higher sensitivity and NPV and fewer false negative results for the nested PCR assay than for histopathology suggest that PCR is suitable for screening clam populations with low levels of parasitization. In addition, PCR is useful to guarantee that a clam stock slated for introduction into a *Perkinsus olseni*-free area is in fact free of individuals with low levels of infection, which could not be correctly diagnosed with histology. However, as none of the techniques is 100% accurate, the risk of movement of *P. olseni* remains. Therefore, clams should be quarantined at the site of introduction and, prior to releasing these clams into the environment, PCR screening should be repeated to ensure that they are *P. olseni* free. Histology resulted in a higher PPV, suggesting that this technique is suitable for confirmatory diagnosis of *P. olseni*. In addition,

histology can give a general impression of the health status of an animal stock. Also, it is important to bear in mind that histology can detect pathogens causing real disease, whereas RFTM and PCR can detect surface contamination or cells not causing disease. As PCR can detect non-viable cells or even naked nucleic acids, PCR is not suitable for confirmatory diagnosis of infection (Burreson 2008).

PCR diagnostic methods have been widely used in mollusk aquaculture (Ko et al. 1999, Carnegie et al. 2000, Cochennec et al. 2000, Penna et al. 2001) and have 4 major advantages. First, PCR is less expensive than histopathology. Calculations made in our laboratory show that the price of processing a stock of 30 bivalves by PCR is about half that of processing it for histopathology. Second, PCR is faster than histopathology. Processing samples for histology takes at least 5 d, versus between 1 and 2 d for the DNA extraction and PCR assay. Third, PCR is more sensitive than histopathology, and finally, even when technical expertise is necessary to carry out this technique, it is easier to train a technician to conduct PCR tests than the other diagnostic methods. Another important application of PCR is the possibility of detection of the parasite in water. In this way, PCR is suitable for monitoring *Perkinsus olseni* in the effluents of a mollusk depuration facility, or for monitoring ballast water, and is thus a useful tool for limiting the spread of the disease (Harvell et al. 1999). RFTM could become a useful tool if positive detection is corroborated by PCR (Audemard et al. 2008) and, as this method has important advantages in terms of speed, low cost, and simplicity, it remains a useful technique for monitoring diseases.

Agreement between laboratories could be used for purposes of validation of techniques when a standard technique is lacking. Comparison among all techniques and between research groups in the present study showed that similarity was good when comparing histology and PCR, independently of the group considered, with all simple matching coefficients above 0.95. Comparison of the analysis with RFTM results decreased the similarity, which ranged from 0.85 to 0.91. This result is a product of the lower repeatability and the number of false positives of the RFTM assay. The κ-value, considering only PCR and histology, showed almost perfect agreement and substantial agreement (Fegan 2000), suggesting that PCR analysis is a suitable screening method for perkinsosis.

Acknowledgements. We thank E. Amorín, D. Pose, M. Pazos, B. Villaverde, and L. Amo for their technical assistance and A. Ordás for statistical advice. This research was supported by Xunta de Galicia (Spain) (PGIDT01MAR40203PR), Secretaría Xeral de Investigación e Desenvolvemento. We also acknowledge Sanidad Animal, Ministerio de Agricultura y Pesca, Spain. P.B. is supported by an I3P fellowship of the Consejo Superior de Investigaciones Científicas (CSIC).

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Editorial responsibility: Eugene Burreson, Gloucester Point, Virginia, USA

Submitted: July 27, 2009; Accepted: December 22, 2009
Proofs received from author(s): May 27, 2010